Hiroto S. Watanabe
Editor

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HORIZONS IN CANCER RESEARCH

VOLUME 56

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HORIZONS IN CANCER RESEARCH

VOLUME 56

HIROTO S. WATANABE
EDITOR

New York

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Preface

This book presents original results on the leading edge of cancer research. Topics discussed include malignancy-related ascites; targeting cathepsin b for cancer therapies; cancer stem cells; metallomics of brain tumors; hepatitis b virus and Hepatitis C virus infections and risk of pancreatic ductal adenocarcinoma; the risk and prognostic factors associated with undifferentiated nasopharyngeal carcinoma; widespread expressions of TCRs in cancer cells and the implications in cancer immunology; hormone therapy in young cancer survivors; and inhibitory effects of ribosome inactivating proteins and compounds on choriocarcinoma cells.

Chapter 1 – Malignancy-related ascites, a frequent cause of significant morbidity and distress in many cancer patients, is often a marker of poor prognosis. Malignancies, mainly cancers of the colon, breast, ovary, endometrium, pancreas and stomach, account for nearly 10% of all cases of ascites. Between 10-15% of gastrointestinal cancer patients can develop ascites as a part of their disease course; the ascites are frequently recurrent and difficult to manage. The pathogenesis is multifactorial including both an increased production and a decreased clearance of peritoneal fluid. Cancer cells responsible for ascites formation can invade lymphatic channels or hepatic veins resulting in mechanical obstruction and fluid accumulation. Decreased lymphatic drainage can also result in intravascular volume depletion, which in turn activates renin-angiotensin-aldosterone system and causes sodium and fluid retention. Additionally, cancer cells also show increased expression of vascular endothelial growth factor (VEGF) and vascular permeability factor, which may lead to increased microvascular permeability.

Patients with malignant ascites frequently present with signs and symptoms of underlying malignancy and features of elevated intra-abdominal pressure such as abdominal distension, anorexia, nausea, and dyspnea. Malignant ascites, particularly related to peritoneal carcinomatosis, can contribute to bowel obstruction, decreased oral intake and cachexia. The presence of ascites can be diagnosed using clinical and imaging modalities, however, ascitic fluid analysis is needed to differentiate malignant from benign causes. Cytologic analysis remains the gold standard for diagnosis despite its poor sensitivity. Individual tumor markers in ascitic fluid such as carcinoembryogenic antigen, cancer antigen 125 or carbohydrate antigen 19-9 have modest sensitivity; however, a combination of tumor markers may serve as an adjunct to cytology. Additionally, higher levels of fibronectin, cholesterol, sialic acid and telomerase activity in the ascitic fluid have shown excellent specificity and varying sensitivity for the diagnosis of malignant ascites; these tests can potentially be useful in the future to differentiate between malignant and benign ascites. The management of malignant ascites can

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be complex and should incorporate cancer-directed therapies in suitable patients. However, with malignant ascites frequently indicating the terminal stage of malignant process, the management is mostly palliative and consists of the use of diuretics, paracentesis, peritoneovenous shunts and intraperitoneal chemotherapy. All these strategies have shortcomings, and improved therapies are warranted. Novel treatments such as VEGF inhibitors, matrix metalloproteinase inhibitors and monoclonal antibodies have shown promising results but remain investigational.

Chapter 2 – Cathepsin B is a member of the papain family of cysteine proteases normally present in the lysosome, but it can translocate and function to degrade components of the extracellular matrix. It exhibits carboxyopeptidase, peptidyldipepidase, and endopeptidase activity. Aberrant overexpression of cathepsin B has been reported in invasive and metastatic cancers, including breast cancer, melanoma and colorectal cancer. It has been shown that oncogenic activation, such as the signaling of the ErbB pathways, can lead to cathepsin B overexpression. The degradation of the extracellular matrix is a key factor for cathepsin B to contribute to development and metastasis of tumors. An example of substrates for cathepsin B is E-cadherin, which is involved in adherens junctions, and the downregulation of E-cadherin in cancer is directly linked to invasion and metastasis. Recent studies also point to a role for cathepsin B in macrophages in the tumor microenvironment. The structure of cathepsin B is crystallographically solved, and several highly selective and potent inhibitors for cathepsin B have been developed. Yet it remains to be a challenge to demonstrate the clinical utility or benefit of any cathepsin B inhibitor. As cathepsin B is required for a cellular process called lysosomal membrane permeabilization (LMP), inhibition of cathepsin B would protect cancer cells from cell death induced by chemotherapeutic agents. It is expected that combining cathepsin B inhibitors with other approaches, such as nanoparticles, to direct the inhibition to the extracellular space may lead to better clinical approaches to treat cancers and metastasis.

Chapter 3 – For many years, the problem of the tumour has afflicted humanity, resulting in thousands of deaths. The main obstacle to overcome is the variability of this malignancy, which does not allow the discovery of a specific therapy to prevent cancer re-expansion and tumour metastasis. In recent years, the cancer stem cells (CSCs) theory has acquired remarkable importance. The CSCs are a subpopulation of cells with self-renewal and differentiation capacity within tumours, and exhibit high resistance to chemo and radio-therapies therefore this was thought to be the cause of the failure of current therapies. In fact, the conventional therapies target cancer cells, pertaining to tumour bulk, but are unable to destroy CSCs. However many still show several doubts about the CSCs theory and their real existence, thus new and ongoing research is being carried out on them. Herein we want to validate the CSCs theory, by analysing their features, the microenvironment in which they live, possible markers, processes and signal pathway in which they are involved. Moreover, attention is focused on new drugs which target CSCs and their respective clinical trials, confirming the effectiveness of this new approach, and the prospective of finding a specific drug that can totally eradicate tumours.

Chapter 4 – Brain tumors are the leading cause of cancer-related death in the US in patients under the age of 35. They are divided into primary and secondary (metastatic) that are more frequent in adults (melanoma, lung carcinoma, renal cell carcinoma and thyroid carcinoma as into brain mostly metastasizing).

Metallomics can be defined as comprehensive analysis of the entirety of metal and metals containing proteins within cells and tissues. Approximately 1/3 of proteins are associated with

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some metals. Moreover, some drugs contain a metal in their structure and are referred to as metallodrugs.

Up-to-date, the greatest knowledge has been gathered in the field of metalloproteins containing zinc because it has been shown to be not only a structural component, but also a signaling substance in number of cascades and a cofactor of many important enzymes. On cellular level, key cellular processes such as proliferation, differentiation and apoptosis have been connected with its signaling. In the field of metallomics of brain tumor, there is still little known but our knowledge is rapidly growing. On other metals in brain tumors, there are only limited reports as the grade of glioma correlated with the Fe(II) to Fe(III) ratio in the tissue. However, this relationship cannot be explained by occurrence of hypoxic regions in the tissue because of lack of correlation between the average oxidation state of iron and hypoxia.

In the case of non-essential metallomics, the cytotoxic effect of platinum drugs consists of DNA adducts formation, especially to the most nucleophilic bases - guanine and adenine. This phenomenon causes DNA strands crossing and subsequent interference with normal transcription and/or replication. Moreover, platinum drugs influence the cell cycle of tumor cells, which can also influence their effect. In addition, many potential platinum-binding molecules are available including RNA and sulfur-containing biomolecules such as glutathione and metallothioneins in the cytoplasm. Binding of platinum cytostatics to MT results in their inactivation and is one of the mechanisms of chemoresistance to those cytostatics. The exact mechanism by which MT prevent "non-platinum" cytostatics induced cell death has not been established until now.

The aim of this review is to evaluate the current state of research on metals and metalloproteins in brain tumors and report new insights into the diagnostics, therapy by metal containing drugs, including resistance to those cytostatics and potential of metalloproteins-targeted therapy and drug nanocarriers containing metals to improve the treatment efficiency in treatment of brain tumors.

Chapter 5 – Pancreatic ductal adenocarcinoma (PADC) represents a highly lethal cancer with a very dismal prognosis. Absence of early symptoms, advanced stage at diagnosis, aggressive biological behaviour and lack of effective systemic treatment are the most important factors, explaining its elevated mortality rate and its low overall five-year survival (< 5%).

Until now, the causes of this malignancy remain still largely unknown and further efforts are underway to reach a better knowledge of PADC aetiology and to improve our understanding of mechanisms involved in carcinogenesis of this organ. In the last years it has progressively emerged that viruses play a key role in human carcinogenesis. Unfortunately, some host and viral factors have contributed to make the study of the pancreas extremely difficult and to hamper the identification of pathogenetic processes involved in cancer development, including its retroperitoneal localization as well as the small size of precursor cancer lesions. However, in the past and more recently, some histological investigations suggested that both antigens and genome of hepatitis B (HBV) and hepatitis C (HCV) viruses, two pathogens with well-known high liver tropism and pro-oncogenic properties may be detected also in extra-hepatic tissues, such as pancreas. In addition, some epidemiological articles have suggested that HBV and HCV might be involved even in pancreatic carcinogenesis. Here we review the results of available reports, evaluating the possible association between HBV or/HCV infections and risk of pancreatic cancer development as well as to discuss the limiting factors of these researches.

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Chapter 6 – Nasopharyngeal carcinoma (NPC) is a unique head and neck cancer with characteristic geographic distribution. In endemic area, keratinizing undifferentiated carcinoma is the major histological type and the cancer is closely associated with Epstein-Barr virus infection. NPC is a rare disease in Western countries. The incidence of undifferentiated NPC is very much higher in Southern China in comparison with other parts of the world. Oncogenic gene mutations are rarely found in NPC suggesting other risk factors are associated with the disease. Genetic predisposition is a major risk factor at the high incidence areas. Other risk factors including environmental exposure and viral infection could also contribute to the disease. The mainstay treatment for early NPC is radiation therapy as the tumor is very sensitive to radiation. Functional imaging represents a noninvasive method to provide metabolic and clinical information for the guideline of NPC management. Tumor stage and tumor histology have major influence on the treatment outcomes and are significant prognostic factors for local or regional control of NPC. EBV, HPV and a number of oncogenic proteins could serve as critical molecular biomarkers for the monitoring of patients. Comprehensive evaluation on the latest findings on risk factors and prognostic factor may help to identify high-risk community earlier and predict treatment outcome.

Chapter 7 – RP215 is a monoclonal antibody which specifically recognizes a carbohydrate-associated epitope located predominantly on the heavy chains of immunoglobulins, as well as other immunoglobulin superfamily (IgSF) proteins, including TCRs (TCR) and cell adhesion molecules, all of which are expressed among most cancer cells but not among normal immune cells. These cancerous glycoproteins are designated, in general, as CA215. Molecular biological analysis, including RT-PCR and cDNA sequencing of mRNA isolated from cancer cells, revealed that as many as 80% of cancer cell lines express significant levels of TCR-α and/or TCR-β genes. In contrast, co-receptors and co-stimulators of TCR, such as CD3, CD4 and CD8, were rarely expressed in these cancer cells, suggesting undefined roles for these receptors. Consistent with TCR expression as detected by RT-PCR, both immunohistochemical staining and Western blot assay also indicated significant levels of TCR expressions in various cancer cells of different tissue origins. Similar to anti-human IgG and RP215, anti-TCR monoclonal or polyclonal antibodies were also shown to induce apoptosis and complement-dependent cytotoxicity (CDC) to OC-3-VGH ovarian cancer cells, as well as several other cancer cell lines. Upon treatments with anti-IgG and anti-TCRs, the gene expression patterns of cancer cells were compared and found to be highly correlated. Based on the results of cell-based functional assays, these RP215 epitope-specific antigen receptors on the cancer cell surface may act as potential targets for RP215-based anti-cancer drugs for future therapeutic applications in humans.

Chapter 8 – As the overall survival rates of cancer treatment have increased, the short- and long-term sequelae faced by the growing number of young survivors have become more of a concern. Among these sequelae, hypogonadism is of particular importance and demands specialized medical care. However, a scarcity of evidence leaves no ideal solution. Hypogonadism caused by cancer therapy leads to impairment of pubertal development, hormonal regulation, fertility and sexual function in childhood and adolescent cancer survivors, and produces menopausal symptoms such as vasomotor and urogenital symptoms, and osteoporosis in young adult survivors. In the majority of cases except hormone receptor-positive cancers, hormone therapy is the most effective option for hypogonadism-induced problems and should be age-specific. For the prepubertal survivors, timing of hormone-therapy is crucial to ensure acceptable growth. For the postmenarchal who cease menstruating

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during or after cancer therapy, monitoring of menstrual resumption for one year is an acceptable management strategy. Those who remain amenorrheic, have symptoms of gonadal failure, or have elevated gonadotropin levels should be offered individualized hormone-therapy options. For the young adult survivors, sparing ovarian reserve and fertility as well as reduction of menopausal symptoms should be the main objectives of hormone therapy. In this review, we focused on effective hormone therapy according to these age groups.

Chapter 9 – The aim of this article is to review the mechanisms of action of compounds with inhibitory activity toward choriocarcinoma cells. Two isomers of linolenic acid, alpha- and beta-calendic acid, inhibited invasion of human choriocarcinoma JEG-3 cells in vitro, and enhanced oxidative stress in the cells as witnessed by elevated levels of the lipid peroxidation product malondialdehyde and reactive oxygen species. The soybean phytoestrogen genistein stimulated the MTA3/Snail/E-cadherin regulatory pathway by binding with estrogen receptor-β, thus suppressing JAR cell invasion. A translocator protein ligand (initially referred to as a ligand for the peripheral benzodiazepine receptor), 1-(2-chlorophenyl-N-methylpropyl)-3-1-(2-chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide (PK11195), reduced the percentage of cells in the S phase of the cell cycle and raised the proportion of cells in the G0/G1 phases, and triggered apoptosis in choriocarcinoma BeWo cells. Tubeimoside I, a triterpenoid saponin, isolated from *Bolbostemma paniculatum* tubers, induced apoptosis in JEG-3 cell, cell cycle arrest at G2 phase and decline in mitochondrial transmembrane potential, mitochondrial cytochrome c release and augmented caspase-3 expression. Tubeimoside I upregulated Bcl-2 associated X protein (Bax) expression, downregulated Bcl-2 expression, suppressed nuclear factor-κ-B (NF-κB) function and affected phosphorylation of p38 mitogen-activated protein kinase (p38/MAPK), extracellular signal-regulated kinases (ERK)1/2 and protein kinase B (Akt). Tubeimoside I produced its apoptosis-inducing effects, at least partially, by induction of mitochondrial dysfunction and regulation of the p38/MAPK, ERK1/2 and PI3K/Akt signaling pathways. Flax seed fractions exhibited antiproliferative activity toward choriocarcinoma Jeg3 cells. Elm bark extract, mainly composed of triterpenes, phytosterols, free fatty acids and suberins with smaller amounts of lipids and dilignols, reduced the viability of Jeg3 and BeWo cells. The quinolinone derivative vesnarinone induced expression of c-Myc gene in choriocarcinoma cells, the product of which may be associated with cell growth suppression and apoptosis induction. All-trans retinoic acid exhibited antiproliferative activity toward four choriocarcinoma cell lines. Co-administration of all-trans retinoic acid and methotrexate or actinomycin-D produced an augmented effect. The aminopeptidase inhibitor Ubenimex (bestatin) suppressed the growth of choriocarcinoma NaUCC-4 cells *in vivo* and *in vitro,* not via potentiation of effector cells, but through its direct cytostatic activity, Paclitaxel, a taxane analog isolated from bark of the western yew (*Taxus brevlfolla*), is highly potent against choriocarcinoma cell lines. The activity of VP16-213 (etoposide) against the three human choriocarcinoma cell lines, SCH BeWo, and HCCM-5, was similar to or superior to those of actinomycin D and methotrexate.

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Chapter 1

Malignancy-Related Ascites: Diagnostic and Therapeutic Strategies

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Abstract

Malignancy-related ascites, a frequent cause of significant morbidity and distress in many cancer patients, is often a marker of poor prognosis. Malignancies, mainly cancers of the colon, breast, ovary, endometrium, pancreas and stomach, account for nearly 10% of all cases of ascites. Between 10-15% of gastrointestinal cancer patients can develop ascites as a part of their disease course; the ascites are frequently recurrent and difficult to manage. The pathogenesis is multifactorial including both an increased production and a decreased clearance of peritoneal fluid. Cancer cells responsible for ascites formation can invade lymphatic channels or hepatic veins resulting in mechanical obstruction and fluid accumulation. Decreased lymphatic drainage can also result in intravascular volume depletion, which in turn activates renin-angiotensin-aldosterone system and causes sodium and fluid retention. Additionally, cancer cells also show increased expression of vascular endothelial growth factor (VEGF) and vascular permeability factor, which may lead to increased microvascular permeability.

Patients with malignant ascites frequently present with signs and symptoms of underlying malignancy and features of elevated intra-abdominal pressure such as

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abdominal distension, anorexia, nausea, and dyspnea. Malignant ascites, particularly related to peritoneal carcinomatosis, can contribute to bowel obstruction, decreased oral intake and cachexia. The presence of ascites can be diagnosed using clinical and imaging modalities, however, ascitic fluid analysis is needed to differentiate malignant from benign causes. Cytologic analysis remains the gold standard for diagnosis despite its poor sensitivity. Individual tumor markers in ascitic fluid such as carcinoembryogenic antigen, cancer antigen 125 or carbohydrate antigen 19-9 have modest sensitivity; however, a combination of tumor markers may serve as an adjunct to cytology. Additionally, higher levels of fibronectin, cholesterol, sialic acid and telomerase activity in the ascitic fluid have shown excellent specificity and varying sensitivity for the diagnosis of malignant ascites; these tests can potentially be useful in the future to differentiate between malignant and benign ascites. The management of malignant ascites can be complex and should incorporate cancer-directed therapies in suitable patients. However, with malignant ascites frequently indicating the terminal stage of malignant process, the management is mostly palliative and consists of the use of diuretics, paracentesis, peritoneovenous shunts and intraperitoneal chemotherapy. All these strategies have shortcomings, and improved therapies are warranted. Novel treatments such as VEGF inhibitors, matrix metalloproteinase inhibitors and monoclonal antibodies have shown promising results but remain investigational.

Keywords: malignancy-related ascites, gastrointestinal cancer, peritoneal carcinomatosis, cytology, paracentesis, peritoneovenous shunts, intraperitoneal chemotherapy

Introduction

Malignancy-related ascites, a significant cause of morbidity in many cancer patients, is common in cancers of the colon, breast, ovary, endometrium, pancreas and stomach. These malignancies can cause secondary peritoneal carcinomatosis. Similarly, primary peritoneal carcinomatosis can cause ascites. Ascites can be an early presentation in ovarian cancer. In most malignancies, however, it heralds the terminal phase of the cancer, and frequently indicates a poor prognosis [1]. Often recurrent and difficult to manage, malignant ascites can require frequent hospital visits, compromise quality of life and cause serious complications. In symptomatic patients, successful management of malignant ascites has the potential to enhance quality of life and potentially prolong survival. In this chapter, we discuss the epidemiology and pathogenesis of malignant ascites, its presentation, diagnostic modalities as well as therapeutic options for the management.

Epidemiology

Malignancies account for nearly 10% of all cases of ascites [2, 3]. While most cases of malignant ascites result from ovarian (25%), breast (16%), gastric (13%) and colorectal cancers (13%), up to one fifth of these cases have unknown primary tumors (Table 1) [4, 5].

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Table 1. Primary site of malignancy in malignant ascites

Malignancy	Percent of cases of malignant ascites	
	Ayantunde and Parsons [5]	Sears and Hajdu [6]
Ovarian	25	29
Breast	16	13
Gastric	13	6
Colorectal	13	5
Esophageal	5	-
Pancreatic	5	4
Uterine	4	-
Primary peritoneal tumor	3	-

The frequency of ascites varies by cancer types, stage of cancer, duration of follow-up and other factors [5, 6]. Overall, ascites is common in ovarian cancers (occurring in 37% of cases), gastrointestinal (26% of cases), pancreatobiliary (21% of cases) and breast cancers (3% of cases) [5]. In other studies, 10-15% of gastrointestinal cancer developed ascites as a part of their disease course [7].

Pathogenesis

A variety of pathological processes in malignancy can alter the delicate balance between production and clearance of peritoneal fluid. In peritoneal carcinomatosis, which can account for up to two-third of the cases of malignancy-related ascites [8, 9], invading cancer cells may obstruct the diaphragmatic lymphatic channels [10, 11], thus preventing clearance of peritoneal fluid. The obstruction of main thoracic duct can result in the development of chylous ascites [12].

Several mechanisms can increase production of peritoneal fluid [13]. Liver metastases or tumor invasion into the hepatic vein can result in portal hypertension. Similarly, increased expression of vascular endothelial growth factor (VEGF) and vascular permeability factor by the tumor cells leads to increased neovascularization and microvascular permeability, which is central to the pathogenesis of malignant ascites [14-18]. In peritoneal carcinomatosis, the seeding and attachment of tumor cells in the peritoneal lining involves VEGF and similar growth factor-mediated neoangiogenesis, along with increased permeability of these newly-formed vessels [19]. Additionally, the extravasation of protein-rich fluid to the peritoneal cavity decreases plasma to peritoneal oncotic pressure gap. This decreases reabsorption of peritoneal fluid into intravascular compartment, and may even favor fluid movement into peritoneal cavity.

Lastly, a hormonal basis for the development of malignant ascites has also been described. Decreased lymphatic drainage, for example, due to obstruction, depletes intravascular volume, which in turn, activates renin-angiotensin-aldosterone system [7]. This results in sodium and fluid retention, thus contributing to the accumulation of ascites.

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Clinical Manifestations

Ascites may be the first sign of malignancy in as many as 7-49% of cases [20-22]. Nearly half of patients with intra-abdominal malignancies such as ovarian, pancreatic or colon cancer may have obvious ascites at the time of cancer diagnosis [17]. In general, malignant ascites and associated symptoms develop gradually over months and are accompanied by additional symptoms of underlying malignancy.

Irrespective of the underlying etiology, malignant ascites leads to an elevated intra-abdominal pressure, which can result in symptoms such as abdominal distention, discomfort, anorexia, nausea, dyspnea, reduced mobility and distortion of body image (Table 2). Malignant ascites, particularly related to peritoneal carcinomatosis, can result in bowel obstruction [23]. Weight loss related to malignancy may offset the weight gain that occurs with fluid accumulation.

Table 2. Symptoms at presentation of malignant ascites [5]

Symptoms	Frequency
Abdominal swelling	55%
Abdominal pain	53%
Nausea	37%
Anorexia	36%
Vomiting	25%
Fatigue	17%
Dyspnea	11%
Early fullness	6%
Weight change	5%
Ankle swelling	3%
Heartburn	1%

Table 3. Sites of metastases at diagnosis of malignant ascites [5]

Site of metastasis	Frequency
Peritoneal	87%
Liver	27%
Bone	12%
Lung	8%

Physical findings include flank dullness, shifting dullness and a fluid wave, which may occur with any cause of ascites. Additionally, malignant ascites may have signs resulting from underlying malignancy or from other metastases (Table 3). Rarely, an umbilical nodule, named Sister Mary Joseph nodule, may accompany ascites in gastric, colon, and hepatocellular cancers [24].

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Diagnosis

The presence of significant ascites can be diagnosed clinically in most cases. Imaging studies such as ultrasonography and abdominal computed tomography have the sensitivity to detect even small amount of ascitic fluid. However, these clinical and imaging techniques cannot accurately distinguish malignant from benign ascites. Ascitic fluid analysis is essential for the diagnosis of malignant ascites, particularly in patients without a cancer diagnosis or with an early cancer. The need to confirm the diagnosis of malignant ascites may not be as important in patients with an advanced or metastatic cancer, where a presumptive diagnosis of malignant ascites is largely sufficient for most practical purposes. In women, the outcomes of malignant ascites associated with ovarian cancers are significantly better than other causes. Therefore, intensive attempts to detect the primary tumor may be justifiable in many women [5, 21].

Diagnostic abdominal paracentesis is a safe and quick procedure [25]; the aspirated fluid is subjected to chemical and cytologic analysis. The ascitic fluid is usually turbid, and can be blood stained in about one fifth of these cases [9]. Black-colored ascites can be present in cases of malignant melanoma [26]. The pH is lower than in non-malignant ascites [27, 28]. Truly chylous ascites is characterized by a milky appearance and the presence of chylomicrons and a high concentration of triglycerides (> 200 mg/dL). Chylous ascites indicates a malignant process in nearly four-fifth of cases (29, 30). Though malignant ascites is mostly exudative with a total protein content of ≥2.5 g/dL, nearly 18% of the cases may have protein content in transudative level [31, 32]. While a high lactate dehydrogenase activity in ascitic fluid suggests an exudative process such as malignancy, low activity does not rule it out [8].

High serum ascitic albumin gradient (SAAG) (≥11 g/L) indicates increased portal pressure as in cirrhosis and congestive heart failure. Conversely, a low SAAG value indicates an increase in vascular permeability as in most malignancies. Low SAAG has a high diagnostic accuracy (~ 90%) and specificity (~ 99%) for conditions such as malignancy (ascites from causes other than portal hypertension) but suffers from poor sensitivity (~ 62%) [33-37] (Table 4). This is in part due to the heterogeneous nature of malignant ascites, with peritoneal seeding causing low gradient ascites and hepatic metastases causing high gradient ascites.

Table 4. Diagnostic value of serum ascitic albumin gradient (SAAG) in malignant ascites

Reference	Accuracy	Sensitivity	Specificity
Chen et al. [36]	90%	62%	99%
Bala et al. [38]	-	60%	87%
Rana et al. [28]	86%	88%	84%
Akriviadis et al. [39]*	98%	-	-
Runyon et al. [31]*	97%	-	-

These studies utilized a cut-off of SAAG <11 g/L.
*These studies provide diagnostic accuracy for ascites from causes other than portal hypertension such as malignancy but are not specific for malignancy only.

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Ascitic fluid cytology is the gold standard of diagnosis of malignant ascites, particularly in peritoneal carcinomatosis, because of its high specificity (Table 5). However, cytology suffers from poor sensitivity since malignancies may cause ascites without shedding large burden of tumor cells in the fluid [2, 9, 32]. A combination of cytology and immunohistochemistry can increase sensitivity, and may also help in the diagnosis of the primary tumor [40]. Some of the commonly used immunohistochemical stains include cytokeratins (epithelial cancers), vimentin (mesenchymal tumors), leukocyte common antigen (hematopoietic tumors), carcino-embryogenic antigens (colon cancers and carcinoids) and S100 protein (melanoma, schwannoma, and glial tumors) [40].

Table 5. Diagnostic value of cytology in malignant ascites

Reference	Sensitivity	Specificity	PPV	NPV
Karoo et al. [41]	60%	100%	-	-
Siddiqui et al. [32]	55%	100%	-	-
Motherby et al. [42]	63%	98%	100%	88%
Jha et al. [43]	57%	100%	100%	64%
Gerbes et al. [44]	70%	100%	100%	82%
Tangkijvanich et al. [45]	40%	100%	-	-
Rana et al. [28]	64%	100%	-	-

NPV indicates negative predictive value; PPV positive predictive value.

Malignant ascites, compared to benign ascites, have a higher level of tumor markers such as carcinoembryogenic antigen (CEA), cancer antigen 125 (CA-125) and carbohydrate antigen 19-9 (CA 19-9). The tumor markers in ascitic fluid show greater diagnostic value than those in the serum [36, 46]. While the sensitivity for individual tumor markers in ascitic fluid is rather poor (69% for CEA and 66% for CA 19-9 in one study) [36], combination of tumor markers yields a higher sensitivity (86%), which increases further when combined with cytology [36, 46]. In one study, the diagnostic accuracy reached 99% with a combination of CEA, carbohydrate antigen 15-3 (CA 15-3) and cytokeratin 19 fragments (CYFRA-21.1) [47]. So, while no single tumor marker in ascitic fluid is accurate enough for routine use, a combination of tumor markers, especially as an adjunct to cytology, may represent a helpful tool to rule in malignancy as a probable cause of ascites.

In recent years, several novel markers have been explored to diagnose malignant ascites. Higher levels of fibronectin, cholesterol, sialic acid and telomerase activity in the ascitic fluid have shown excellent specificity and varying sensitivity for the diagnosis of malignant ascites. These tests may potentially be used to differentiate between malignant and benign ascites in the future [32, 48, 49]. Some studies have shown elevated ascitic fluid cholesterol (at a cut-off of 70 mg/dL) to be highly sensitive (88%), specific (100%) and accurate (94%) in discriminating malignant from benign ascites [28]. Conversely, other studies found normal cholesterol level in malignant ascites from massive hepatic metastasis [50, 51]. Fibronectin in the ascitic fluid are derived from either the malignant cells or adjacent connective tissues. In some studies, elevated fibronectin level in the ascitic fluid was found to have a greater diagnostic accuracy than other parameters in differentiating malignant and benign ascites [32, 52]. Another study revealed a higher concentration of sialic acid in malignant ascites, compared to benign ascites [53]. However, both fibronectin and sialic acid were less useful in the diagnosis of ascites resulting from hepatic metastasis and hepatocellular carcinoma.

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Telomerase enzyme is expressed in large number of cancer cell lines and can be detected using polymerase chain reaction-based assays [54, 55]. Detection of telomerase activity in the cells obtained from ascitic fluid is a highly accurate (89%) method of differentiating malignant from benign ascites [45]. Telomerase assay is also more sensitive than conventional cytology in diagnosing ascites related to hepatocellular carcinoma (67% vs. 11%) [45].

Diagnostic laparoscopy is an important tool, which may clinch the diagnosis in patients with unexplained ascites. It may be useful in cases where ascitic fluid analysis and imaging modalities fail to confirm a pathological diagnosis [56, 57]. Diagnostic laparoscopy can also be helpful in identification of the primary tumor site and in assessing operability of certain tumors. However, laparoscopy has the potential risk of tumor implantation, visceral injury, intestinal perforation, ascitic leakage and subcutaneous emphysema [57]. Closure of only skin rather than all layers of the abdomen at the end of the procedure (58% vs. 2%), advanced primary tumor, peritoneal carcinomatosis, larger ascites and longer interval to the start of chemotherapy or cytoreductive surgery are potential risk factors for tumor recurrence at the implantation site [58, 59]. The diagnostic utility of several tests are compared in table 6.

Table 6. Diagnostic value of different tests in malignant ascites

Test (Cutoff value)	Study	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Total protein (> 3 g/dL)	Rana et al. [28]	56	88	82	66	72
pH (<7.45)	Rana et al. [28]	48	84	75	63	66
LDH (200 U/L)	Gerbes et al. [44]	85	89	85	89	88
SAAG (<1.1 g/dL)	Rana et al. [28]	88	84	84	87	86
Cholesterol (>70 mg/dL)	Rana et al. [28]	88	100	100	89	94
Cytology	Rana et al. [28]	64	100	100	73	82
Fibronectin (≥110 μg/mL)	Siddiqui et al. [32] Scholmerich et al. [52]	100	100	100	100	100
Sialic acid (≥300 mg/L)	Colli et al. [53]	70	90	-	-	82
Laparoscopy	Han et al. [57]	100	94	-	-	-
Telomerase assay	Tangkijvanich et al. [49]	76	96	91	88	89
CEA (2.5 ng/ml)	Gerbes et al. [44]	45	100	100	72	77

CEA indicates carcinoembryonic antigen; LDH lactate dehydrogenase; NPV negative predictive value; PPV positive predictive value; SAAG serum ascitic albumin gradient.

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Management

The presence of malignant ascites frequently indicates a poor prognosis, except in ovarian cancer [60]. Therefore, in most instances, management is palliative and aimed at improvement in symptoms and quality of life. The use of definitive therapy for the underlying cancer, including palliative chemotherapy or targeted agent, may result in tumor shrinkage with improvement in ascites. This is the preferred approach in patients, who are candidates for cancer-directed therapy. Other patients should receive therapy for the symptomatic management of ascites (Table 7), as described below.

Table 7. Comparison of different treatment modalities in malignant ascites

Treatment Modality	Symptomatic relief	Complications	Reference
Diuretics	43%	Hyperkalemia, hypovolemia, circulatory and renal failure	Becker et al. [62]
Paracentesis	94%	Hypotension (3%), pulmonary embolism (1.5%), peritonitis (0.8%), intestinal perforation (0.8%)	Becker et al. [62] Appelqvist et al. [92]
Peritoneovenous shunts	78%	Subclinical DIC (76%), clinical DIC (2%), pulmonary edema (12%), pulmonary emboli (7%), tumor dissemination (7%), shunt block (26%), sepsis (5%) (7)	Becker et al. [62]
Permanent drain	100%	Infection (38%), catheter blockage (30%) hypotension (95%)	Smith and Jayson [7]
Intraperitoneal chemotherapy	47%	Fever and abdominal pain (21%), sepsis, intestinal obstruction (5.5%)	Smith and Jayson [7]
Intraperitoneal radioisotopes	54%	Transient GI upset (10-35%), transient fever (13%), abdominal pain (5%), reaction at injection site (2%)	Ariel et al. [125] Smith and Jayson [7]
OK-432	60%	Nausea, malaise, chills and fever	Katano and Morisaki [117]
Intraperitoneal interferon	40%	Fever (40%), mild and transient abdominal pain (20%)	Gebbia et al. [114] Stuart et al. [113]
Radioimmunotherapy (2G3 with ^{131}I)	75%	Painless erythema (22%)	Buckman et al. [121]
Matrix metalloproteinase inhibitors	70%	Fatigue (39%), fever (30%), vomiting (26%), abdominal pain (22%)	Beattie and Smyth [109]
Tumor necrosis factor- alpha	76%	Fever, chills, nausea, vomiting, fatigue	Rath et al. [103]
Octreotide	66%	Abdominal pain, hyperkalemia, dehydration, cognitive dysfunction, fatigue, anemia, neutropenia	Cairns and Malone [123]
Corynebacterium parvum	75%	Transient fever/chills (53%), transient nausea/vomiting (47%), transient pain (53%), ileus (40%), injection site cellulitis (27%)	Mahler et al. [124] Currie et al. [126]

DIC indicates disseminated intravascular coagulation; GI gastrointestinal.

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Diuretics

Diuretics are easy to administer and non-invasive, hence frequently used as a first line agent. A physician survey reported the use of diuretics in 61% of malignant ascites, however, malignant ascites usually show inconsistent response to diuretics [61].

In general, less than half of the cases respond to diuretic therapy [62]. Malignant ascites resulting from massive hepatic metastases may respond to spironolactone; the efficacy may relate to the plasma rennin-aldosterone ratio [63, 64]. SAAG may also prove to be a useful predictor of response to diuretics, with SAAG >11 g/L showing better response [63].

Paracentesis

Therapeutic paracentesis can cause a significant but temporary improvement in symptoms. It is the preferred initial therapy and frequently the mainstay of management [61]. Available data demonstrate that more than 90% of patients show symptomatic improvement with the use of paracentesis [61, 62]. However, rapid re-accumulation of fluid, within 11 days on average, may necessitate repeated paracentesis [7]. Instillation of *Viscum album* (medicinal plant) extract into the peritoneal cavity following paracentesis may reduce fluid reaccumulation and the frequency of paracentesis [65]. Potential complications of paracentesis include the risk of ascitic fluid leak, plasma fluid depletion with hypovolemia, hypotension, hypoalbuminemia, visceral and vascular injury, and infections including peritonitis and sepsis [66]. Clinically significant hypovolemia with a significant drop in mean arterial pressure is thought to result from increased arterial vasodilation secondary to an abrupt decrease in intra-abdominal pressure [67, 68]. Even though up to 5 L of ascitic fluid can be quickly removed without any intravascular volume replacement, slower rate of fluid draw and simultaneous plasma volume expansion have been shown to decrease the incidence of severe hypotension in large-volume paracentesis [67, 69, 70]. Studies in benign ascites due to liver disease have shown albumin to be superior to other plasma expanders in preventing circulatory dysfunction; however, such a study is lacking in malignant ascites [71].

Peritoneovenous Shunts

Peritoneovenous shunts continuously drain the peritoneal fluid to the superior vena cava, decompress the abdomen, and relieve symptoms without repeated paracentesis [72, 73]. However, these shunts can be complicated by the development of shunt occlusion, infection including sepsis and varying degree of coagulation disorder [74-76]. Although subclinical disseminated intravascular coagulation (DIC) is frequent, clinically significant DIC is seen in <2% of patients with peritoneovenous shunts [7]. The shunts also have the potential to disseminate tumor cells [77, 78], however, the clinical significance of this potential risk is unclear [79]. Though severe complications may be seen in as many as 6% of cases, the shunts continue to be an important palliative option for patients with refractory malignant ascites, who have a life expectancy of 3 or more months [62, 80, 81]. In fact, a study among patients with malignant ascites showed higher postoperative performance, earlier discharge and increased median survival with peritoneovenous shunts compared to paracentesis [82].

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Permanent Peritoneal Drains

Indwelling peritoneal drains relieve increased intra-abdominal pressure and associated symptoms without the need of repeated paracentesis. A retrospective study among patients with malignant ascites demonstrated similar rate of complications between permanent drains and repeated paracentesis [83]. Unlike peritoneovenous shunts, the drains are free of the potential risks of tumor dissemination and coagulopathy. However, the drains may be complicated by sepsis (38%), catheter blockage (30%) and significant hypotension (5%) [7, 84]. Overall, the peritoneal drains offer an alternative management option in patients, who want to avoid repeated paracentesis or the placement of peritoneovenous shunts.

Intraperitoneal Chemotherapy and Radioisotopes

Intraperitoneal chemotherapy, as opposed to systemic chemotherapy, achieves greater dose intensity within the peritoneum with possibly lesser systemic toxicity [85-89]. This is related to the extensive first pass metabolism of these drugs in the liver before the drugs enter systemic circulation. Furthermore, intra-peritoneal chemotherapy induces a fibrotic process, which may reduce peritoneal fluid accumulation. Some of the commonly utilized intraperitoneal chemotherapeutic agents include cisplatin, 5-fluorouracil, bleomycin, mitomycin C and etoposide. However, intra-peritoneal chemotherapy may have variable drug distribution and limited tissue penetration [90, 91]. Widespread intra-abdominal adhesions can lead to intestinal obstruction [92]. Several studies have shown the use of hyperthermia can improve tissue penetration and reduce resistance of intraperitoneal chemotherapeutic agents, alleviate symptomatic ascites and prolong overall survival [93, 94]. Further improvement in survival is observed when such hyperthermic intraperitoneal chemotherapy (HIPEC) follows debulking tumor surgery [95]. Additional studies are needed to determine the role and safety of this strategy in malignant ascites as well as optimal drug selection based on the primary tumor.

Radioisotopes such as radioactive gold (AU-138) and phosphorus (^{32}P) have been used intraperitoneally for the management of malignant ascites [96-98]. Though the use of these radioisotopes reduces ascitic fluid production, bowel complications including intestinal obstruction are common [99]. In fact, the use of radioactive gold is hazardous and no longer advised. Radiophosphorus is contraindicated in patients with intra-abdominal adhesions due to the risk of intestinal necrosis [100].

Novel Treatments

In recent years, several new therapeutic strategies have been investigated to manage malignant ascites. These emerging novel therapies, although not ready for clinical use, have the potential to offer additional therapeutic options in the future.

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Vascular Endothelial Growth Factor Inhibition

With recent studies elucidating a central role of VEGF in the pathogenesis of malignant ascites, VEGF inhibition is an emerging therapeutic strategy. Several animal studies have shown PTK-787, a protein blocking the tyrosine kinase activity of the VEGF receptors, to be effective in malignant ascites [101, 102]. Similarly, tumor necrosis factor (TNF) inhibits the interaction between VEGF and its receptors. The use of intra-peritoneal TNF has been shown to reduce or eliminate ascites in a phase I/II study [103] but not in another study [104]. Specific VEGF-antibodies, which neutralize the effect of VEGF, have potential to offer some benefits based on mouse models [105]. However, further human studies are needed to establish their roles in clinical practice.

Matrix Metalloproteinase Inhibitors

Inhibition of matrix metalloproteinase, enzymes that degrade the extracellular matrix, blocks angiogenesis and reduces the potential of tumors to invade and metastasize [106-108]. While batimastat is the agent most studied in malignant ascites, a newer matrix metalloproteinase inhibitor, marimastat has also been developed. Several phase I/II studies have shown significant clinical improvement with intraperitoneal batimastat [109, 110], including synergism with conventional chemotherapy [108]. Though recent clinical trials have shown no survival advantage with marimastat in colorectal or pancreatic cancer, their role in palliation of malignant ascites needs further investigation [111, 112].

Immunotherapy

Intra-peritoneal alpha or beta interferons can augment the activity of natural killer cells against the tumor cells and improve symptomatic ascites [113]. In a clinical trial, the use of intra-peritoneal interferon was well tolerated and resulted in complete or partial resolution of ascites in almost half of the recipients [114].

OK-432

OK-432 is a lyophilized powder of su-strain of *Streptococcus pyogenes* A_3, which can potentially cause regression of peritoneal metastasis and ascites through activation of cell-mediated immunity [115, 116]. In a study, OK-432 resulted in reduction in the malignant ascites in more than half of the cases, tumor shrinkage and prolongation of overall survival [117]. Improved reduction in malignant ascites may be achieved by combining OK-432 with interleukin-2 [118]. Common side effects reported with OK-432 include nausea, malaise, chills and fever [117].

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Radioimmunotherapy

A few studies have shown promising roles of monoclonal antibodies in malignant ascites [119, 120]. In a study, 2G3, an anti-mucin monoclonal antibody, given intra-peritoneally with radioactive ^{131}Iodine resolved ascites in most of the recipients, and avoided the need for paracentesis or diuretics [121].

Octreotide

Octreotide is a somatostatin analogue, which decreases peritoneal secretion and increases reabsorption of fluid and electrolytes by intestinal mucosa. It may also have anti-VEGF effect [122]. In one study, the use of octreotide relieved ascites and reduced the need of paracentesis [123].

Corynebacterium Parvum

Intraperitoneal administration of *Corynebacterium parvum* can produce fibrinous peritonitis and reduce ascitic fluid production [124]. However, further data supporting its use in malignant ascites is not available.

Prognosis

The development of malignant ascites is frequently a marker of poor prognosis. In a study, the median survival after the diagnosis of malignant ascites was approximately 6 months. The survival ranged from approximately 2 months for unknown primary cancers to 24 months for ovarian cancers. The presence of non-ovarian primary cancer, hepatic metastases and low serum albumin level were independent poor prognostic factors. Females with malignant ascites survived longer than males, but this was likely related to better prognosis associated with ovarian and breast cancers [5].

The availability of newer chemotherapy and targeted therapies, however, has improved the outcomes in more recent years. Similarly, cytoreductive surgery and HIPEC use have improved survival in primary and secondary peritoneal carcinomatosis [95] (table 8).

Table 8. Median survival in peritoneal carcinomatosis following cytoreductive surgery and HIPEC

Study	N	Underlying cancer	Median survival
Mahteme et al. [127]	18	Colorectal cancer	32 months
Verwaal et al. [128]	105	Colorectal cancer	22 months
Yonemura et al. [129]	105	Gastric cancer	19 months
Glehen et al. [130]	49	Gastric cancer	10 months
Cotte et al. [131]	81	Ovarian cancer	28 months
Yan et al. [132]	405	Diffuse malignant peritoneal mesothelioma	53 months

HIPEC indicates hyperthermic intraperitoneal chemotherapy; N number of patients.

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Conclusion

Malignant ascites, a frequent cause of significant morbidity and distress in many malignancies such as ovarian, breast, gastric and colon cancers, is often a marker of poor prognosis. The pathogenesis is multifactorial including both an increased production and a decreased clearance of peritoneal fluid. The elucidation of central role of VEGF and vascular permeability factor in the pathogenesis has important therapeutic implications. Although the presence of ascites can be diagnosed using clinical and imaging modalities, ascitic fluid analysis is needed to differentiate malignant from benign causes. Since no single test consistently and accurately identifies malignant ascites, a combination of different tests including cytology is utilized for diagnostic purposes. The preferred palliative management includes paracentesis; however, cancer-directed therapies should be incorporated in suitable patients. Novel therapy options such as VEGF inhibitors, matrix metalloproteinase inhibitors and monoclonal antibodies have shown promising results but remain investigational. High morbidity, diagnostic challenges and poor overall survival associated with malignant ascites highlight limitations of current armamentarium and needs for enhanced diagnostic and therapeutic strategies.

Conflict of Interest/Funding

None.

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Chapter 2

Targeting Cathepsin B for Cancer Therapies

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Abstract

Cathepsin B is a member of the papain family of cysteine proteases normally present in the lysosome, but it can translocate and function to degrade components of the extracellular matrix. It exhibits carboxyopeptidase, peptidyldipepidase, and endopeptidase activity. Aberrant overexpression of cathepsin B has been reported in invasive and metastatic cancers, including breast cancer, melanoma and colorectal cancer. It has been shown that oncogenic activation, such as the signaling of the ErbB pathways, can lead to cathepsin B overexpression. The degradation of the extracellular matrix is a key factor for cathepsin B to contribute to development and metastasis of tumors. An example of substrates for cathepsin B is E-cadherin, which is involved in adherens junctions, and the downregulation of E-cadherin in cancer is directly linked to invasion and metastasis. Recent studies also point to a role for cathepsin B in macrophages in the tumor microenvironment. The structure of cathepsin B is crystallographically solved, and several highly selective and potent inhibitors for cathepsin B have been developed. Yet it remains to be a challenge to demonstrate the clinical utility or benefit of any cathepsin B inhibitor. As cathepsin B is required for a cellular process called lysosomal membrane permeabilization (LMP), inhibition of cathepsin B would protect cancer cells from cell death induced by chemotherapeutic agents. It is expected that combining cathepsin B inhibitors with other approaches, such as nanoparticles, to direct the inhibition to the extracellular space may lead to better clinical approaches to treat cancers and metastasis.

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1. Introduction

Cathepsin B belongs to the cathepsin family of lysosomal hydrolases. According to their active site amino acid, cathepsins can be divided into three sub-groups: cysteine (B, C, H, F, K, L, O, S, V, W and X/Z), aspartate (D and E) and serine (G) cathepsins (Rawlings et al., 2012). The lysosome relies on these protein hydrolases and other enzymes to carry out intracellular degradation before recycling cellular constituents. In addition to its localization in the lysosome, cathepsin B can be released from the cell and function to degrade components of the extracellular matrix (Sloane, 1990). Overexpression of cathepsin B has been observed in malignant tumors and has been found to be closely correlated with an array of cancers (invasive and metastatic). Aberrant regulation of cathepsin B can lead to amplified degradation of the extracellular matrix, and thereby attributes to the infiltrative nature of tumor cells. Cathepsin B is also found to participate in various intracellular processes such as autophagy and immune response.

2. Structure and Functions of Cathepsin B

The human cathepsin B gene is located on chromosome 8p22 and contains 12 exons (Fong et al., 1991). Cysteine cathepsins are synthesized as inactive precursors. For pro-cathepsin B, it has a N-terminal domain to cover the active site and binding sites (Figure 1A). Pro-cathepsins are normally activated in the acidic environment of lysosomes, where they are initially believed to function primarily as intracellular proteases that mediate proteolysis (Turk et al., 2001). The matured cathepsin B composes of a heavy chain of 25-26kDa and a light chain of 5kDa (Frlan and Gobec, 2006). Cathepsin B is different from other cathepsins with unique enzyme characteristics. Most cysteine cathepsins are endopeptidases, whereas cathepsin B has both endopeptidase and carboxyopeptidase activity (Turk et al., 2001).

Like other cysteine cathepsins, cathepsin B shares a conserved active site that is formed by cysteine (Cys29), and histidine (His199) residues (Figure 1B). The substrate binding cleft exists next to the active site, which is controlled by the occluding loop, an 18 residue long insertion. In addition, the occluding loop contains two His residues (His110 and His111) that can interact with the C-terminus carboxylic group of the substrate peptide and facilitate the access of substrate into the active site (Mohamed and Sloane, 2006). The interaction between the two His residues and the carboxylate group explains the carboxy dipeptidase activity of cathepsin B at an acidic pH (Mohamed and Sloane, 2006). The flexible nature of the occluding loop allows for cathepsin B to act as an endopeptidase as well when the occluding loop moves from the active site cleft and cleaves internal peptide bonds (Mort and Buttle, 1997). Cathepsin B, however, is less effective as an endopeptidase compared to other proteases in the papain family due to the large amount of energy needed to alter the conformation of the occluding loop (Cygler et al., 1996).

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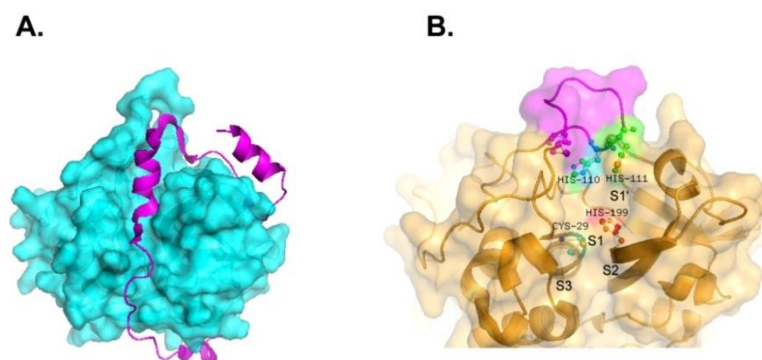

Figure 1. Crystal structures of pro-cathepsin B and cathepsin B. (A) The crystal structure of pro-cathepsin B (PDB:1MIR) (Cygler et al., 1996). The 62-residue pro-peptide region (magenta) is folded along the surface of mature cathepsin B (cygans) and covers the active site cleft. (B) The crystal structure of cathepsin B (PDB:1HUC) (Musil et al., 1991). The occluding loop (108-122) is shown in magenta on the surface of cathepsin B (orange). Two cysteine residues from the occluding loop form a di-sulfide bridge. Residues Cys29 and His199 form the catalytic dyad, and two histidines, His110 and His111, are positioned within the active site cleft. These functional residues are represented as green ball-and-stick model on the molecular surface model of cathepsin B. The S1, S2, S3 and S2' active pocket are highlighted with bold labeling. The figure is prepared with PYMOL using reported structures in PDB.

3. Overexpression of Cathepsin B in Cancers

Recent studies have revealed that cysteine cathepsins have profound functions beyond the protein turnover in normal cells and play roles in the development of heart, brain and skin (Reinheckel et al., 2001), bone resorption (Saftig et al., 1998) and antigen presentation (Shi et al., 1999).

Cathepsin B is produced constitutively, which classifies this protease as a housekeeping protein. However, it is highly upregulated in malignant tumors. Tumor cells are reported to first produce pro-cathepsin B, which is secreted but tethered to the cell surface in a Ca^{2+} dependent manner via the Annexin II tetramer complex (Mai et al., 2000). Once pro-cathepsin is converted to cathepsin B, it no longer binds to Annexin II and is released into the tumor microenvironment.

Overexpression of cathepsin B has been linked to breast, cervix, bladder, stomach, colon, ovary, bladder, lung, prostate, and thyroid cancers (Kuester et al., 2008). Levels of cathepsin B and C were significantly higher in cystic fluid of malignant ovarian tumors (Kolwijck et al., 2010). Many studies have demonstrated the association of elevated levels of cathepsin B with enhanced angiogenesis, invasion and metastasis. In colorectal carcinomas, elevated expression of cathepsin B in the tumor epithelial cells was associated with a significantly shorter survival of the patients (Campo et al., 1994). Expression of cathepsin B is usually the strongest in the advancing edge of tumors but is also detectable in stromal cells and normal epithelial cells adjacent to the tumors (Hirai et al., 1999).

By manipulating the cathepsin B expression levels with either overexpression or antisense oligos, Szpaderska et al. observed a correlation between cathepsin B levels and

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invasiveness of several tumor cell lines (Szpaderska and Frankfater, 2001). In a mouse model of pancreatic islet cell carcinogenesis, cathepsin B was upregulated together with several other cathepsins (cathepsin L, S, and C), and a significant association between increased levels of cathepsins B and L and tumor malignancy was observed (Gocheva et al., 2006). Conversely, inactivation of cathepsins B by mutation or knockout impaired tumor growth. Cathepsin B deficient mice demonstrated reduced tumor vascularity, increased tumor cell death, lowered cell proliferation and impaired tumor invasion. RNAi of cathepsin B was shown to reduce glioma cell invasion and angiogenesis both *in vitro* and *in vivo* (Gondi et al., 2004). In a study of IL-8 mediated endothelial cell migration, cathepsin B was found to be critical for IL-8 induced transactivation of EGFR (Schraufstatter et al., 2003).

We have detected significantly elevated serum cathepsin B levels in breast cancer patients (n=24) as compared with healthy controls (n=13) (Figure 2, $p = 0.015$). This observation is consistent with other reports on overexpression of cathepsin B in the cytosol of breast cancer tissue specimens and the correlation between higher cathepsin B content and risk of recurrence in breast cancer (Thomssen et al., 1995). It is not clear at this point if serum cathepsin B levels correlate with disease stages and metastasis status.

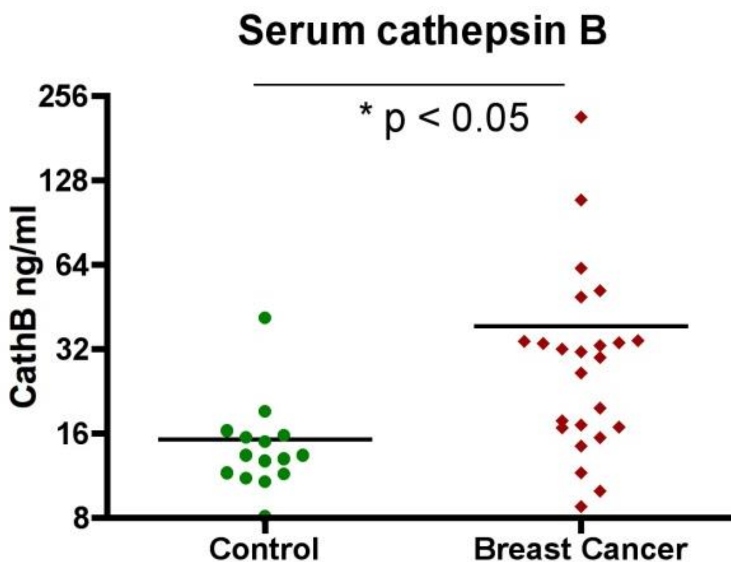

Figure 2. Serum levels of cathepsin B are elevated in breast cancer patients. Cathepsin B levels from both healthy volunteers (control) and breast cancer patients were determined by ELISA. The average levels of cathepsin B in breast cancer patients is significantly higher than that in the control ($p < 0.05$).

4. Activation of Cathepsin B in Malignant Cells

As studies have indicated that transformation of cells leads to changes in lysosomal activity, which include increases in lysosomal volume and protease activity, it is speculated that the expression of cathepsins such as cathepsin B is regulated by oncogenic pathways (Kirkegaard and Jaattela, 2009). Endogenous cathepsin inhibitors, such as stefin A (Chambers

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and Tuck, 1993), can also be regulated by oncogenes in an opposite way, but here we will only focus on the regulation of cathepsin B.

4.1. Activation of Cathepsin B by HER2 Signaling

Her2/*neu* has been implicated in the development of tumors and now the tyrosine kinase receptor has been validated as a clinical target for breast and stomach cancers (Zhang et al., 2007). Studies have shown that cathepsin B as well as cathepsin L are elevated in HER2 positive primary human breast cancer (Rafn et al., 2012). This outcome is closely related to the invasiveness of HER2-tumors. In the same study, it is also demonstrated that Cdc42-binding protein kinase beta, extracellular regulated kinase 2, p21-activated protein kinase 4, and protein kinase C alpha are essential mediators of HER2-induced cathepsin expression and breast cancer cell invasiveness. The HER2 signaling leads to the activation of myeloid zinc finger-1, which is a transcription factor that can bind to a HER2-responsive enhancer element in the first intron of the cathepsin B gene (CTSB)(Rafn et al., 2012).

HER2 and EGFR belong to the same family of receptor tyrosine kinases, and HER2 has been involved to form heterodimers with EGFR to mediate cell growth (Kokai et al., 1989). In CXCR2 positive microvascular endothelial cells (HMECs), EGF signaling is critical for IL-8-mediated cell migration (Schraufstatter et al., 2003). This process is determined to be highly dependent on the activation of cathepsin B, as inhibition of cathepsin B ablates the IL-8 effect on migration. These studies indicate that cathepsin B could be a good target to reverse oncogenic cellular changes that are induced by HER2/EGFR signaling.

4.2. Cathepsin B and Resistance to Chemotherapies

There is evidence that non-tumor cells in the tumor microenvironment can produce cathepsin B and contribute to invasion and metastasis. Indeed, in some highly invasive tumors, large numbers of myeloid cells (Gr-1^{+}CD11b^{+}) that secret cathepsin B are found at the leading edge of tumor margins (Shchors et al., 2013).

In a mammary tumor model, treatment with chemotherapeutic agent paclitaxel (Taxol) increased macrophage infiltration into tumors (Shree et al., 2011). These tumor-associated macrophages (TMAs) expressed cathepsin B and protected against Taxol-induced tumor cell death in co-culture. These macrophages also protect tumor cells against death induced by other chemotherapeutics, specifically etoposide and doxorubicin. The tumor-protective activity of these macrophages was dependent on cathepsin, as it can be fully reversed by cathepsin inhibition (Shree et al., 2011).

In addition, it is reported that chemotherapeutic agents (e.g. gemcitabine and 5-fluorouracil) trigger lysosomal permeabilization in myeloid-derived suppressor cells (MDSCs) and the release of cathepsin B directly activates the Nlrp3 inflammasome to produce active caspase-1 (Bruchard et al., 2013). As a result, MDSCs secret IL-1β and limits the anti-tumor activity of these chemotherapies.

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5. Substrates of Cathepsin B in the Extracellular Matrix

5.1. Cleavage Sites of Cathepsin B

Using a proteomic approach called “proteomic identification of protease cleavage sites” (PICS), Biniossek and colleagues have studied the cleavage sites of cathepsin B as an endopeptidase and compared the motif with that of cathepsins L and S (Biniossek et al., 2011) (Figure 3). The enzyme cleaves between the P1 and P1’ residues. According to the PICS study, cathepsin B prefers a glycine residue at the P3’ as well as the P1 position, and a phenylalanine at the P1’ position. It is noted that cathepsin B differs from cathepsin L and S by preferring an aromatic residue at the P1’ position.

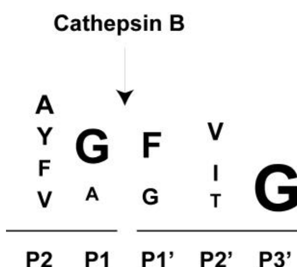

Figure 3. Motifs for cathepsin B substrates. The cathepsin B substrate peptide motifs based on the PICS study (Biniossek et al., 2011) are shown.

5.2. Active Roles of Cathepsin B in Tumor Microenvironment

Increased expression of proteinases or a decrease in levels of proteinase inhibitors have been thought to facilitate the ability of tumor cells to metastasize and invade tissues (Campo et al., 1994). In addition to degrading the basement membrane during extravasation and intravasation, proteinases can also detach cells from the primary tumor and allow the tumor cells to invade surrounding as well as remote tissues via vascular channels. Several studies have shown that cathepsin B staining is inversely correlated with basement membrane staining in lung, bladder, gastric, and colon carcinomas (Tan et al., 2013). This indicates that the confinement of tumor cells by the basement membrane could be broken by activation of cathepsin B and other proteases.

As stated above, overexpression and translocation of cathepsin B to the periphery of the tumor cell can degrade components of the extracellular matrix, mainly laminin, fibronectin, and collagen IV (Buck et al., 1992). In addition, studies have identified several critical substrates for cathepsin B that are particularly relevant to tumor invasion and metastasis.

E-Cadherins as Cathepsin Substrates

The N-terminal extracellular domains of E-cadherin proteins on adjacent cells bind to each other in a homophilic manner to establish cell-cell junctions (Beavon, 2000). E-cadherin has been identified as an epithelial marker and its expression is greatly downregulated during

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the epithelial-mesenchymal transition, a critical process in embryogenesis, and in breast cancer stem cells (Mani et al., 2008). Thus, proteolysis of its extracellular domain represents an alternative to the well-documented mechanisms of mutational inactivation or transcriptional downregulation as a means of losing E-cadherin function. Several proteases have been shown to disable E-cadherin function by proteolysis: MMP3, MMP7, ADAM10 and plasmin (Lochter et al., 1997; Noe et al., 2001; Ryniers et al., 2002; Maretzky et al., 2005). Recently, Gocheva et al. identified E-cadherin as a target substrate of cathepsin B, L, and S, but not C. At the invasive tumor front of the islet tumor, cathepsin B localization correlated with downregulation of E-cadherin (Gocheva et al., 2006).

Pro-Urokinase Plasminogen Activator (pro-uPA)

Cathepsin B and pro-uPA co-localize in the caveolae of tumor cells. Cathepsin B can activate pro-uPA to become uPA. Once activated, uPA converts plasminogen to plasmin, which subsequently activates MMPs. Downregulation of caveolin-1, the main structure protein of caveolae, leads to reduced secretion of both procathepsin B and pro-uPA (Cavallo-Medved et al., 2005). Meanwhile, downregulation of caveolin-1 also reduces degradation of the extracellular matrix protein collagen IV and the invasion of these cells through Matrigel.

Tissue Inhibitors of Metalloproteinases (TIMP)

As indicated by their names, TIMPs are inhibitors of matrix metalloproteinases (MMPs) and can prevent degradations of extracellular matrix. Cathepsin B has been shown to deplete the tissue inhibitors of matrix metalloproteinases TIMP-1 and TIMP-2 (Kostoulas et al., 1999) and thus will lead to higher MMP activity to promote cell invasion. In addition, TIMPs are also angiogenesis inhibitors, and cathepsin B and MMPs are found at sites of neovascularization in cancer as well as in osteoarthritis (Kostoulas et al., 1999). By degrading TIMPs, cathepsin B raises the activity of the MMPs, even in the absence of over-production of these enzymes, and stimulates angiogenesis. This will prepare blood vessels for invasion in cancer development and metastasis. However, the exact mechanism of each TIMP is not clearly defined since a reverse correlation of TIMP-1/TIMP-2 with MMPs was not observed in several reports (Naka et al., 2008; Sameni et al., 2008). In a prostate cell line, increased secretion of TIMP-3, but not TIMP-1 and TIMP-2, was observed when β1-integrin was knocked down and extracellular matrix degradation was decreased (Sameni et al., 2008).

6. Cathepsin B Inhibitors

Current cathepsin inhibitors are mostly active site inhibitors derived from epoxysuccinyl (Murata et al., 1991), vinyl sulfone or nitrile based compounds (Vasiljeva et al., 2007; Mirkovic et al., 2011). For cathepsin B, both epoxysuccinyl and nitrile inhibitors with potent activity have been identified, while peptide vinyl sulfone inhibitors in general have poor activity and selectivity. E-64 is an irreversible epoxysuccinyl-based inhibitor with broad-spectrum inhibitory activity to cathepsin and other proteases such as calpains. JPM-OEt is structurally related to E-64 but with more specific activity to cathepsins. This pan-cathepsin inhibitor has been shown to enhance chemotherapy regimens in a multistage cancer mouse model (Bell-McGuinn et al., 2007). CA-074, a specific inhibitor for cathepsin B, is also

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derived from the E-64 scaffold (Towatari et al., 1991). A methyl ester derivative of CA-074, CA074Me, was further developed to improve cell permeability. However, CA074Me has compromised cathepsin B selectivity and shows good inhibition against cathepsin L (Montaser et al., 2002; Mihalik et al., 2004).

The complex crystal structure of human cathepsin B with a dipeptidyl nitrile inhibitor (compound 3) was first obtained (Greenspan et al., 2001). Compound 3 had an IC_{50} of 45nM for cathepsin B and was an improvement after molecular modeling from an initial inhibitor, Compound 19, which had a weak IC_{50} of 62 μM. The high-resolution 1.9 Å crystal structure demonstrated that the nitrile inhibitor binds the enzyme at Cys29 in the active site by forming a reversible thioimidate ester intermediate. An analysis of the co-crystal structure led to the addition of a carboxylated aromatic ring (Compound 39) to form a salt-bridge with His110 in the S2' pocket of the enzyme, which improved the IC50 to 1.8 nM. Removing a bulky ring from the inhibitor close to Tyr75 in the S3 pocket of the enzyme (Compound 10) barely had any effect on inhibitory activity against cathepsin B but produced ~100 fold selectivity over other cathepsins (Greenspan et al., 2001).

Epoxysuccinyl inhibitors have also been co-crystalized with cathepsin B and the crystallographic studies confirm the binding of inhibitors to the active site. This family of inhibitors, such as E64c (Yamamoto et al., 2002), CA030 (Turk et al., 1995), CA074 (Yamamoto et al., 2000), and NS134 (Stern et al., 2004), are irreversible inhibitors derived from the natural compound E64.

These inhibitors form a covalent bond with the SH group of Cys29 in cathepsin B. Comparison of the potency of these epoxysuccinyl inhibitors also reveals that binding to both S1' and S2' sites in the enzyme is required for a potent inhibition (IC50: 20-30 nM) (Watanabe et al., 2006). CA074 (Figure 4) has a very high selectivity for cathepsin B over other enzymes since its free carboxylic acid can interact with the two histidine residues in the occluding loop structure.

7. Cathepsin B As a Target for Cancer Therapies

Although a variety of cathepsin B inhibitors have been developed and investigated for the inhibition of tumor invasion to treat different cancers, none has yet succeeded in demonstrating clinical evidence to treat cancers. However, what has been learned from the use of inhibitors to block cathepsin B function is still valuable and will provide insights to the development of clinically useful agents.

As cathepsin B has been confirmed to play a vital role in invasion and metastasis, specific cathepsin B inhibitors will be more valuable for late stage cancers. In an immunocompetent model of breast cancer with spontaneous bone metastasis, intraperitoneal administration of the highly selective cathepsin B inhibitor CA-074, but not the broad spectrum cysteine cathepsin inhibitor JPM-OEt, reduced metastasis in tumor-bearing animals (Withana et al., 2012).

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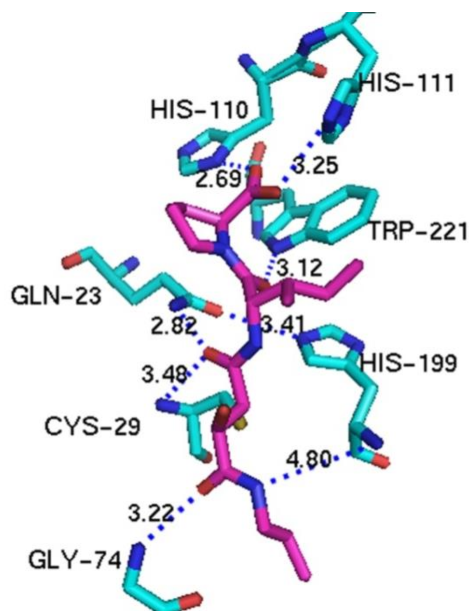

Figure 4. Interaction of CA-074 with cathepsin B. The crystal structure of the CA-074 and cathepsin B complex (PDB: 1QDQ) has revealed the mode of binding of CA-074 (magenta) to the active site (cygans) of cathepsin B. The side chain of interacting residues are labeled using the stick model. Interatomic distances (less than 3.5 Å) between the functional groups of CA-074 and cathepsin B active sites are shown as dashed blue lines. Double hydrogen bonding between the two carboxyl oxygen atoms of CA-074 and the nitrogen atoms of His110 and His111 of the occluding loop are important for cathepsin B-specific inhibition of CA-074. The figure was prepared with PYMOL based on published structures in PDB (Yamamoto et al., 2000).

Cathepsin B inhibitors may also help potentiate current chemotherapies. Since cathepsin B was reported to protect tumors against cell death induced by chemotherapeutic agents such as Taxol, combination of Taxol and cathepsin inhibitors *in vivo* significantly enhanced efficacy against primary and metastatic tumors (Shree et al., 2011).

For cathespin B inhibitors, there have been two issues that hamper the advancement of clinical application. One is the specificity of the compound. The other is the complex role of cathepsin B in cancer treatment. As we mentioned above, advances have been made for the first issue after the atomic resolution of cathepsin B crystal structures were solved in complex with its inhibitors. For the second issue, recent studies have shed light on this problem and are leading towards more promising ways to use inhibitors in cancer treatment.

It turns out cathepsin B is required for a cellular process called lysosomal membrane permeabilization (LMP). LMP could be induced by a variety of reagents including TNF and chemotherapeutic agents and will lead to apoptosis as well as necrosis of cells. In cathepsin B deficient hepatocytes, TNF fails to induce LMP (Groth-Pedersen and Jaattela, 2013). Pharmacological and genetic inhibition of cathepsin B are also able to rescue WEHI-S murine fibrosarcoma cells from apoptosis triggered by TNF or TRAIL (Foghsgaard et al., 2001). These studies demonstrate that cathepsin B is essential for TNF-induced LMP. In addition, in non-small lung cancer cells, blocking of cathepsin B activity can also prevent the appearance of multinucleated cells, which is an early characteristic of cell death induced by microtubule-

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disturbing agents. Furthermore, cathepsin was also shown to protect cancer cells against paclitaxel induced cell death (Broker et al., 2004).

Since cathepsin B plays a critical role in LMP (Kirkegaard and Jaattela, 2009) induced cell death, complete inhibition of cysteine cathepsin activity in cancer treatment may accidentally interfere with drug-induced lysosomal cell death and result in poorer responses to therapies. A better cathepsin B targeting strategy is to limit the inhibitor effect to the extracellular space or prevent lysosomal exocytosis.

7.1. Nanoparticle or Liposome Inhibitors for Cathepsin B

Mikhaylov and colleagues reported a cathepsin B inhibitor conjugated to a highly biocompatible liposomal nanocarrier (Mikhaylov et al., 2014). This inhibitor, termed LNC-NS-629, is designed to target extracellular cathepsin B from tumor and stromal cells in the tumor microenvironment. The lipidated inhibitor NS-629 can form a covalent bond with the catalytic Cys29 of cathepsin B, thereby this nanoparticle is also an irreversible inhibitor. Treating *in vivo* mammary tumor model, LNC-NS-629 shows clear strong staining on tumor cells and stromal cells. The staining in the macrophages was much stronger, possibly due to the capacity of phagocytosis in this type of cells. The staining on tumor cells was dominantly pericellular, confirming the selective targeting of extracellular cathepsin B (Mikhaylov et al., 2014). Most importantly, LNC-NS-629 was able to function as a drug delivery system. When doxorubicin was encapsulated in LNC-NS-629, its potency against tumor cells was improved 22-fold (Mikhaylov et al., 2014).

8. Pro-Drugs That Utilize Cathepsin B Activity

Doxorubicin is a popular and effective anticancer drug used to treat vast numbers of solid tumors. In its salt form, doxorubicin can disseminate into tissues and intracellular compartments through either passive diffusion or active transport (Zhong, et al., 2012). Doxorubicin must be administered at low doses due to its indiscriminative toxic effects on cells, which include but are not limited to bone marrow toxicity, cardiotoxicity, nephrotoxicity, and hepatotoxicity. Pro-drugs, derivatives of drugs that are inactive except when metabolized at the site of action, are therefore synthesized to reduce the toxicity of the drug.

Several cathepsin B cleavable pro-drugs have been developed (Zhong et al., 2013). These pro-drugs contain the tetrapeptide linker that is cleavable by cathepsin B: Gly-Phe-Leu-Gly (see Figure 3). Two pro-drugs, PK1 and PK2, have advanced to phase II clinical trials (Seymour et al., 2009).

PK1

PK1 comprises doxorubicin covalently bound to N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer by the cathepsin B cleavable linker. In phase I trial, PK1 showed better

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maximum tolerated dose (MTD) of 320 mg/m^2 (doxorubicin equivalent), which is 4-5 fold higher than that of free doxorubicin (Vasey et al., 1999). In a phase II trial in patients with breast, non-small cell lung and colorectal cancers, 6 out of 62 patients showed partial responses (PR) (Seymour et al., 2009). Compared with doxorubicin, PK1 demonstrated reduced non-specific organ toxicity in both phase I and phase II trials.

PK2

PK2 is also a HPMA copolymer with the cathepsin B cleavable linker but contains both doxorubicin and galactosamine (Julyan et al., 1999). The inclusion of galactosamine is designed to target the asialoglycoprotein receptor (ASGPR) that is selectively expressed in hepatocytes and hepatoma cell lines (Zhong et al., 2013). In a clinical trial with a total of 31 primary or metastatic liver cancer patients, 3 patients showed partial responses, with one in continuing partial remission 47 months after therapy (Seymour et al., 2002). However, study of tissue distribution revealed that more PK2 was retained in the normal liver than the tumor tissue (16.9% *vs* 3.3% of administrated dose).

Currently, neither PK1 nor PK2 is under an active development program. Clearly, the accumulation of PK2 in normal liver tissue is a serious concern. For PK1, although the pro-drug might be less toxic to normal tissues as indicated in clinical trial, the therapeutic efficacy over free doxorubicin is not obvious. As revealed in two animal models, PK1 requires co-administrated cathepsin B to release doxorubicin (Satchi et al., 2001), and PK1 alone is inferior to free doxorubicin in the xenografted tumor growth control.

9. Perspectives

In summary, due to the extensive study of cathepsin B functions and progress in the atomic understanding of cathepsin B structure, a variety of inhibitors have been developed to combat this enzyme in cancer. Since cathepsin B has some overlapping functions with other members of cathepsins, the ideal selectivity for cathepsin B inhibitors needs to be carefully defined to be broad enough to sufficiently block similar enzymes while sparing others to minimize unintended toxicity. A novel approach termed activity-based protein profiling (ABPP) was developed to screen compound libraries for activity to inhibit multiple cathepsins in defined tissues simultaneously (Sadaghiani et al., 2007). This approach has highlighted a particular compound, ASM7, which has good activity against both cathepsin B, H and X (IC_{50}: 40-70 nM), and could be useful to reevaluate available inhibitors for required specificity. In addition, combining cathepsin B inhibitors with other approaches, such as nanoparticles, may lead to better clinical approaches to treat cancers and metastasis.

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Chapter 3

Cancer Stem Cells: The New Objective for the Eradication of Tumours

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Abstract

For many years, the problem of the tumour has afflicted humanity, resulting in thousands of deaths. The main obstacle to overcome is the variability of this malignancy, which does not allow the discovery of a specific therapy to prevent cancer re-expansion and tumour metastasis. In recent years, the cancer stem cells (CSCs) theory has acquired remarkable importance. The CSCs are a subpopulation of cells with self-renewal and differentiation capacity within tumours, and exhibit high resistance to chemo and radio-therapies therefore this was thought to be the cause of the failure of current therapies. In fact, the conventional therapies target cancer cells, pertaining to tumour bulk, but are unable to destroy CSCs. However many still show several doubts about the CSCs theory and their real existence, thus new and ongoing research is being carried out on them. Herein we want to validate the CSCs theory, by analysing their features, the microenvironment in which they live, possible markers, processes and signal pathway in which they are involved. Moreover, attention is focused on new drugs which target CSCs and their respective clinical trials, confirming the effectiveness of this new approach, and the prospective of finding a specific drug that can totally eradicate tumours.

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1. Introduction

The cancer stem cells (CSCs), which are also called ‘tumour-initiating cells’, represent a small population of cancer cells that share common properties with normal CSCs including self-renewal, differentiation potentials, and long lifetime and that could be responsible for tumour initiation, rapid growth, resistance to therapy, recurrence, and metastasis [1]. The first evidence of the role of stem cells in cancer came in 1994 with a study of human Acute Myeloid Leukemia (AML) [2]. An AML-initiating cell population was identified from AML patients by transplantation into severe combined immune-deficient (SCID) mice. These leukaemia-initiating cells presented surface markers expressed as CD34+/CD38−, and they were able to recreate the heterogeneity of the original tumour through serial transplantations in xenograft models. In 2003, human CSCs were identified in solid tumours, including breast and brain cancer. The subsequent reports identified CSCs in a variety of tumours, including colon, pancreas, lung, prostate, melanoma, and glioblastoma [1].

2. The CSCs Theory and Its Characteristics

To explain the solid tumour origin and heterogeneity, two cancer models were manifested: the ‘clonal evolution’ and the ‘CSCs model’. The conventional clonal evolution model is a non-hierarchical model in which it proposed that all cells within a tumour have an equal chance of acquiring the genetic mutations necessary for stimulating tumour growth. Tumours cells gradually and stochastically acquire many mutations so that the most aggressive cells induce tumour propagation, progression and possibly therapy resistance. Instead, the CSCs model is a hierarchical model, in which only a subset of cells, or rather CSCs, at the apex of hierarchy, could propagate the growth of tumours behaving as multi-potent progenitors, having the ability to recapitulate the molecular and phenotypic heterogeneity of the original tumour. A feature of this model is its apparent unidirectional nature whereby CSCs undergo symmetric division to replenish the CSCs pool and irreversible asymmetric division to generate daughter cells (non-CSCs) with low tumourigenic potential. Recent evidence supports a new model of tumourigenicity, a ‘plastic CSCs model’ in which the existence of plasticity between the non-CSCs and CSCs compartments is denoted, so that non-CSCs could reacquire a CSCs phenotype. These bidirectional conversions are common and essential components of tumourigenicity [3].

2.1. Features of the CSCs Niche

‘Niches’ are specialized microenvironments, located within each tissue wherein stem cells reside [4]. In recent years interest has greatly increased in the concept of a niche for the maintenance of CSCs. This specific microenvironment is one of the intrinsic properties of CSCs, which protect them through the induction of a quiescent state in tissues thus avoiding the consequences of chemotherapy. Therefore, the niche is defined as the microenvironment where CSCs are located, a dynamic system with anatomic and functional features, and in which are found a variety of cell types such as mesenchymal stem and endothelial cells,

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cytokines, growth factors, and signalling pathways [1]. In addition a CSCs niche includes a specific barrier called extracellular matrix (ECM), which performs a different regulatory function and maintains the tissue structure. It plays a critical role in the resistance against chemotherapies [5]. Another important contribution to the maintenance of a CSCs niche could be its low oxygen level; in fact, hypoxia is a critical factor in the retention and self-renewal in the CSCs niche, and supports the interaction between the CSCs niche and the tumour microenvironment. Moreover, regarding CSCs generation, two different possibilities are presented: either they are originated by tumour cells, or CSCs usurp existing tissue-specific stem cell niches. The theory of nascent CSCs niches is rather problematic because there are no common anatomical characteristics among tumours, while the usurpation of niches results amply observed in early stages of cancer progression [4].

2.2. Surface Markers

With the new discoveries, it has been possible to identify some CSCs surface markers, whose expression has been revealed in many tumours, such as breast, lung, pancreas, prostate, colorectal, renal, and ovarian: these markers are CD44, CD24, CD133, CD166, and EpCAM [6].

CD44 is a multifunctional class I transmembrane glycoprotein, which generally operates as a specific receptor for hyaluronic acid, promoting migration in normal cells. It is highly expressed in every cancer cell in its standard or variant form; in fact, CD44 genes often show alternative splicing to encode different proteins, based on different cancer subset, displaying its multifaceted expression. It is mainly associated with proteins that monitor the extracellular changes and regulates cell functions such as adhesion, proliferation, growth, survival, motility, migration, angiogenesis, and differentiation. Moreover, CD44 interacts with osteopontin, collagen, laminin, and fibronectin, acts as a ligand binding receptor, coreceptor and organizer in the cortical actin skeleton, and aids the haematogenous spread, interacting with P- or L-selectins. It is also involved in numerous complex signalling cascades enhancing tumour initiations by interacting with neighbouring receptors like tyrosine kinase [6].

CD24 is a small cell surface protein molecule anchored by glycosyl-phosphotidyl-inositol in a wide variety of cancer cells. It is heavily glycosylated and coordinates cell-cell and cell-matrix interactions. CD24 was discovered in mice as a heat-stable antigen and was used as a marker to differentiate hematopoietic cells and neuronal cells. Distinct functions are attributed to CD24 based on variable glycosylation in different cells. It acts as a versatile ligand in various cells including cancer cells, controlling diverse physiological functions [6].

EpCAM (Epithelial Cell Adhesion Molecule), initially described in 1979 as a tumour associated antigen in human CRC, is a 30-40 kDa transmembrane glycoprotein, which shows high-level expression in a variety of human epithelial normal and cancer tissues. It has been also located on normal stem and progenitor cells and in cancer-initiating cells isolated from colon, breast, pancreas and prostate carcinomas. Ample evidence demonstrates that EpCAM is involved in cell adhesion, proliferation, differentiation, and migration in cancer [7].

ALCAM (Activated Leukocyte Cell Adhesion Molecule), also known as CD166, is a trans-membrane glycoprotein that belongs to the immunoglobulin superfamily, and is found in a large number of tumours including breast, lung, colon, and prostate cancer and melanoma. Moreover, a recent work shows that CD166 expression is a positive prognostic

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marker for the survival in CRC patients. In fact, CD166 seems to be involved in cell-cell and cell-matrix adhesion and its loss could cause a reduction of cell adhesion, with a higher metastatic potential of tumours [7].

CD133 is a pentaspan transmembrane glycoprotein, also known as Prominin-1. It has been identified as a marker of normal stem cells in several organs and cancer population cells in many tumour types including brain, pancreas, colon, lung, and prostate. Moreover it has being reported that CD133-1 and CD133-2 were both expressed in human ovarian tumours at higher frequency than in normal ovaries and metastatic omental lesions. CD133-1 and CD133-2 may be useful, therefore, to select the population of CD133+ ovarian tumour cells that are characterized by a higher clonogenic efficiency and proliferative potential [8,9].

2.3. Genes Involved in the Regulation of CSCs

In addition to cell surface markers, many investigators have focused their attention on the overexpression of certain genes normally present in progenitor cells [10], because it is possible that these genes participate in the development and regulation of tumour and hyperplastic tissues [11].

Among these, **Octamer 3/4** (Oct 3/4), a member of the **POU** (Pituitary-specific Pit-1 family; Octamer transcription factor proteins Oct-1 and Oct-2; Neural Unc-86 transcription factor) is considered to be an important stem cell marker and essential transcription factor during human embryogenesis. In recent years, the Oct-4 expression has been found in human breast, liver, pancreas, colon, kidney, gastric cancers, and bladder tumours.

Another gene considered is **Sox2**, a member of the Sox gene family that encodes transcription factors with a single HMG DNA-binding domain. Sox2 controls neural progenitor cells, blocking differentiation and is expressed in several malignant tissues.

For its function and in conjunction with other factors such as Oct-4 and Sox2, **Nanog** is believed to form an embryonic SC identity, and its expression as breast carcinoma, and osteosarcoma in human neoplasms has recently been noted.

Nucleostemin is expressed at high levels in human placental tissues, and in many cancer tissues [12]. It seems to be localized in the nucleolus, but in some cancer cell lines nucleostemin exhibited both cytoplasmic and nucleolar localizations. This distribution of nucleostemin has recently been observed in gastric, prostate, and renal cell carcinomas.

Bmi is a potent repressor of the retinoblastoma and the p53 pathways. Its role in tumourigenesis is very important; indeed, it is a transcriptional repressor that belongs to the polycomb-group family of proteins, which are involved in haematopoiesis, regulation of proliferation, and senescence. Moreover, the Bmi gene is widely expressed in diverse human tumours including lymphomas, bladder and breast carcinomas, and neuroblastoma.

Zfx is a zinc finger protein of the Zfy family and normally suppresses apoptosis in embryonic stem cells; the Zfx gene is amply expressed in the glioma tissue and cell lines. In addition, it can directly activate cell type-specific targets such as Tbx3 and Tcl1 in embryonic stem cells.

Tbx3 seems overexpressed in breast, liver, and ovarian cancers and also in endometrioid adenocarcinomas.

T-box genes encode transcription factors involved in the regulation of the cancer developmental process.

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Tcl1 has been extensively studied as an oncogene in T-cell leukemia and plays an important role in early mouse embryos and embryonic stem cells (ESCs).

Esrrb in coordination with Nanog and Oct-4 activate the internal system of ESCs.

Dppa4 protein is located in the embryonic stem cell nucleus and is associated with chromatin. It regulates the differentiation of ES cells into a primitive ectoderm lineage, and its expression in cancer requires further investigation.

The expressions of Dppa4, Esrrb, Tcl1, Tbx3, and Zfx in human bladder, colon, and prostate cancer cell lines have been reported [12].

2.4. Signal Pathway

Since CSCs possess many common features with normal stem cells, it has been thought that likewise to them, CSCs had a possible connection with signalling pathways, such as Wnt, Hedgehog (Hh), and Notch, found in neural stem cells (NSCs). Aberrant genetic mutations, due to several environmental factors and carcinogens such as cigarette smoke, radiations, and reactive oxygen species (ROS), may cause the reprogramming of the epigenetic machinery and vast changes in the DNA structure. This could deregulate the genes involved in these signalling pathways with consequent hyperactivation, causing accordingly tumourigenesis [7].

WNT family are a family of growth factors which bind to G protein coupled receptors of the Frizzled (Fzd) family and other coreceptors such as the low-density lipoprotein (LDL) receptor-related, also known as LRP 5/6. In canonical Wnt signalling, Wnt binding to Fzd activates dishevelled (Dsh), inactivating GSK3β, and stabilizes the transcriptional co-activator, named β-catenin, by inhibiting its degradation and the phosphorylation steps that lead to the ubiquitination and transport to the proteasome. Thus, β-catenin can operate a regulation of genes expression, especially for activities such as cell proliferation, survival, and differentiation, replacing transcriptional repressors in the nucleus. Additionally, the Wnt pathway operates in establishing the mitotic spindle, and can regulate asymmetric cell division that may lead to the preservation of an adult SC and the production of differentiated progeny [11]. Many studies have shown an important role of Wnt signalling in CSCs; in fact, it promotes genomic instability after irradiation, thus allowing the tumour cells to survive and to develop additional adaptive mutations, and possibly promotes the conversion of normal stem cells to CSCs in gliomas. Moreover, Malanchi et al. ablated the β-catenin during *in vivo* tumour formation in dermal CSCs and this arrested tumour growth. In summary, the role for Wnt signalling in CSCs is far reaching and most certainly complex because the same pathway can promote differentiation and elsewhere, proliferation in normal SCs.

The **Hedgehog** pathway regulates important functions during embryogenesis through the secretion of morphogenes, called Hh-type ligands, such as Indian hedgehog (Ihh), desert hedgehog (Dhh), and Sonic hedgehog (Shh), which is the most important in the regulation of CSCs. Shh bind to Patched (Ptch1), a transmembrane receptor, which is thus released and inhibits Smoothened (Smo), a seven transmembrane domain receptor, allowing the transduction of the Shh signal. It results in the nuclear translocation of cytoplasmic transcription factors of the Gli family to modulate the target gene expression. HH signalling has been associated with diverse tumour histology and their CSCs, in particular breast cancer, glioma, basal cell carcinoma, gastric cancer, and colon carcinoma. CSCs express functional

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HH components and the modification in this signal pathway could cause sphere formation and/or tumourigenicity.

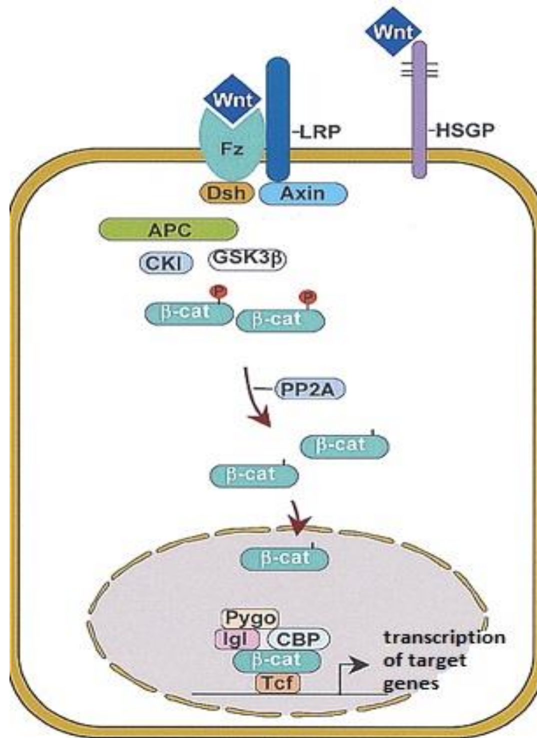

Figure 1.

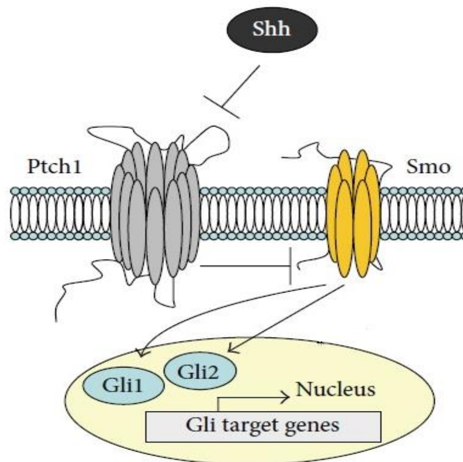

Figure 2.

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The **Notch** pathway is involved in developmental processes, especially with respect to the morphogenesis cell through two important bound membrane receptors, Delta and Notch. It is a cell-cell communication system in which cells expressing a transmembrane receptor bind cells expressing membrane-associated ligands, which are members of the Delta-like and Jagged families. The bond with the receptor leads to the release of the intracellular domain of Notch (NICD) by the action of proteases, TNF-α converting enzyme and g-secretase, which regulate the cell fate. Once in the nucleus, it causes the depression of transcription factors and other epigenetic regulators, promoting target gene expression [11]. An up-regulation of Notch signalling correlated with CSCs has been revealed in various solid and hematologic tumours such as acute myeloid leukemia, breast cancer, pancreatic cancer, embryonal brain tumours, and colon carcinoma. Moreover, Notch signalling seems be in intimate contact with both Wnt and HH signalling. Its up-regulation could promote differentiation interceding at certain points, such as in the inactivation of glycogen synthase kinase 3b, a kinase that plays an important role in suppressing Wnt and HH, therefore promoting tumourigenesis. Hence, it is vitally important to study this pathway since Notch signalling may be considered as oncogenic or suppressive.

These are the well-known signalling pathways but others have been found such as the **PI3K/AKT** signalling pathway, which is involved in numerous cancers, including acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). AKT is a serine-threonine protein kinase, which is implicated in survival signalling in a wide variety of cells, including fibroblastic, epithelial, and neuronal cells [13]: its activation is very important for cellular transformation and in the tumourigenesis. In fact, it has been observed that AKT1 containing an E17K mutation could increase the protein levels of Bcl-2 and the phosphorylation of the proapoptotic protein BAD, resulting in enhanced resistance to apoptosis [14].

Another important member of this pathway is **PTEN** (phosphatase and tensin homolog deleted on chromosome 10, also called MMAC1 or TEP1) a tumour suppressor gene, which is frequently deleted or mutated in a wide range of human cancers, including glioblastoma, melanoma, and prostate, breast, and endometrial cancers. PTEN increases sensitivity to cell death in response to several apoptotic stimuli by negatively regulating the PI3K/AKT pathway [13] and also inhibits growth factor induced Shc phosphorylation and suppresses the MAP kinase signalling pathway. Hence, hyperactivation of AKT causes resistance to apoptosis; increases cell growth, cell proliferation, metastasis, angiogenesis, and cellular energy metabolism.

The **JAK/STAT** signalling pathway is also involved in tumour initiation. Aberrations in the JAK/STAT pathway have been revealed in many cancers, especially leukemia.

Indeed v-Abl, a tyrosine kinase dependent to JAK/STAT pathway, could induce the malignant transformation of pre-B cells, activating Pim-1 and Pim-2, two kinases, which play important roles in cellular transformation. In addition, the JAK2-V617F mutation has been shown to be a factor which contributes to the malignant transformation of hematopoietic cells [14]. An important mechanism for a negative regulation of the JAK/STAT signalling pathway is mediated by proteins that belong to the suppressor of cytokine signalling (SOCS) family, SOCS-1 and SOCS-2 especially seem to be involved in the inhibition of JAK/STAT signalling pathway activation, being inhibited by the phosphorylation of several oncogenes such as Bcl-Abl, a tyrosine kinase that causes CML. Therefore, SOCS proteins have been considered necessary for tumour formation for their regulatory effects on JAK/STAT pathway [14].

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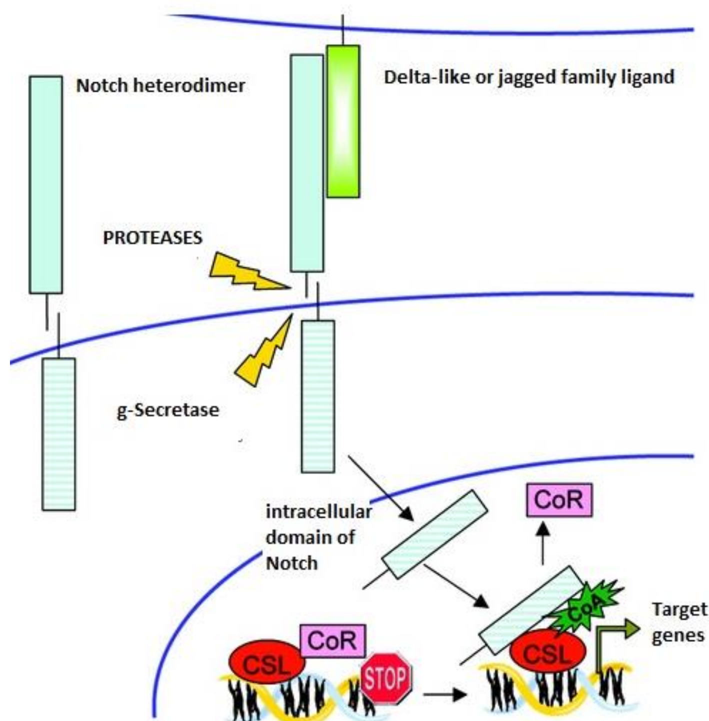

Figure 3.

NF-κB (Nuclear factor kappa B) is a transcription factor that is involved in the expression of several apoptosis related proteins, such as Bcl-xL, Bcl-2, survivin, cellular inhibitors of apoptosis (cIAPs), TRAF, and cell cycle regulatory components. An up-regulation of NF-κB could cause cancer development and progression, chemoresistance, chronic inflammation, and autoimmune diseases [14].

3. Identification Methods of CSCs in Solid Tumours

In order to better understand the characteristics of the CSCs and evaluate their role in tumourigenesis, it is necessary to isolate them from the heterogeneous tumour cells, using the following different techniques:

a) Isolation by flow cytometry according to CSCs-specific cell surface markers.
b) Detection of side-population (SP) phenotypes by Hoechst 33342 exclusion.
c) Determination of ability to grow as floating spheres in serum-free medium.
d) Assessment of aldehyde dehydrogenase (ALDH) activity [15].

Obviously, no absolute method exists to isolate the CSCs of each solid tumour, in view of the heterogeneity of cancers.

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a) The identification and isolation of these CSCs using surface markers have been an important step in the research of cancer, because has confirmed the CSCs theory. However, the identification of specific CSCs surface markers for each tumour type requires further investigations. In fact, it is obviously difficult to discover unique markers because of the heterogeneity of cancer cells, and in addition surface markers show variable expression levels at different stages of a tumour.
b) SPs are considered as cell subsets exhibiting CSCs properties with a distinctly low Hoechst 33342 dye staining. Goodell et al. first identified this population of cells in 1996. They noticed that on analyzing a distinct population of murine bone marrow cells, some internal cells stained poorly with Hoechst 33342. These cells were termed SPs due to their position in the flow cytometry dot plot, and they seem to possess the majority of the characteristics of hematopoietic stem cells. Moreover, the researchers discovered that the cause for the low Hoechst staining was the efflux of the dye mediated by ATP-binding cassette (ABC) transporters. SPs have been used to identify both normal cells and CSCs but this technique has some defects, such as toxicity of the dye [15].
c) A characteristic of CSCs is their ability to form colonies from a single cell more efficiently than their progeny and to grow as spheres in non-adherent culture conditions. To assess clonogenic potential, assays on forming colonies are carried out, starting from a single cell that is plated in soft agar. Colonies derived from CSCs, usually after 21 days of incubation, are stained with crystal violet or nitro-blue-tetrazolium (NTB), counted and measured and compared with colonies derived from non-CSCs fractions. Generally, colonies derived from CSCs result greater in number and size than colonies derived from non-CSCs [15].
d) ALDH is a cytosolic isoenzyme involved in the detoxification of intracellular aldehydes through the oxidation of retinol to retinoic acid, conferring resistance to chemotherapeutic agents, such as cyclophosphamide. If the levels of ALDH1A1 decreased, it would cause an increase of the tissue levels of acetaldehyde, a potent carcinogen, with a greater risk of tumour formation. High ALDH activity has been shown in CSCs of breast, colon, and lung cancers. Visus et al. were the first to highlight a possible use of ALDH1A1 as a CSCs marker in HNSCC [15].

4. The Role of Micro-RNA in Tumourigenesis

MicroRNAs (miRNAs) are a large family of small non-protein-coding RNAs with about 18-24 nucleotides in length, which act as potent post-transcriptional regulators of genes, binding to their specific binding sites in the 3’ untranslated region (3’-UTR) of their target mRNAs and causing the degradation of mRNA or the inhibition of protein translation [16]. miRNAs are amply recognized as regulators of the expression of, at least, one-third of human mRNAs, and hence are involved in a wide range of biological processes, such as cell differentiation, proliferation, cell death and metabolism. Recent studies have suggested that an altered expression of miRNAs might be involved in tumourigenesis, associated with a clinical prognosis of tumour, resistance to chemo-radiation therapy and metastasis. The most important miRNAs are:

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Let-7: It has been shown that the let-7 family could be involved in tumour development and progression; in fact, let-7 acts as a tumour suppressor by repressing several oncogenes and targeting many transcription factors that play important roles in the regulation of the cell cycle, cell differentiation, and apoptosis. Recent research has revealed the underexpression of let-7 or even the deletion of the chromosomal region of human let-7 in many cancers [17]. Hence its level expression could be an effective marker to reveal tumour formation, and its restoration to normal levels could be a new therapeutic strategy. This has been shown, for example in breast CSCs in which forced over expression of let-7a inhibited cell proliferation, mammosphere formation, tumour formation, and metastasis in the mouse xenograft tumour, and also led to a reduction of undifferentiated cells *in vitro* [16]. Moreover many of the factors controlling the expression of let-7, (as LIN28, POU5F1, SOX2, NANOG, TLX1, HMGA2, MYC, and IMPs) form a double negative feedback loop which plays an important role in the maintenance of pluripotency and in cancer progression. Indeed to demonstrate this concept, it has been proposed that during carcinogenesis, the down-regulation of let-7 leads to the up-regulation of oncofetal genes such as HMGA2 and LIN28, which are otherwise not expressed in adult tissues, and this reprogramming promotes dedifferentiation and cancer progression [17]. In addition it has also been showed that let-7 inhibits the expression of the enhancer of zeste homolog 2 (EZH2). This is a major epigenetic component of the polycomb repressive complex 2 (PRC2) that functions as a regulator for maintaining the phenotype and function of CSCs [16]. It also has been shown that let-7 targets protooncogene N-Myc, inhibit the proliferative and clonogenic growth of neuroblastoma cells.

miR-21: It has been demonstrated be an oncogenic miRNA, which targets multiple signalling pathways, and clinical studies proved the increased expression of miR-21 in a wide variety of tumours such as cancer of the pancreatic, prostate, breast and brain. It also has been demonstrated that the miR-21 has antiapoptotic, proliferative, invasive and angiogenic effects on cancer cells, and its expression is increased in CSCs. For example, breast CSCs show the increase expression of miR-21 and HIF-1α, a potential positive mediator of CSCs phenotype and function. Hence, it may be possible to contrast tumour growth with the use of inhibitor of miR-21: the functional loss of miR-21 and consequent decreased formation in pancreatospheres of human pancreatic cancer cells has been demonstrated with this treatment [16].

miR-22: It also appears to play an important role in tumourigenesis: the expression of miR-22 has been demonstrated in a variety of cancer cells as those of colon, liver, ovarian, and breast in low levels [16]. It seems that it acts as a tumour suppressor. In fact, the over-expression of miR-22 reduces cell growth, invasion, and metastasis in several cancer cells by targeting PTEN, p21, and p53. However, a recent study has revealed a potential role of miR-22 as an oncogene. The analysis of the relation between PTEN and miR-22 with UV led to the over-expression of miR-22-protected cells to apoptosis by repressing the PTEN expression, which caused the hyperactivation of AKT [18] and inactivated caspase signalling cascade, leading to increased cell survival upon UV radiation. Therefore, the use of a miR-22 inhibitor may be able to rescue the PTEN repression and promote cell apoptosis.

miR-26: It is considered to be a tumour suppressor. In fact several reports have shown that miR-26a modulates the cancer epigenome by repressing the polycomb group protein EZH2, a potential regulator of CSCs characteristics, associated with tumour angiogenesis, the self-renewal capacity, the genic expression of CSCs, and tumour metastasis. Its down-regulation, resulting in the reexpression of miR-26a, causes the inhibition of tumour

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metastasis and invasion. Moreover, other studies have found that the reexpression of miR-26a caused the down-regulation in the expression of EZH2, Oct-4, Notch-1, and EpCAM in pancreatic cancer cells, which are important factors involved in the regulation of the CSCs phenotype. Therefore, targeting miR-26a shows a novel therapeutic approach for cancer treatment [16].

miR-34a: Evidence clearly suggests that miR-34a functions as a potent suppressor of tumourigenesis, inhibiting cell survival, proliferation, invasion, and metastasis through the activation of p53 and inactivation of Cyclin D1, E2F1/2, and CDK6 in cancer. It has shown that the re-expression of miR-34a decreases the expression of some CSCs genes such as CD44, CD133, and Notch-1. This causes the down-regulation of the CSCs self-renewal capacity in various cancer cells, and the inversion of the EMT phenotype by the down-regulation of the EMT mesenchymal markers ZEB1, Snail, and Slug. Moreover, clinical evidence shows that miR-34a decreases the formation of pancreatospheres by inhibiting the expression of CSCs cell surface proteins CD44 and EpCAM. These data suggest that miR-34a is very important in CSCs regulation [16].

miR-101: It is considered as a tumour suppressor. Several *in vitro* and *in vivo* studies show that miR-101 plays a protective role in tumour aggressiveness through the inhibition of EZH2, an epigenetic regulator of CSCs [16]. The loss of miR-101 expression by its inhibitor could cause the activation of the EZH2 signalling network, increasing the capacity of CSCs self-renewal and leading to the tumour aggressive phenotype. However, the precise role of miR-101 in CSCs characteristics during tumour development and progression requires further in depth investigation.

miR-146a: It seems to function as a potent tumour suppressor through the decrease NF-κB activity, suppressing the expression of NF-κB target genes such as IL-1β, IL-6, IL-8, and TNF-α. In fact, several studies have showed that the activation of NF-κB signalling is involved in the enrichment of CSCs characterized by the regulation of CSCs genes such as Nanog, Sox2, and Lin28. Recently it has been demonstrated that the reexpression of miR-146a in pancreatic cancer cells, in which miR146a was lost, causes decreased capacity of tumour cell invasion, consistent with the inactivation of EGFR and NF-κB and the down-regulation of NF-κB targets. However, one study showed that oral squamous cell carcinoma tissue samples had high levels of miR-146a and this caused increase of the oncogenicity. These results suggest that the role of miR-146a appears to be cell lineage specific [16].

miR-200: miR-200 family members seem to be involved in the development and progression of tumours. In fact it has been shown that miR-200 decreases the expression of Bmil-1, Suz12, and Notch-1, regulators of CSCs and EMT phenotypes and functions in various cancer cells, causing the inhibition of CSCs development. It has also been reported that the expression of miR-200 is lowered in a vast variety of tumours such as prostate, pancreatic, colon, gastric, and breast cancers, and this reduction is often caused by drug-resistance. Hence, these data clearly suggest that the miR-200 family plays a key role in the regulation of CSCs [16].

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5. The Role of Epithelial-to-Mesenchymal Transition (EMT)

It has been extensively demonstrated that EMT play an important role in the increase of tumour aggressiveness and metastasis formation [19]. Primarily Epithelial-to-Mesenchymal Transition (EMT) was retained as a very essential step of embryogenesis, which is a vital process for morphogenesis during embryonic development. The process of EMT involves a disassembly of cell-cell junctions, actin cytoskeleton reorganization, and increased cell motility and invasion. This happens due to down-regulation and relocation of E-cadherin and zonula occludens-1 (ZO-1), as well as down-regulation and translocation of β-catenin from the cell membrane to nucleus, and up-regulation of mesenchymal molecular markers such as vimentin, fibronectin and N-cadherin. During the processes of EMT, non-motile epithelial cells lose their cell-cell junctions and adhesion, and convert themselves into individual, motile and invasive mesenchymal phenotypic cells. Recently this process has also been associated with the conversion of early stage tumours into invasive cancer metastasis [19]. This idea that EMT is relevant in cancer was initially seen with skepticism but new evidence has demonstrated that the process of EMT is vitally important in the development of cancer, because tumour cells acquire the capacity to infiltrate surrounding tissues and break the structural constraints imposed by tissue architecture, thus metastasizing in distant sites. In fact, in the tumour centre cells keep the expression of E-cadherin and cytoplasmic β-catenin, while the tumour cells in the periphery display loss of surface E-cadherin and the up-regulation of vimentin as well as nuclear β-catenin staining, the typical characteristics of the EMT phenotype. Moreover recent evidence suggests that the process of EMT is caused by a molecular interaction between extracellular signals such as collagen and growth factors including transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) A, B, and D. In addition CSCs generated from EMT induction, are well known to be highly drug resistant. It is necessary to identify the factors triggering EMT and uncover their mechanistic role during tumours progression, in order to develop novel and targeted therapies for the complete eradication of cancer [19].

6. The Drug-Resistance of CSCs

CSCs exhibit several genetic and cellular mutations that often confer resistance to classical therapeutic approaches. Radiotherapy and most types of chemotherapy exert their antineoplastic function by disrupting cancer cell DNA integrity. Therefore, it is possible that the oncogenic resistance of CSCs is caused by the increased expression of DNA integrity-maintenance systems [11], and the increased expression of drug efflux pumps, which may promote oncogenic resistance against cytotoxic chemotherapeutic agents. One of the most important factors of CSCs, associated with relative radioresistance, is their quiescent status. In general, quiescent cells stand in late S phase, less radiosensitive than cells in G2/M phase, and it has been shown that the loss of tumour bulk due to radiation, leads to the reactivation of the CSCs [20]. Other important factors, which increase resistance include efficient DNA repair: normal stem cells possess well-fortified DNA mutation defence systems that serve to

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prevent potential mutations. Unfortunately potential mutations can happen, originating the putative CSCs, which result very resistant to DNA damage. In fact they used the above mentioned defence system to save themselves from DNA-targeting chemo- and radio-therapy, repairing damages more rapidly [11]. Some modulators of CSCs resistance to DNA damage belong to the family of checkpoint kinases 1/2 (Chk 1/2 kinases), which are activated after genotoxic stress and arrest the cell cycle, allowing DNA repair. These kinases have higher basal and inducible activities in CSCs than in non-stem cells [11]. In addition to increase DNA repair systems CSCs may also exhibit changes in telomerase function, which is an enzyme that maintains telomeric repeats at the end of chromosomal strands, causing resistance to chromosomal degradation. Telomerase function was recently shown to be down-regulated in brain CSCs and several drugs that interfere with the telomerase function are already in clinical trials. Moreover, high expression levels can be found in the CSCs of O6-methylguanine-DNA-methyltransferase (MGMT), which repairs O6-alkyl lesions in DNA, rendering CSCs resistant to DNA alkylating agents such as temozolomide (TMZ) [5]. Another form of resistance is the resistance to drug penetration. Usually in normal stem cells, there is a relatively high expression of efflux transporters of the ATP-binding cassette (ABC) gene family. These pumps allow normal stem cells to preserve their genome against chemical mutagens and can function as receptors, channels, and multidrug transporters. ABC membrane transporters use ATP to efflux small endogenous molecules such as ions, peptides, bile acids, and cholesterol across cell membranes [11]. In CSCs, there are also high expressions of these pumps, which detoxify cells by expelling cytotoxic agents such as chemotherapy drugs, suggesting a mechanism for drug resistance [15]. Therefore, drugs that block these transporters or down-regulate their expression and function may be able to destroy CSCs.

Moreover, a very important factor also is the resistance to apoptosis. The activation of the Akt pathway [11] and the amplification of apoptosis inhibitor proteins could be the key to the CSCs resistance. This fact can be verified in chemo-resistant hepatocellular carcinoma CSCs, which showed resistance to apoptosis, activating Akt/PKB and bcl-2 cell survival pathways. This suggests that the characterization of Akt and bcl-2 expression in CSCs may have significant clinical utility. Another promising molecular target to promote apoptosis in CSCs is the nuclear factor NF-κB, a transcription factor believed to be intricately involved in the development and progression of certain cancer types. Even the microenvironment plays an important role, especially the oxygenation status of CSCs during radiation. It has long been postulated that areas of low oxygen concentration within tumours create microenvironments that are relatively protected from radiation-induced damage. However it was unexpectedly discovered that CSCs reside along perivascular areas, and so they are well oxygenated, because the hypoxic state, typical characteristic of the CSCs niche, stimulates oxigensensitive transcriptional factors, called hypoxia-inducible factors (HIFs), which serve to prevent cell differentiation, promoting aberrant angiogenesis and regulating apoptosis. HIFs induce enzymes to repair DNA and induce the development of tumour cells resistant to DNA-targeting therapeutic agents [1]. This approach may help to explain the efficacy of antiangiogenic therapies such as bevacizumab, because theoretically these therapies induce hypoxia conferring radio-resistance. Still, it presumes that radiations might be more efficacious if any antiangiogenic therapies are administered beforehand [11].

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7. Therapeutic Implication

In spite of the numerous uncertainties of the existence CSCs hypothesis, it is evident that in cancer there exists a portion of cells named exactly CSCs, on which depends the growth and survival of tumours. Therefore the new therapeutic strategies aim to complete eradication of this subpopulation within cancer. In fact, the suppression of residual CSCs after initial tumour debulking may be curative. This approach sustains remissions, and increases the survival of patients receiving CSCs suppressive therapy. Hence, it appears that specific CSCs-targeted therapies could be an effective complement to conventional treatments such as surgery, chemotherapy, and radiation therapy, which damage only the tumours bulk sparing CSCs, responsible for cancer regeneration and the resulting cancer recurrence and metastasis. This happens because CSCs possess the ability to develop resistance after induction chemo and radiation therapy due to their heterogeneity, which represent the real obstacle. In fact, the identification of a specific drug is complicated because of the continual mutations that cancer may exhibit fluctuating phenotypes, frequencies, and biological properties within an individual patient. Therefore, the investigation on drugs against CSCs shows the necessity to recognize factors, intrinsic and extrinsic, which are involved in the maintenance and regulation of CSCs such as the signal pathway, various markers, oncogene genes, and microenvironment, thus finally with the hope to eradicate tumours [11]. Nowadays, further investigation in strategies targeting CSCs is being carried out, an arduous way in which there are not yet absolute certainties but only many theories. In the last fifteen years, many agents have been evaluated and some of them, although not yet approved, have shown good effects in the inhibition of the CSCs self-renewal, both *in vitro* and *in vivo* models. Moreover, it has often been observed that a combination of these agents and conventional chemotherapy drugs can significantly inhibit tumour growth, metastasis, and recurrence.

7.1. Inhibitors of CSCs Signal Pathways

As we have seen previously, Wnt/β-catenin, Hedgehog, Notch, JAK/STAT, and PI3K/AKT are the most important signalling pathways, with which CSCs can act. Therefore, in order to inhibit and kill them, many drugs act on these signal pathways, by down- or up-regulating, or inhibiting some factors involved.

7.1.1. Metformin

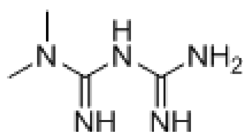

Figure 4.

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Metformin (1,1-dimethylbiguanide hydrochloride) is a FDA approved biguanide anti-diabetic drug, derived from French lilac (*Galega officinalis*), a plant used in folk medicine for several centuries. In addition to its function as a gluconeogenesis suppressor [21], it has recently been shown that metformin, both *in vivo* and *in vitro* models, could inhibit cancer cell growth of breast, ovarian, endometrial, prostate and pancreatic cancers [21]. In many preclinical studies, it was observed that metformin caused the decrease of proliferation, the induction to apoptosis, cell cycle arrest, and the reduction incidence and growth of potential tumours *in vivo* [22]. Additional confirmation to this observation has been shown in some reports that metformin improved the response of human breast tumour xenografts to conventional chemotherapy by eradicating CSCs in the tumour. In a recent study by Hirsch et al., contrary to the chemotherapeutic drug doxorubicin, metformin selectively killed CD44high/CD24low breast CSCs while it had less effect on CD44low/CD24high breast non-CSCs cells [21], and had synergistic effect with doxorubicin on killing breast CSCs *in vitro* and *in vivo*. In fact, the treatment with doxorubicin and metformin in combination rapidly decreased the CSCs proportion to undetectable levels. After the block of treatment, the animals remained in tumour remission for at least 60 days, meaning that the tumours had been eliminated. Moreover, it had been demonstrated that metformin could also act synergistically with FuOx, a combination of 5-FU and oxaliplatin, which is the mainstay in colon cancer chemotherapy. This combination appears to block the growth of colon CR cells *in vitro* and *in vivo*, as well as their migration via down-regulating miR21, and inhibits the Wnt/b-catenin signalling pathway, which plays a critical role in the growth and maintenance of colon spheres, and increase cell death [22]. Moreover, it has been shown that in cancer cells, metformin could cause the activation of the AMP-activated protein kinase (AMPK) enzyme [21], arrests cell cycle, decreases cyclin D1 expression, increases p21 protein expression, attenuates mTOR-S6RP phosphorylation, inhibits protein-translational and lipid biosynthetic pathways, and disrupts crosstalk between the insulin/IGF-1 receptor and GPCR signalling pathway. The correct dose for metformin to kill CSCs in humans is unknown, so although metformin is a promising agent in targeting CSCs, more research is necessary.

7.1.2. Salinomycin

Salinomycin, a member of the family of the monocarboxylic polyether antibiotics [23], is a highly selective potassium ionophore and an antibiotic extensively used for coccidiosis [21].

It has been shown to selectively kill breast CSCs. In a study by Gupta et al., among different chemical compounds, salinomycin was observed to be able to target CSCs, especially CSCs of breast cancer. It killed breast CSCs at least 100 times more effectively than paclitaxel in mice. In an experimental study on breast cancer using immortalized Human Mammary Epithelial Cells (termed HMLER) with knockdown of the E-cadherin, the generation of cells that displayed characteristic properties of CSCs was observed. They were capable of forming tumour spheres in suspension cultures, showed high CD44 and low CD24 expression, and exhibited resistance to chemotherapeutic drugs and cytotoxic agents, such as paclitaxel, doxorubicin, actinomycin D, campthotecin, and staurosporine [23]. Only salinomycin selectively reduced the ratio of CD44high/CD24low CSCs in cultures of mixed populations of HMLER-shEcad cells. Moreover, it inhibited tumour sphere formation, eliminated ALDH1+ and Sox2 expressing breast CSCs from tumour spheres, and supported tumour regression and depletion of breast CSCs in MCF-7 xenograft mice. Furthermore, the

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tumourigenicity of breast cancer cells, pre-treated with salinomycin, decreased more than 100-fold compared with cells pretreated with paclitaxel.

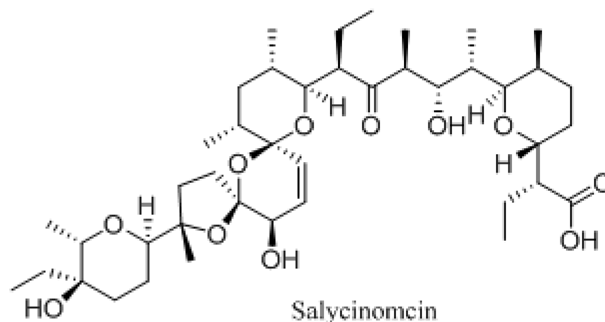

Figure 5.

In animal models, salinomycin intraperitoneal injections inhibited mouse xenografts' growth, induced apoptosis, necrosis, epithelial differentiation and suppressed stemness gene expression of cancer cells. In addition, salinomycin could overcome ATP-binding cassette (ABC) transporter-mediated multidrug resistance and apoptosis resistance of KG-1a cells (human leukemia stem-like cells), restoring the sensitivity of these cells to chemotherapeutic drugs [21]. Salinomycin was first therapeutically administrated in man in 2010, in the context of a pilot clinical trial with a small cohort of patients with metastatic breast, ovarian, and head and neck cancers. With intravenous administration of 200–250 μg/kg every second day for three weeks resulted in partial regression of tumour metastasis and showed fewer side effects than chemotherapy [21]. Moreover, salinomycin inhibited tumour sphere formation and expression of Oct-4, Nanog, and Sox2 of A549 in lung adenocarcinoma CSCs. It also blocked the growth of CD133 expressing pancreatic CSCs in tumour spheres formation assays, and in combination with gemcitabine, a conventional cytostatic drug that inhibits the growth of CD133-non-CSCs it eliminates the engraftment of human pancreatic cancer in xenograft mice more effectively than the single agent. Moreover, salinomycin first appeared to induce apoptosis and inhibit Wnt signalling pathway in CLL (Chronic Lymphocytic Leukemia) cells by reducing the levels of the Wnt co-receptor LRP6 and by down-regulating the expression of the Wnt target genes LEF1, cyclin D1, and fibronectin. Systemic salinomycin treatment induces apoptosis in metastatic cells, causing regression of metastases, partial clinical response, and decrease of the tumour markers in Ca 15-3 and CEA cells lines in metastatic triple negative invasive ductal breast cancer. However, it is not at present clear whether salinomycin will be used routinely for the treatment of cancer patients in the coming years [23].

7.1.3. Deca-14

In a study by Smith et al., DECA-14 (Dequalium analogue C-14 linker) was identified among other compounds because it selectively targeted patient-derived neuroblastoma TICs (NB TICs) [21]. It selectively inhibited neuroblastoma (NB) CSCs survival *in vitro* and significantly reduced both NB xenograft cancer volume and self-renewal, and tumour-initiating capacity *in vivo*. To examine whether DECA-14 was effective against NB CSCs *in*

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vivo, xenograft tumours were established by the subcutaneous injection of NB12 cells into NOD/SCID mice.

Animals were treated with intraperitoneal injections of DECA-14 every other day and they received supplemental hydration with daily subcutaneous injection of lactated Ringer's solution. This treatment significantly reduced the percentage of sphere-forming cells, and reduced both tumour growth and the tumour-initiating capacity of NB CSCs *in vivo*. Moreover it appeared that DE-CA-14 induced apoptosis through effects on the mitochondria, and in fact inhibited Complex I, increasing the ROS production and thus inducing apoptosis [24]. In addition, it reduced the formation of secondary tumours. DECA-14 is also considered as a good therapeutic drug because it is administered in nanomolar doses, implying low toxicity, and a potential use in combination with other agents [21, 24].

Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, has been identified as a leukemic stem cell-selective agent [24] and is being currently tested in clinical trials for various solid tumours.

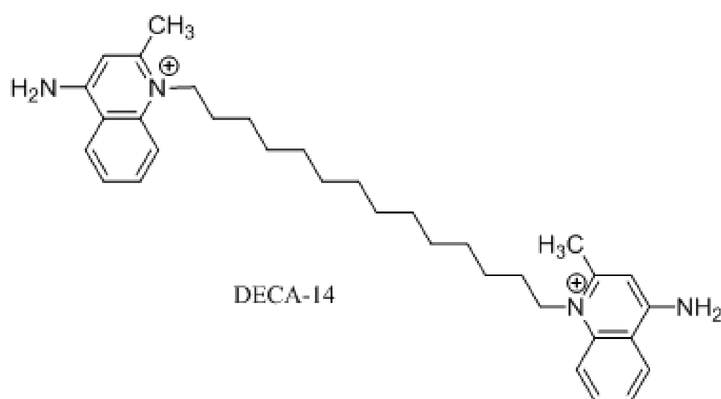

Figure 6.

7.1.4. Rapamycin

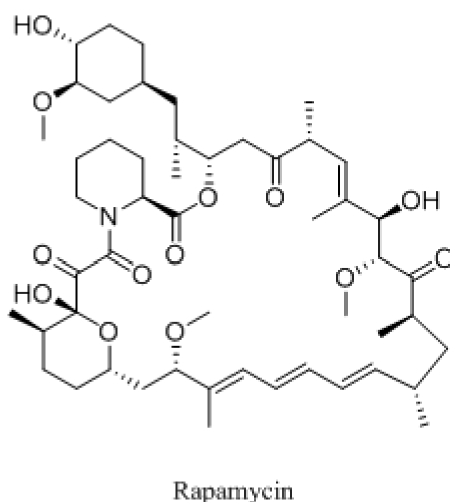

Figure 7.

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Recent study demonstrated that rapamycin exerts its antitumour effects by inhibiting both angiogenesis and cell proliferation [25].

Rapamycin treatment resulted in a decrease of cell viability in as early as 24 hours, and it was also associated with decreased proliferation of NB CSCs isolated from multiple patients. Moreover, it was observed that rapamycin inhibited phosphorylation of p70S6K and S6 ribosomal protein (S6RP), two important proteins in the mTOR signalling pathway. This caused an up-regulation of mTOR and AKT signal in NB CSCs [24], suggesting that this pathway is constitutively activated in NB TICs. This treatment induced apoptosis in NB12 and NB122R cells, and daily rapamycin treatment reduced tumour weight by 82.6% for NB12 tumours and 74.0% for NB88 tumours. *In vivo* rapamycin treatment targeted the NB CSCs and showed the reduction of sphere-forming ability 7.75-fold, demonstrating that rapamycin targeted and destroyed the tumour cell population capable of self-renewal. In addition, a combination of rapamycin and vinblastine is effective against NB12 xenograft tumours; in fact, a North American multicentre has started a phase I trial to evaluate rapamycin in combination with vinblastine for pediatric solid tumours. These findings suggest that rapamycin and rapamycin analogues may be ideal therapeutics for patients [26].

7.1.5. Cucurbitacin I

Cucurbitacin I (also known as JSI-124) is a triterpenoid compound, and belongs to the *Cucurbitaceae* family of plants.

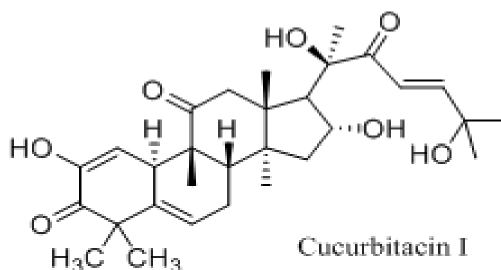

Figure 8.

Cucurbitacin I have been used as medicine for their anti-inflammatory and analgesic effects, but recent studies have reported that cucurbitacin I potently inhibited cell growth via the selective repression of STAT3 (p-STAT3) tyrosine phosphorylation in various human cancer cell lines, especially in anaplastic thyroid cancer (ATC). Friedman et al. evaluated the tumour initiation potential of these CD133+ ATC cells in an animal model. Their results suggest that the CD133 subsets within ATC may account for tumour progression, resistance to chemotherapy, and the aggressiveness of ATC. Cucurbitacin I can improve the efficacy of treatment targeting ATC-CD133+ cells, reducing their resistance to radiation and chemotherapeutic drugs, and showing a significant suppression of tumourigenicity. In fact, it has been demonstrated that 100 nM cucurbitacin I, which is a specific JAK-STAT inhibitor, efficiently inhibited the proliferation of ATC CD133+ cells, induced apoptosis in them and also significantly interfered with sphere formation, colony formation, and invasion. Furthermore, the treatment with 150 nM cucurbitacin I can suppress the tumourigenic, invasive, and self-renewing abilities of ATC-CD133+ cells. This suggests that the STAT3

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and the p-STAT-related pathway play a crucial role in maintaining the CSCs-like property of ATC-CD133+ cells. Fluorescence analysis also showed that cucurbitacin I decreased the number of ATC-CD133+ cells in a dose-dependent manner. In addition Cucurbitacin I treatment rendered ATC-CD133+ cells more sensitive to chemotherapeutic agents such as cisplatin, 5-fluorouracil, and doxorubicin. This fact was proved by the administration of doses of ionizing radiation, which showed that CD133+ cells were more radioresistant than CD133- cells, and so, silencing STAT3 signalling with cucurbitacin I, it sensitized CD133+ cells to radiation by driving apoptosis through both the inactivation of p-STAT3 and the downregulation of effectors as survivin, Bcl-2, and MMP9. Hence, IR and chemotherapeutic drugs treatment were able to effectively inhibit only the growth of ATC CD133- cells, while the combination dramatically diminished sphere formation and colony formation and invasion in both CD133- cells and CD133+ cells. Most important is the new triple treatment, cucurbitacin I-cisplatin-IR, which effectively reduced the number and volume of lung metastases *in vivo*. CD133+ cell-transplanted mice treated with the triple treatment showed a greater survival rate compared with CD133+ cell-transplanted mice that had received other treatments. Overall, these *in vivo* results show that the effectiveness of conventional chemo/radiotherapy can be significantly improved with the addition of cucurbitacin I treatment [27].

7.1.6. Phosphosulindac

Phosphosulindac is a novel compound (PS, OXT-328), derivate of sulindac, a non-steroidal anti-inflammatory drug, which is more effective than the conventional sulindac in the prevention of cancer and inhibition of tumourigenesis, because long-term administration of sulindac is associated with significant side effects.

Recent studies have shown how, both *in vitro* and *in vivo*, that PS can selectively kill breast CSCs and reduce their tumour-initiating ability, inhibiting Wnt/β-catenin signalling and EMT. This effect provides a strong rationale for the evaluation of PS in the treatment of breast cancer. *In vitro* two different cell lines were used to evaluate the effect of PS: doses of 50 μM PS induced cycle arrest after 24 hours of treatment, while a higher concentration of 180 μM induced cell death in CSCs. Moreover, PS impaired the ability of CSCs to form mammospheres in a cytotoxicity-independent manner because it showed a more potent effect in the down-regulating of genes as Oct-4, BMI-1, Sox2, and Nanog, with similar action to salinomycin. The mechanism of action of PS is the inhibition of the Wnt signalling cascade, reducing the levels of β-catenin in the nucleus where it exerts its transcriptional activity. The low levels of β-catenin reduce phosphorylation of GSK-3β on serine 9, which enhances its kinase activity, and so can form the degradation complex with APC which is involved in the cytoplasmic-nuclear shuttling of β-catenin.

Moreover, PS blocked the induction of EMT by TGFβ1, which enhance Snail expression and abolished the generation of CD44high/CD24low cells: this result was also confirmed on detecting the expression levels of E-cadherin and vimentin. The effect of PS on Wnt signalling is not restricted to cultured cells, but it has been evaluated *in vivo*. PS treatment in mice strongly reduced β-catenin level and most of the remaining β-catenin relocalized to the cell membrane [28].

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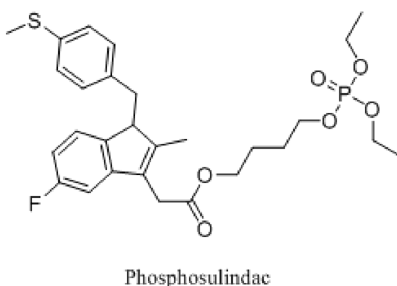

Figure 9.

7.1.7. Sorafenib

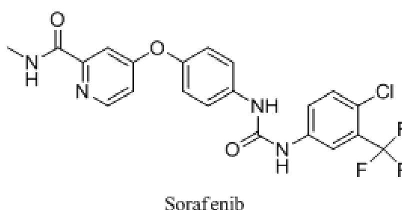

Figure 10.

Sorafenib (SO), also named BAY 43-9006 or Nexavar®, is a multi-kinase inhibitor, which seems to possess a good anti-tumour activity. In fact it has been demonstrated in several clinical phase I, II, and III trials with SO alone or in combination with other chemotherapeutic drugs, and these studies are still ongoing. SO inhibits vascular endothelial growth factor (VEGF) receptor-2 and VEGF receptor-3. Recent studies showed that using SO reduced significantly colony and spheroid formation in a concentration of 20 μmol/L in pancreatic cancer cells. However, some small colonies survived, resulting in the regrowth of spheroids 30 days later and in fact, it was observed that some clones acquired resistance owing to the strong induction of NF-κB signalling. These data indicated that SO alone could reduce CSCs properties *in vivo*, though not completely. Indeed it needed a co-treatment with sulphoraphane, which down-regulated NF-κB signalling and increased the targeting of CSCs characteristics, involving colony and spheroid formation, ALDH1 activity, and apoptosis resistance. *In vivo* SO diminished the growth of CSCs inhibiting proliferation and angiogenesis. In conclusion, SO does not lead to total elimination of CSCs, but with the combination with an NF-κB inhibitor such as SF may be a therapeutic option to improve the therapeutic effect [29].

7.1.8. Cyclopamine

Cyclopamine is a natural steroidal alkaloid and inhibits the Hh pathway by directly binding to a membrane receptor Smoothened (SMO), suppressing SMO and its downstream activities, eventually leading to apoptotic cell death. Despite this premise, its application for cancer treatment is limited by the high hydrophobicity. Therefore, recent studies decided to

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synthesize a water-soluble molecular drug carrier based on HPMA copolymer, by RAFT (Reversible Addition-Fragmentation chain Transfer) copolymerization, thus enabling the evaluation of the CSCs inhibitory effects of the HPMA copolymer-cyclopamine conjugate in an *in vitro* prostate cancer epithelial cell model, RC-92a/hTERT cells, with stem cell properties.

To identify prostate CSCs-enriched population in RC-92a/hTERT cells, surface markers were considered, such as CD133+, integrin α2β1hi, CD44+ with enhanced colony and sphere forming ability *in vitro* and increased tumourigenicity *in vivo* but of the three cell surface markers, CD133+ was the most specific to define cancer stem-like cells in this cell line. Therefore, if prostate CSCs population changes following drug or conjugate treatment, also should be observed changes of CD133 expression level. HPMA copolymer-cyclopamine conjugate treatment showed a decrease of the fraction of CD133+ cells in a dose-dependent manner.

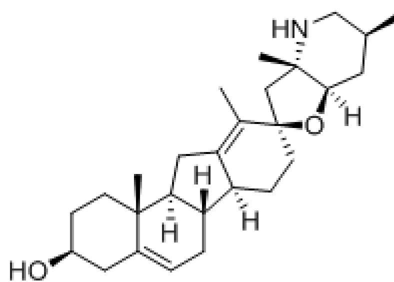

Figure 11.

Moreover, the different response of prostate cancer cells to various drugs could be evaluated by comparing the sensitivity of RC-92a/hTERT cells to HPMA copolymer-cyclopamine conjugate and to docetaxel, a traditional firstline chemotherapeutic for prostate cancer. The data obtained *in* a *vitro* model suggested the greater sensitivity to cyclopamine and HPMA copolymer-cyclopamine conjugate than to traditional first line chemotherapeutics docetaxel. In fact with cyclopamine treatment the growth of prostate CSCs was suppressed and bulk cancer cells appeared to survive, whilst following docetaxel treatment, bulk prostate cancer cells could be eradicated, but prostate CSCs survived. In addition HPMA copolymer-cyclopamine conjugate has the potential to shrink the CSCs population, which contributes to refractory and metastatic prostate cancer, thus improving the outcome of prostate cancer treatment. Hence, the selective cytotoxic effects of conjugated cyclopamine and docetaxel on different subpopulations of prostate cancer cells suggest a potential combination therapeutic strategy to eliminate both bulk and cancer stem cell [30].

7.1.9. Oncostatin M

Oncostatin M (OSM), is a pleiotropic cytokine that belongs to the IL-6 family which includes IL-6, IL-11, and leukemia-inhibitory factor [26] and it has been shown to inhibit the proliferation of various solid tumours [21], such as hepato-cellular carcinoma (HCC) which is one of the most common malignancies. In HCC EpCAM+ often show altered expression, causing the activation of Wnt/β-catenin signalling and therefore seems to be a potentially

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useful marker for the isolation of liver CSCs in hepatic stem cell-like HCC. To induce the differentiation of CSCs or to eradicate CSCs, the signalling pathway responsible for self-renewal needs to be inhibited. In the study by Yamashita et al., high frequency of OSMR receptor in EpCAM+ hepatocellular carcinoma (HCC) cells [26] has been detected. *In vitro* OSM treatment on HuH1 and HuH7 HCC cells led to a decrease in stemness gene expression, EpCAM expression, α-fetoprotein and cytokeratin protein expressions and an increase in albumin protein expression, indicating the differentiation of the OSM-induced HCC cells. In fact, OSM induced the activation of the signal transducer and activator of the transcription 3 (STAT3) pathways. The incubation of HCC cells with OSM for 10 days resulted in the induction and nuclear accumulation of phosphorylated STAT3: thus the differentiation of EpCAM+ HCC cells happens in a STAT3-dependent manner [21]. Moreover OSM could cause an increase of chemosensitivity. The effect of combining OSM treatment with conventional chemotherapy is to target both dormant CSCs and amplify non-CSCs. In fact it has been shown that 5-FU treatment alone could diminish EpCAM− non-CSCs which results in the enrichment of EpCAM+ CSCs in HCC, while the effects of 5-FU in combination with OSM on EpCAM+ HCC effectively show suppression of cell proliferation in HuH1 and HuH7 cells. This combination also shows increased apoptosis, through induction of the activation of caspase-3 and Annexin V binding to the membrane by 5-FU. Moreover, OSM treatment alone showed limited and weak tumour-suppressive effects *in vivo*, while the combination with 5-FU showed a marked inhibition of tumour growth [26]. Hence, the combination of some agents targeting CSCs and conventional chemotherapies may produce a better effect [21].

7.1.10. Other Molecule Inhibitors

Other inhibitors of signal pathway could be **GDC-0449** (Genentech), a small molecule SMO inhibitor, resulting in no dose-limiting toxicities in patients with advanced solid tumours. A study in GDC-0449 showed two complete and 16 partial responses out of 33 patients with Basal Cell Carcinoma (BCC), and common adverse effects included hair loss, nausea, vomiting, anorexia, dyspepsia, weight loss, hyponatremia, and fatigue. This molecule is in phase II trial and is efficacious in patients with ovarian cancer in remission, advanced colorectal cancer, and advanced BCC.

It is also being tested in combination with various conventional drugs such as oxoplatin and others. **IPI926**, a cyclopamine-derived compound, inhibitor of the Hh pathway, has been evaluated in clinical trials for advanced stage solid tumours and metastatic pancreatic cancer, and is in phase II trial in collaboration with gemcitabine.

PF04449913 (Pfizer), a SMO inhibitor, is currently being examined in a phase I study as a single agent or in combination with dasatinib in patients with CML.

ICG001 (Institute for Chemical Genomics) is a small molecule, which selectively inhibits Wnt/β-catenin signalling by interrupting β-catenin binding to the transcriptional cofactor cyclic AMP. ICG001 treatment can be used in colon carcinoma cell lines and results in apoptosis, while sparing normal colonic epithelial cells [31].

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7.2. Natural Agents Targeting CSCs

In the last decade, the naturally occurring agents and their structurally-derived compounds have attracted remarkable attention due to their potential role in the prevention and treatment of human malignancies, especially in the regula-tion of CSCs. Considering the non-toxic characteristics of these natural agents, they could provide a novel, safe, and effective approach for controlling tumour aggressiveness.

7.2.1. Diflourinated-Curcumin (CDF)

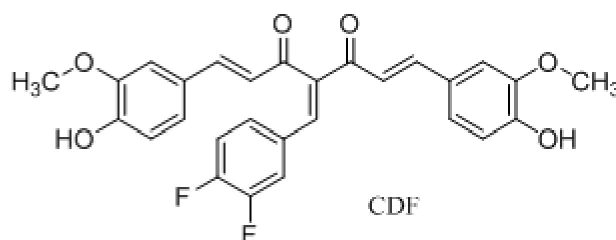

Figure 12.

CDF is a novel synthetic derivative of curcumin, a known natural anti-tumour agent. Recent studies have demonstrated the action of CDF on cell survival, clonogenicity, sphere-forming capacity, cell migration and invasion, CSCs self-renewal capacity, and the regulation of EZH2 by miRNAs in pancreatic cancer cells *in vitro* and *in vitro* [32]. EZH2 is a histone methyltransferase enhancer of the polycomb group, which is a class of chromatin-modifying enzymes capable of methylation of both DNA and core histones to regulate gene expression. Emerging evidence suggests that polycomb group proteins play an important role in stem cell maintenance, X-inactivation, development, differentiation, and proliferation, so the altered expressions of many polycomb group proteins have been found to be associated with the development of cancer. Recent studies show that the decreased EZH2 expression led to the decrease of cell proliferation, migration, and expressions of CSCs markers such as Nanog, CD44, and Notch-1. In addition, it increased the expression of miRNAs such as let-7, miR-26a, miR-101, and miR-200 in pancreatic cancer cells [16,32], suggesting that these miRNAs are involved in the regulation of EZH2. It has especially been shown that the inhibition of EZH2 led to an increased expression of let-7 and miR-200 in pancreatic cancer cells, which would be highly valuable in reverting tumour cell aggressiveness or in the destruction of CSCs. Moreover, miR-26a and miR-101 are also known to be potential tumour suppressors. CDF could modulate the cancer epigenome, reexpressing these miRNA and re-pressing EZH2 [16]. Furthermore, to examine the effect of CDF on the self-renewal capacity of CSCs, a sphere formation assay has been conducted of the cells derived from the xenograft using pancreatospheres of MiaPaCa-2 cells. CDF caused the inhibition of pancreatospheres in a dose-dependent manner, attenuating the CSCs function. Hence, it has been proved that CDF could be a useful agent for the prevention of tumour progression and treatment of pancreatic cancer by attenuating CSCs function and overcoming therapeutic resistance [32].

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7.2.2. Genistein

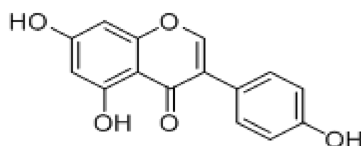

Figure 13.

Soy Isoflavones belong to the flavanoid group of compounds, the largest class of polyphenolic compounds especially found in the *Leguminosae* family. Genistein, daidzein, and glycitein are the three major components, but genistein (4,5,7-trihydroxyisoflavone) is the most studied of these bioactive compounds. Several studies have demonstrated that genistein treatment increased the expression of let-7, miR-26a, miR-101, miR-146a, and miR-200, and decreased the expression of miR-21, markers such as CD44 and EpCAM, and the formation of pancreatospheres, thus showing its anti-tumour activity against human pancreatic cancer cells [16,33]. Moreover other studies show that genistein down-regulated Wnt signalling by inhibiting Wnt-5a expression and enhancing Sfrp-2 expression (secreted frizzled-related protein-2), an extracellular Wnt receptor antagonist; also reduced Notch-2 expression in rat mammary epithelial cells *in vivo* [34]. Recent studies have demonstrated that genistein inhibited the gastric cancer cell stem-like properties, such as self-renewal ability, drug resistance, and tumourigenicity. In fact, *in vitro* it has been seen that genistein inhibited the colony formation capacity, which is an important characteristic of CSCs, in a dose-dependent manner, and the level of gastric CSCs markers was greatly decreased in the spheres treated with 15μM genistein compared with the untreated spheres. As regards chemoresistance, the genistein treatment down-regulated ABCC1, ABCC5 and ABCG2 expression, and especially inhibited the ABCG2 mRNA expression and ERK 1/2, which have been shown to play an important role in regulating the ABCG2 expression: this enhanced gastric cancer cell chemosensitivity to 5-FU and cisplatin. Furthermore, genistein reduced tumourigenicity *in vivo* after the inoculation of gastric cancer cells in mice, confirming that the size and weight of xenograft tumours treated with genistein were significantly less than the control tumours. These results thus demonstrated that genistein efficiently attenuated the tumourigenicity of gastric cancer cells [33].

7.2.3. Epigallocatechin-3-Gallate

Epigallocatechine 3-gallate (EGCG) is the most abundant catechin in green tea and is considered a potent chemoprevention agent against certain types of cancer [34]. In fact, several experimental studies have demonstrated that EGCG has an anti-tumour activity in vitro and in vivo, potentially related to its down-regulation of NF-κB, hedgehog and Wnt pathways in a variety of cancers, such as lung, oral, colon, prostate, colon, pancreatic, gastric, ovarian, and breast cancer [16]. The regulatory effects of EGCG on CSCs phenotype have been reported by several *in vitro* and *in vivo* studies.

In fact, it has been noted that EGCG treatment inhibits the expression of androgen-induced miR-21 in prostate cancer cells, and it is also reported to increase the expression of

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let-7 and miR-34a in human hepatocellular carcinoma cells and neuroblastoma cells [16]. A recent study has demonstrated that EGCG regulated self-renewal capacity and early metastasis of human pancreatic CSCs. In fact EGCG inhibited the growth of spheroids in suspension in a dose-dependent manner and the CSCs's viability, and it inhibited the growth of colonies. Moreover, EGCG inhibited the expression of Nanog, c-Myc, and Oct-4 in pancreatic CSCs, but had no effect on the expression of Sox2. It seems that the inhibition of Nanog further enhanced the anti-proliferative effects of EGCG on CSCs, thus suggesting a strong engagement in the regulation of self-renewal capacity of CSCs, and inhibiting the factors required for maintaining pluripotency in CSCs. As regards the effect on the Shh pathway, EGCG inhibited the expression of Snail, ZEB1 and Slug, EMT markers, thus inhibiting cell migration and invasion of CSCs, and furthermore it inhibited the expression of smoothened (SMO), Patched-1, and Patched-2, and the expression of transcription factor Gli1 and Gli2, which seem to play important roles in maintaining stemness and tumourigenesis [35,36]. Moreover, it is shown that quercetin enhances the inhibitory effects of EGCG on self-renewal, migration and invasion of pancreatic CSCs, thus demonstrating a synergistic effect with chemopreventive agents, such as paclitaxel [37]. These findings suggest that this bioactive compound is involved in the regulation of CSCs characteristics.

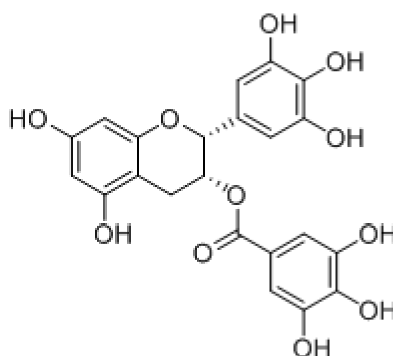

Figure 14.

7.2.4. Resveratrol

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is another type of dietary polyphenolic compounds most abundant in the skin of grapes.

It has been recognized that resveratrol has antioxidant and anti-inflammatory effects, and furthermore increased evidence from *in vitro* and *in vivo* experimental studies have indicated that resveratrol could suppress many types of cancers by the regulation of cell proliferation, apoptosis, angiogenesis and tumour metastasis mediated through deregulation of multiple cellular signalling pathways such as Akt, Wnt, hedgehog, NF-κB.

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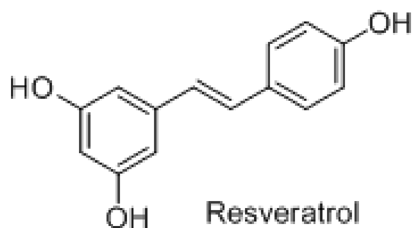

Figure 15.

In a recent study NPCCSCs was used as a model to analyze the regulation of resveratrol in stemness, EMT, and metabolic signatures of CSCs and to explore the potential therapeutic targets in NPC CSCs. It showed that resveratrol significantly affects cell viability in a concentration higher than 50 μM and that resveratrol could suppress the long-term self-renewal and tumourigenicity, and reduce the migration and invasion capacity in CSCs directly through the induction of p53, found in lower levels in CSCs. Moreover, resveratrol diminished the expression of genes characteristic of stemness such as Oct-4, Sox2, Klf4, c-Myc, Nanog, and Lin28, and it caused the downregulation of EMT as Twist, Snail, Zeb-1, and vementin. Chemoresistance, resveratrol-suppressed ABCG2, thus increases drug sensitivity. *In vivo*, after injecting GFP-CSCs into the hypoglossal region of NOD/SCID mice, resveratrol treatment caused the total eradication of CSCs: the mice reverted to good health [16].

It was reported that resveratrol could also increase the p53 protein level in breast cancer cell lines without altering the p53mRNA levels, suggesting a resveratrol treatment in tumours with a loss of normal p53 function [38]. Besides, resveratrol could significantly activate intracellular Notch-1 and restore wild-type p53 expression in glioblastoma cells.

There are also limited reports showing that resveratrol treatment could decrease the level of miR-21 expression in lung and colon cancer cells [16], and one study showed that resveratrol decreases the expression of miR-146a in cancer cells. Therefore, the potential impact of resveratrol against CSCs may be warranted for future exploration.

7.2.5. Indol-3-Carbinol and Diindolylmethane

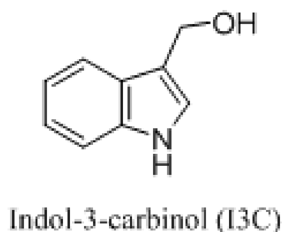

Figure 16.

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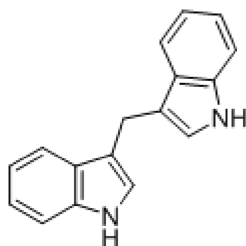

Figure 17.

Indol-3-carbinol (I3C) is a compound produced by members of the *Cruciferae* family. Under acidic conditions as in the stomach, I3C is chemically unstable so it is converted with a self-condensation reaction to a series of oligomeric products among which 3,3'-diindolylmethane (DIM) is the major component. DIM is the product responsible for the biological effects *in vivo* [16]. Several experimental studies *in vitro* and *in vivo* have shown that DIM inhibited tumourigenesis and cancer cell growth, and induced apoptosis in cancer cells, functioning as a potent antitumour agent for the prevention and treatment of tumours. It also seems that DIM inhibited multiple cellular signalling pathways such as NF-κB, Akt, and Wnt, and induced G1/S arrest of the cell cycle and apoptosis by downregulating anti-apoptotic gene products, including Bcl-2, Bcl-xL, survivin, inhibitor of apoptosis protein (IAP), X chromosome-linked IAP (XIAP), and Fas-associated death domain protein-like interleukin-1-b-converting enzyme inhibitory protein (FLIP), and up-regulating pro-apoptotic protein Bax. Moreover, another study showed that I3C treatment decreased the expression of miR-21 and miR-146b in vinyl carbamate-induced mouse lung tumour, along with the inhibition of tumour growth [16]. DIM treatment also increased the expression of let-7 and miR-146a, and decreased the expression of miR-21 and miR-22b, inhibiting cell growth and invasion of human pancreatic cancer cells [40]. *In vitro* it has been shown that I3C potentiates the effects of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) through the induction of death receptors and synergizes with chemotherapeutic agents through downregulation of P-glycoprotein (P-gp). *In vivo*, I3C results to be a potent chemo-preventive agent for hormonal-dependent cancers such as breast and cervical cancer, because it possesses the ability to induce apoptosis, to inhibit DNA-carcinogen adduct formation, to suppress free-radical production, and to inhibit invasion and angiogenesis. Furthermore, initial clinical trials in women have shown that I3C is a promising agent against breast and cervical cancers [39,40].

7.2.6. Sulforaphane

Figure 18.

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Sulforaphane (SFN) is an active compound, found in cruciferous vegetables, and shows great promise for development as a chemo-preventive and therapeutic agent against tumour. It has been demonstrated that sulforaphane treatment down-regulated NF-κB function in prostate and colon cancer cells, as well as the Akt pathway in ovarian, prostate and colorectal cancers, or that it suppressed the expression of Wnt-9a in ApcMin/+ mouse adenomas. Moreover, sulforaphane has been shown to induce the down-regulation of β-catenin in human cervical carcinoma and hepatocarcinoma [34], and very recently, it has been considered as an effective compound in targeting breast cancer stem cells *in vitro* and *in vivo*. In fact, SFN inhibited breast CSCs at concentrations of 0.5–5 μM, approximately 10-fold lower than that exhibiting an antiproliferative effect on cancer cell culture, and *in vivo*, inoculating NOD/SCID mice with tumour cells, inhibited tumour re-growth up to 33 days; furthermore, a down-regulation of the Wnt/β-catenin self-renewal pathway is also observed. It has also been confirmed that SFN can regulate pancreatic carcinogenesis in pancreatic CSCs by inhibiting the Shh pathway and its targets such as Nanog and Oct-4. In addition it can induce apoptosis of pancreatic CSCs by inhibiting Bcl-2, Cyclin D2, and activating caspase-3 and 7, in a dose-dependent manner [41]. In addition it showed high activity if it was used in combination with a chemotherapic agent such as cisplatin, gemcitabine, doxorubicin, and 5-flurouracil, increasing apoptosis, and *in vivo* it abolished the growth of CSCs [42].

7.2.7. Arsenic Trioxide

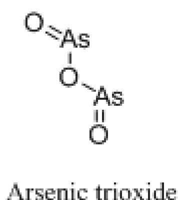

Figure 19.

Arsenic trioxide (As_2O_3) is a traditional Chinese medicine and is effective as treatment for relapsed acute promyelocytic leukemia. It can inhibit Shh signalling, targeting the Gli protein level. Kim et al. demonstrated the antagonistic effect of As_2O_3 in Shh signalling primarily through interference with Gli2. Moreover, Beauchamp and colleagues show that arsenic trioxide could inhibit the growth of Ewing sarcoma and medulloblastoma cells by targeting Gli1. These finding suggest that arsenic trioxide could be used as a therapeutic agent in malignant diseases associated with the Shh pathway activation, thus regulating the CSCs property [43].

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7.3. All-Trans Retinoic Acid (ATRA)

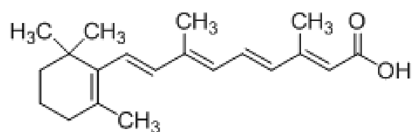

Figure 20.

All-trans retinoic acid (ATRA) is a natural compound derived from vitamin A, which plays a role in cell growth, differentiation and apoptosis. It has been applied in the therapy of hematological malignancies and some solid tumours. It is a potent differentiating agent and is associated with CSCs-enriched gene expression signatures, so it is considered as a promising drug in the eradication of CSCs. It has been demonstrated that low concentrations of ATRA such as 10 μM can induce differentiation into glioblastoma multiform CSCs and high doses of ATRA (40 μM) can cause apoptosis of glioblastoma multiform CSCs in an MAPK-dependent manner.

In head and neck squamous carcinoma CSCs ATRA can suppress the expression of the stem cell markers Oct-4, Sox2, Nestin, and CD44, and inhibit the proliferation of HNSC CSCs *in vitro* and *in vivo*, perhaps down-regulating Wnt/β-catenin signalling. In addition, ATRA treatment can promote the sensitization of HNSC CSCs to cisplatin [21]. Recent studies have shown that ATRA reduced the mammosphere-forming ability of cell lines that expressed higher levels of Sox2 suggesting that only the cancer cells that are dependent on Sox2 for self-renewal are responsive. It also reduced the levels of EGFR, SERPINE1, and SLUG in a cell type-dependent manner. Since the repression of these genes is correlated with diminished CSCs phenotype, the use of these genes as biomarkers is being considered to distinguish ATRA responders from non-responders.

Moreover, Ginestier et al. showed the ability of ATRA to inhibit mammosphere formation by ALDH1-positive CSCs, and Papi et al. recently demonstrated the effect of ATRA and the RXR-specific ligand 6-OH-11-O-hydroxyphenanthrene in reducing CSCs phenotype of breast cancer cells by targeting the NF-κB pathway. Moreover it seems that some cancer-associated mutations such as K-Ras, APC, and KIT mutations cell lines, presented resistance to ATRA but Selumetinib (PD0325901), a MEK inhibitor already in clinical use, and AZ628, a C-RAF inhibitor, were effective against cell lines with K-ras mutation.

A recent clinical trial of ATRA in combination with taxol in patients with recurrent or metastatic disease revealed an overall clinical benefit of 76.4% with a relatively high rate of stable disease; furthermore other results demonstrated that combining ATRA with antihormonal therapy such as fulvestrant or tamoxifen could be a new strategy to eliminate CSCs.

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7.4. Tumour Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL)

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), also named Apo2 Ligand (Apo2L), is a member of the tumour necrosis factor (TNF) super family, discovered in 1995, and is a type II membrane protein of 281 aminoacids. It was observed that TRAIL can be found on the cell surface or soluble, and rapidly induce apoptosis in a wide variety of altered cell lines without affecting normal cells. In fact, TRAIL binding to receptors DR4 or DR5, which have intracellular death domain, leads to the transduction of an apoptotic signal which causes cell death, but sparing normal cells due to the function of the decay receptors, DcR1 and DcR2, which do not possess death domain. When Trail binds a receptor, it causes the formation of DISC complex, leading the consequent activation of procaspase-8 and propagation of TRAIL-induced apoptotic signalling. Hence, TRAIL was originally identified as an attractive candidate in clinical use for its selectivity in apoptosis induction of the cancer cells [44], and for its less toxicity [45]. There are some studies which confirm the capacity of TRIAL to destroy CSCs. One study of Sasportas et al. demonstrated TRAIL-induced apoptosis of CD133-positive glioma cells, and another showed that radioresistant oesophageal cancer cells were 10 times more susceptible to TRAIL therapy. Further study noted an increased sensitivity of colon cancer SP cells with TRAIL therapy [46], and in several breast cancer and ovarian carcinoma cell lines. The percentage of SP cells decreased by at least 50% in the above cancer cell lines and even by more than 90% in the SW480 human colon cancer cell lines because TRAIL receptor DR4 expression in SW480 SP cells is 10-fold higher than non-SP cells. In addition, the expression of c-Myc, an activator of DR4 transcription through E-Box DNA-response elements located in DR4 promoter, also results higher in these SP-cells [21]. These findings suggest that TRAIL or proapoptotic agonist antibodies that target DR4 and c-Myc, could be promising therapeutics for the eradication of CSCs and provide a basis for combining TRAIL with standard chemotherapeutic regimes to target both CSCs and non-CSCs populations [47]. Moreover, in recent experiments the use of mesenchymal cells as a vehicle was very successful. Mesenchymal Stem Cells (MSCs) have been engineered to express TRAIL [46] and this caused significant inhibition of tumour growth and induced significant survival benefits in animal models. Moreover, MSCs expressing-TRAIL could cause apoptosis or death, and reduce colony formation of SP cells in squamous (H357) and lung (A549) cancer cell lines, also acting in synergy with conventional chemotherapy [21]. Unfortunately, numerous studies have demonstrated that many malignancies are completely resistant to monotherapy with TRAIL: it is shown that the high expression of c-FLIP (an inhibitor of death receptor-mediated apoptosis), low levels of caspase-8 expression [21], the overexpression of anti-apoptotic Bcl-2-like proteins as Mcl-1 and Bcl-2, caused this CSCs' TRAIL resistance. An alternative approach has been proposed in which TRAIL is combined with other drugs with the major objective to either synergize the activity of TRAIL or to sensitize TRAIL resistant cells. For example an association with chemotherapeutic agents, including camptothecin, celecoxib and cisplatin, resulted in the down-regulation or the inhibition of c FLIP, and thus sensitized the resistant cancer cells to TRAIL, by inhibiting the formation of the secondary complex which stimulates NF-KB, MAPKS and PI3K/AKT pathways to produce survival signals. Another approach is the association with (R)-roscovitine, a potential candidate drug that targets Mcl-1 to enhance the therapeutic effects of TRAIL. This treatment especially in GBM cells, significantly decreased the diameters of the

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neurospheres, their ability to proliferate and grow in size, increasing the expression of cleaved caspase-3 and enhancing the percentage of cells in the neurospheres undergoing apoptosis.

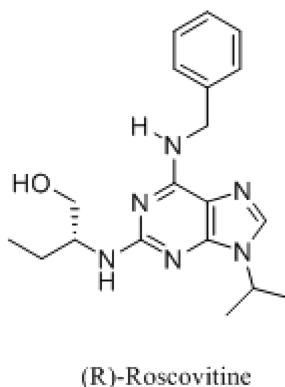

Figure 21.

Moreover, it was also noted that R-roscovitine down-regulated the expression of c-FLIP. Hence, the growing interest in TRAIL-based interventions has led to the development of recombinant human TRAIL (rhTRAIL) as a promising therapy for different types of human cancer [45].

7.5. Monoclonal Antibodies (MAB)

Emergent therapeutic strategies in cancer have been focusing on the use of mAbs to stimulate an immune response against tumours, to block signalling pathways, or to refine the delivery of cytotoxic agents, making CSCs, natural targets for mAbs development. Indeed, mAbs, targeting specific antigens and related pathways altered in cancer stem cells should be more efficient in the destruction of tumour initiating cells, thus improving the clinical outcome. Unfortunately, there are no universal biomarkers for CSCs, because each tumour type possesses a unique combination of cell surface biomarkers that define the cell subpopulation.

EpCAM is one of the best-studied target antigens of human tumours, abundantly expressed in primary tumours and metastasis of many epithelial tumours particularly in adenocarcinoma. Studies show that a novel human EpCAM-targeting monoclonal antibody (3-17I) has been created. It has been demonstrated that this new mAb targets EpCam+ cell lines by flow cytometry and confocal fluorescence microscopy; it presents good affinity, good cross-reactivity profiles, and excellent ADCC and CDC activity, and shows improved properties over MT201 and MOC31, another two mAb anti-EpCam under clinical investigation. Moreover, there is evidence for mAb-sequestration in endolysosomes, suggesting the internalization of 3-17I by receptor-mediated endocytosis. The ribosomal-inactivating toxin saporin was linked to 3-17I, creating the non-toxic immunotoxin 3–17I-saporin, a promising candidate for the drug delivery technology photochemical internalization (PCI), reducing the bond to healthy tissue and increasing selectivity [48]. Other studies make Notch a potential therapeutic target: the Notch1 monoclonal antibodies, specifically bound to the negative regulatory region of human Notch1, lead to a decreased self-renewal ability of

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CSCs and tumour growth in xenograft models derived from triple negative breast cancer (TNBC) Sum149 cell line and TNBC patient primary cancer cells, and have synergistic effect with docetaxel.

One of the most potent Notch inhibitors mAb is 602.101, which inhibited Jag-ged1/Delta-like4-stimulated Notch1 signalling but had no effect on the Notch2 signalling, clearly highlighting its specificity. It inhibited cell proliferation and mammosphere formation up to 3 generations in cell lines, and furthermore, once treated with the antibody, the cells were unable to recover their stemness and could not repopulate even in the absence of the antibody, indicating long-term deleterious effects of the antibody on the putative CSCs. However, the inhibition of Notch signalling alone has been shown to be insufficient to inhibit neurospheres recovery and requires combinatorial therapy with a chemotherapeutic agent such as temozolomide. Moreover, this mAb inhibits the chemo- and radiotherapy-resistant in the CD44High/CD24Low subpopulation and is potentially a strong therapeutic tool to reduce resistance. The antibody also induced apoptotic cell death of the cancer cells and modulated the expression of genes associated with stemness and EMT, further highlighting the therapeutic potential of this antibody in targeting angiogenesis and metastasis [49]. In addition, in human colorectal cancer xenografts, anti-hDLL4 (anti-human Notch ligand Delta-like 4 antibody) significantly decreased the proportion of ESA+/CD44+/CD166+ CSCs in tumours, and the combination of anti-hDLL4 and irinotecan showed synergistic inhibition on tumour growth and recurrence. Moreover CD44, a type I cell-surface glycoprotein, functioning as the major cellular adhesion molecule for hyaluronic acids, could be a credible marker molecule for CSCs. Anti-CD44R1 fully human GV5 mAb, which specifically recognized CD44R1 in the CD44R1 high cells, caused growth inhibition of human cancer xenografts in athymic mice by internalizing of CD44R1. Moreover, the reactivity of GV5 in human normal skin keratinocytes was negative, suggesting low skin toxicity of this anti-CD44R1 fully human mAb. Hence, the cancer therapy with anti-CD44R1 fully human mAb is promising, especially against various human epithelial cancers such as adenocarcinomas of breast, stomach, and colon, transitional cell carcinoma of the bladder, and renal carcinoma [50]. In addition a recent study has shown the cytotoxic effect of anti-CD133 monoclonal on FEMX-I melanoma cells which express CD133, while having no effect on human MA-11 breast carcinoma cells which do not express CD133. *In vitro* it noted that CD133+ cells in glioblastoma, which have cancer stem-like characteristics, were selectively targeted and eradicated, while CD133-cells in glioblastoma remained viable. Moreover, the self-renewal and tumour-initating capability of GBM-CD133+ treated with anti-CD133 mono-clonal was significantly blocked [51].

Another important mAb is CC188, which binds a carbohydrate expressed on the surface of colon CSCs (CD133+); it uses mAb CC188 in humans as a carrier for the specific delivery of chemotherapeutic drugs in both CSCs and differentiated tumour cells. mAb CC118 has an intrinsic ability to suppress invasiveness of colon cancer cells and coupled with a chemotherapeutic drug, caused the eventual eradication of the tumour. Hence, mAb CC188 has been seen to possess significant tumour sensitivity, specificity, and homogeneity, particularly in human colon cancer [52].

Recent studies have also evaluated a new option for therapy, the marker CD24, which is a glyco-sylphosphatidylinositol-anchored membrane protein with a small protein core and a high level of glycosylation. It is over-expressed in many human carcinomas, as pancreatic and ovarian cancer stem cells. The binding of the CD24 specific monoclonal antibody SWA11 to

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tumour cells in situ was accompanied by changes in Src phosphorylation and the expression of STAT3-dependent genes. It reduced tumour cell proliferation, and a significant reduction in blood vessel density was observed. In addition, SWA11 mAb treatment strongly influenced the intratumoural cytokine milieu and increased the infiltration of tumour tissue by macrophages. This effect seems to be associated with the growth retardation of SKOV3ip ovarian and A549 lung tumour models. Effects on the intratumoural cytokine microenvironment were not exclusive to SWA11 mAb treatment, because anti-L1CAM mAb also induced changes in the intratumoural cytokine profile. Moreover, pretreatment of carcinoma xenografts with SWA11 mAb augmented the effects of conventional chemotherapy with gemcitabine *in vivo*.

7.6. Telomerase Inhibitors

Telomerase is a ribonucleoprotein enzyme, which is recognized for its role in de novo telomeric DNA synthesis: it is composed of telomerase reverse transcriptase (TERT) which catalyzes the addition of telomeric repeats at chromosome ends, and the RNA components (TERC) which are used as template. In human stem cells the levels of TERT are low, because it is repressed, while in human tumourigenesis, telomerase is often reactivated via the transcriptional up-regulation of TERT, thus causing the rapid proliferation of cancer cells. It bypasses checkpoint-signalling pathways that are activated during telomere shortening to induce cellular senescence and cell death. This up-regulation of TERT can be stimulated by transcription factors such as c-Myc, NF-kB, and b-catenin. This demonstrated that TERT possesses the telomerase oncogenic functions involved in signalling cascades such as NF-kB and Wnt/b-catenin pathways that influence cancer development and progression [53].

Hence, the new anticancer strategies evaluate as target the oncogenic activities of telomerase in combination with drugs that inhibit the catalytic activity of telomerase, thus preventing the persistent activation of major oncogenic signalling pathways, and concurrently inducing senescence in proliferating tumour cells. It has been shown that CSCs could respond to telomerase-based therapy [54].

Imetelstat sodium, also called GRN163L, is a synthetic lipid-conjugated 13-base oligonucleotide N3'P5'-thio-phosphoramidate, which acts as telomerase template antagonist. In fact, it is complementary to the template region of telomerase RNA and acts as a competitive enzyme inhibitor by binding and blocking the active site of the enzyme. In clinical imetelstat has been used for the treatment of multiple myeloma (MM), chronic lymphocytic leukemia, non-small cell lung cancer and breast cancer. In a study, imetelstat treatment of 2 weeks caused telomerase inhibition, decreased cell colony formation and shortened telomere length in CD138- CSCs cell lines. By short-term imetelstat treatment, the proportion of CD138- CSCs decreased by approximately 40% and the ALDH+ population cells decreased 1.3%. Furthermore, the expression of stem genes OCT3/4, Nanog, Sox2, and BMI1, and Notch target gene HES1 were inhibited in CD138- NCI-H929 CSCs, indicating that short-term imetelstat can suppress the self-renewal and induce differentiation of MM CSCs, although it is independent of telomere length shortening. *In vivo*, imetelstat treatment significantly prolonged the survival of NOD/SCID mice.

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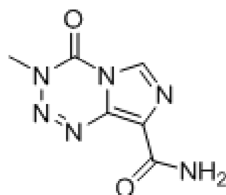

Figure 22.

A study of Marian et al., has shown that a vitro long-term imetelstat treatment on GBM TICs led to telomere shortening, growth arrest and eventual cell death, and had synergic effect with radiation and temozolomide. It also noted the reduction of the volume of subcutaneous tumours derived from glioblastoma TICs and the inhibition of telomerase activity in animals with orthotopic xenograft tumours, since imetelstat penetrated the blood-brain barrier and inhibited telomerase activity. In primary glioblastoma TICs, imetelstat treatment can also produce a dose-dependent inhibition of telomerase. Research also showed that imetelstat treatment caused telomerase inhibition and telomere shortening in MCF7 and MDA-MB231 breast cancer cells and PANC1 pancreatic cancer cells, and *in vitro* it resulted in cell growth inhibition and the depletion of CSCs of breast and pancreatic cancer. Furthermore, pretreatment with imetelstat decreased the tumourigenicity of PANC1 and MDA-MB231 cells *in vivo*. It can also restore the sensitivity of therapy-resistance, acting synergistically with trastuzumab in inhibiting HER2-positive MDA-MB-231 breast cancer cell growth [21], and with Herceptin, paclitaxel, or radiation treatment in breast cancer models *in vitro* and *in vivo*. It was reported that phase I clinical trials in breast cancer patients treated with GRN163L were successfully completed and the agent was selected for phase II clinical trials, testing imetelstat in a combination with standard chemotherapeutic drugs such as bevacizumab and paclitaxel in a group of patients with locally relapsing or metastatic breast cancer. These studies indicate that imetelstat can target CSCs and become a prospective candidate agent for the eradication of cancer.

In addition very recent studies show the formulation of another group of telomerase-targeting drugs. They are telomerase cancer vaccines GRNVAC1 and GRNVAC2, products of mature dendritic cells (antigen-presenting cells) which travel to the lymph nodes and instruct cytotoxic T cells to kill tumour cells that express telomerase.

7.7. Oncolytic Virotherapy

Oncolytic virotherapy is a new anti-tumour strategy which targets CSCs. Oncolytic viruses can infect tumour cells replicating in them, and causing their lysis and probably releasing other viruses to infect other tumour cells. The advantage of this approach consists in the optimal microenvironment offered by tumours for replication of these oncolytic viruses [5], and the restriction of viral entry and replication to CSCs, sparing normal cells.

Among viruses, there is herpes simplex virus-1 [55], a double-stranded, enveloped DNA virus. For the treatment of cancer, several herpes viruses have been genetically engineered.

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Related alterations are the deletion of the γ34.5 genes which, for the infected cell protein ICP34.5, encode a neurovirulence protein that herpes virus uses to promote the host protein synthesis, and mutations of the gene UL39 encoding the large subunit of ribonucleotide reductase. These seem to confer selectivity to cancer cells [55]. These alterations should prevent viral replication in healthy cells, but in some tumours such as gliomas recovery of replication and cell lysis have been observed.

The first variants of viruses oHSV (oncolytic HSV), G207 and HSV1716 to be used, have shown in several phase I trials the increase of survival and no serious adverse effects on patients affected by GBM. Especially G207, which present both mutations, killed CSCs marked by CD133 in human GBM xenolines, and thus it has been demonstrated that all glioma cells, including CD133+ CSCs, are susceptible to γ34.5-deleted oHSV oncolysis in relation to CD111, viral entry cell receptor which predicts sensitivity to HSV therapy.

Moreover, another study evaluates the efficacy of a derivate of G207, G47Δ, which causes suppression of CSCs self-renewal and the increase of CSCs killing and viral replication. It was also been demonstrated that G47Δ was capable of infecting and replicating in CSCs in hypoxic conditions; in fact it noted a decrease of CD133+ CSCs *in vitro* and *in vivo*. Furthermore it has been observed that, arming oHSV with IL-12 and angiostatin, it will likely increase the oHSV efficacy because this will allow the interruption of the recruitment of Tumor-associated macrophages (TAM), which limits the spread of virus by phagocytosis, and the Tregs (regulatory T cells), both very important for the maintenance of immunosuppressive micro-environment. Therefore, it causes a robust immune response against malignant cells, including CSCs. In addition it has been shown that the combination of γ34.5 deletion oHSV with doxorubicin also allows the destruction of the resistant CSCs remaining, enhancing survival in an *in vivo* model.

Another virus to target CSCs is Adenovirus (Ad), a double-stranded, non-enveloped virus which is capable of infecting both dividing and non-dividing cells. They can entry cells during the G_1 phase cellular cycle through the viral immediate-early protein E1A binding to retinoblastoma (Rb) protein, which releases active transcriptional factor E2F. To achieve tumour-specific or conditionally replicating Ad (CRAd), deletions are operated in the Rb-binding site of E1A (Δ24), which abrogates viral replication in normal cells, or it is attached to tumour-specific promoters. Ad H101 has completed a phase III trial for the treatment of squamous cell carcinoma of the head and neck and esophagus, indicating a 78.8% response rate with virus; in addition the combination therapy with cisplatin plus 5-fluorouracil shows a higher response rate.

Recent studies of breast CSCs demonstrate the highest *in vitro* efficacy of Ad5/3-Δ24 and Ad5, showing in a *in vivo* model the reduction of CD44+CD24−/low CSCs. In addition, Bauerschmitz et al. tested a variety of tumour-specific promoters and capsid configurations. The highest oncolytic activity administered at the lowest doses seemed to be owned by the Ad5/3-multidrug resistance (MDR)-Δ24, in which MDR genes encode for a pump that decreases the intracellular accumulation of chemotherapeutic, and Ad5/3-cyclooxygenase Cox-2L-Δ24, in which Cox-2 support tumourigenesis and survival cell [5].

In another study, a telomerase-specific oncolytic adenoviral vector carrying TRAIL and E1A genes, Ad/TRAIL-E1, preferentially showed to target and kill radioresistant human esophageal carcinoma cells, with CSCs characteristics and higher telomerase activity. The *in vivo* model Ad/TRAIL-E1 was injected intratumourally and showed apoptosis of the radio-resistant tumour cells in xenografts. 40% of the mice survived free of tumours for more than

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180 days. Moreover, Ad/TRAIL-E1 did not show significant toxicity on normal cells *in vitro* and *in vivo* [21].

Another oncolytic virus is Measles Virus (MV). MV is an antisense, non-integrating RNA virus. The attenuated Edmonton vaccine strain, oMV-Edm, was first isolated from throat washings of a measles patient and it showed potent anti-tumour properties with a superb safety profile. Allen et al investigated the ability of oMV-Edm to infect and kill glioma CSCs, and it noted syncytia formation of CD133+ CSCs or enhanced infectivity, which must be considered able to totally eradicate tumours. Moreover, to increase selectivity, oMV was fused to a single-chain antibody, scFV, specific to CD133 CSCs, sparing the CD133+ somatic cells and hematopoietic stem cells [5]. In another study oMV was retargeted by using designed ankyrin repeat proteins, DARPins, which exclusively bind the HER2/neu. The DARPin-targeted oMV showed that tumours grew more slowly than tumours treated with scFV-targeted virus, and attenuated toxicity.

The Reovirus also displays severe cytopathic effects and oncolytic potential. It is a double-stranded RNA virus, which employs tumour-specific viral replication and oncolysis through the oncogenic Ras signalling pathways that attenuate the translational arrest associated with antiviral PKR response. Indeed, in a study utilizing reovirus in a CSCs breast mouse model, tumour remission was observed with a decrease in CSCs percentages due to apoptotic cell death [5].

Finally another virus used is Vacciniavirus (VACV) which is a double-stranded, enveloped DNA virus in the poxvirus family. Its oncolytic potential appears with rapid replication and cell death, as well as the cytotoxic immune cell response. Vaccinia JX-594 was used in phase I trial for hepatocellular carcinoma, and showed clinical responses in four out of five patients after intravenous administration, expressing granulocyte macrophage-colony. Furthermore Wang et al. demonstrated that VACV replicates more efficiently in breast CSCs with increased ALDH activity, and above all in the CD24+ subset of ALDH+ cells compared with CD24−/low. *In vivo* it showed the general inhibition of breast cancer, especially in the CD24+ subset. VACV could be systemically administered to treat breast cancer. Moreover, recent reports have shown a new targeting platform in which VACV is used as vector to express BMP-4, which has an important role in regulating cancer, to expedite the differentiation of Glioblastoma multiforme (GBM) CSCs. In a *in vivo* model tumour regression and survival improvement in mice [56].

Next-generation viruses are being developed to enhance direct targeting of CSCs and indirect targeting of the niche through the localized production of small molecules.

7.8. Inhibitors of Protein Kinase C-Delta

Recent studies have shown the importance of Protein Kinase C-Delta (PKCδ) as a lethal target in CSCs. It is a novel class serine/threonine kinase of the PKC family, and functions in a number of cellular activities including cell proliferation, survival or apoptosis [48]. Tumours with aberrant activation of the PI3K pathway or the Raf-MEK-ERK pathway in the setting of wild-type RAS alleles have also been shown to require PKCδ activity for proliferation or survival.

One of the first PKCδ inhibitor was **Rottlerin**, a natural product which induced cytotoxicity in human prostate and pancreatic CSCs cultures in a dose-dependent manner, and

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decreased the clonogenic capacity of PCSC by 90% after an exposure of 48 hours. Subsequently the second and the third generation of PKCδ inhibitors were synthesized with greater specificity.

KAM1, a chimeric molecule, is a second generation inhibitor which showed potent activity against Ras-mutant human cancer cells in culture and *in vivo* animal models while not producing cytotoxicity in normal cell lines, and it induced cytotoxicity in a dose-dependent fashion in both PCSC and PrCSC cultures at concentrations as low as 2.5 μM (PCSC) and 5 μM (PrCSC).

The compound of the third generation is **BJE6-106**, which shows the inhibition of the PCSC culture growth at lower concentrations such as 0.1 μM. By inhibiting PKCδ, it prevents tumour sphere formation and blocks the growth of CSCs in vivo in a mouse xenograft model. These findings suggest that the new PKC isozyme PKCδ may represent a new molecular target for CSCs populations [57].

Conclusion

Recent advances in cancer therapy have led to the improvement of the quality of life and to increase the median survival rates. However the principal barrier of conventional treatment is the habitual relapse of tumours. Therefore, several studies have investigated in this field to understand the origin of this resistance. It has been hypothesised the presence of a subpopulation cells, called CSCs. New research is constantly developing, with the objective to investigate in this type of cells, trying to discover their proprieties and their functions within tumours. This investigation is often realized by experimenting new compounds, which target the principal factors involved in the regulation of CSCs and verify potential consequences in cancer. In this review we want to sustain the CSCs theory, analyzing studies which show the efficacy of some drugs, targeting CSCs, *in vitro* and *in vivo*. Nowadays, all the drugs that kill CSCs are still in phases I, II, and III of clinical trials and it is hoped that in the future a drug will be found to succeed in totally eradicating tumours.

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Chapter 4

Metallomics of Brain Tumors - New Diagnostic and Therapeutic Possibilities

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Abstract

Brain tumors are the leading cause of cancer-related death in the US in patients under the age of 35. They are divided into primary and secondary (metastatic) that are more frequent in adults (melanoma, lung carcinoma, renal cell carcinoma and thyroid carcinoma as into brain mostly metastasizing).

Metallomics can be defined as comprehensive analysis of the entirety of metal and metals containing proteins within cells and tissues. Approximately 1/3 of proteins are

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associated with some metals. Moreover, some drugs contain a metal in their structure and are referred to as metallodrugs.

Up-to-date, the greatest knowledge has been gathered in the field of metalloproteins containing zinc because it has been shown to be not only a structural component, but also a signaling substance in number of cascades and a cofactor of many important enzymes. On cellular level, key cellular processes such as proliferation, differentiation and apoptosis have been connected with its signaling. In the field of metallomics of brain tumor, there is still little known but our knowledge is rapidly growing. On other metals in brain tumors, there are only limited reports as the grade of glioma correlated with the Fe(II) to Fe(III) ratio in the tissue. However, this relationship cannot be explained by occurrence of hypoxic regions in the tissue because of lack of correlation between the average oxidation state of iron and hypoxia.

In the case of non-essential metallomics, the cytotoxic effect of platinum drugs consists of DNA adducts formation, especially to the most nucleophilic bases - guanine and adenine. This phenomenon causes DNA strands crossing and subsequent interference with normal transcription and/or replication. Moreover, platinum drugs influence the cell cycle of tumor cells, which can also influence their effect. In addition, many potential platinum-binding molecules are available including RNA and sulfur-containing biomolecules such as glutathione and metallothioneins in the cytoplasm. Binding of platinum cytostatics to MT results in their inactivation and is one of the mechanisms of chemoresistance to those cytostatics. The exact mechanism by which MT prevent "non-platinum" cytostatics induced cell death has not been established until now.

The aim of this review is to evaluate the current state of research on metals and metalloproteins in brain tumors and report new insights into the diagnostics, therapy by metal containing drugs, including resistance to those cytostatics and potential of metalloproteins-targeted therapy and drug nanocarriers containing metals to improve the treatment efficiency in treatment of brain tumors.

Introduction

Brain Tumors

Brain tumors are divided into two groups: (i) primary that originate and reside within the brain and (ii) secondary (metastatic) that originate from a primary cancer outside the central nervous system and spread into the brain. Metastatic ones are more frequent than primary in adult patients while primary are the most frequent solid tumors of childhood. Primary brain tumors represent a heterogeneous group that is classified according to WHO classification, (see Table 1). This classification is based on morphological, immunohistochemical and molecular genetic examination e.g. 1p and 19q lose in oligodendroglioma or mutation or loss of the SMARCB1 in atypical teraoid/rhabdoid tumors (AT/RT).

According to the Central Brain Tumor Registry of the United States (CBTRUS) 2005–2009 report, the incidence of CNS tumors in the USA is 20.6 cases per 100,000 persons/year, incidence of malignant is 7.3 and of low grade is 13.3. The histological spectrum of brain tumors in children and adolescents differs from that in adults (Table 2).

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Table 1. WHO classification of brain tumors. Adapted from [1]

Tumors of Neuroepithelial Tissue	Tumours of the Meninges
Astrocytic tumors	**Tumours of meningothelial cells**
Pilocytic astrocytoma	Meningioma
Pilomyxoid astrocytoma	Meningothelial
Subependymal giant cell astrocytoma	Fibrous (fibroblastic)
Pleomorphic xanthoastrocytoma	Transitional (mixed)
Diffuse astrocytoma	Psammomatous
Fibrillary astrocytoma	Angiomatous
Gemistocytic astrocytoma	Microcystic
Protoplasmic astrocytoma	Secretory
Anaplastic astrocytoma	Lymphoplasmacyte-rich
Glioblastoma	Metaplastic
Giant cell glioblastoma	Chordoid
Gliosarcoma	Clear cell
Gliomatosis cerebri	Atypical
Oligodendroglial tumors	Papillary
Oligodendroglioma	Rhabdoid
Anaplastic oligodendroglioma	**Mesenchymal tumors**
Oligoastrocytic tumors	Lipoma
Oligoastrocytoma	Angiolipoma
Anaplastic oligoastrocytoma	Hibernoma
Ependymal tumors	Liposarcoma
Subependymoma	Anaplastic (malignant)
Myxopapillary ependymoma	Solitary fibrous tumor
Ependymoma	Fibrosarcoma
Cellular	Malignant fibrous histiocytoma
Papillary	Leiomyoma
Clear cell	Leiomyosarcoma
Tanycytic	Rhabdomyoma
Anaplastic ependymoma	Rhabdomyosarcoma
Choroid plexus tumors	Chondroma
Choroid plexus papilloma	Chondrosarcoma
Atypical choroid plexus papilloma	Osteoma
Choroid plexus carcinoma	Osteosarcoma
Other neuroepithelial tumors	Osteochondroma
Astroblastoma	Haemangioma
Chordoid glioma of the third ventricle	Epithelioid haemangioendothelioma
Angiocentric glioma	**TUMORS OF CRANIAL AND PARASPINAL NERVES**
Neuronal and mixed neuronal-glial tumors	Schwannoma (neurilemoma, neurinoma)
Dysplastic gangliocytoma of cerebellum (Lhermitte-Duclos)	Cellular
Desmoplastic infantile astrocytoma/ganglioglioma	Plexiform
	Melanotic
Dysembryoplastic neuroepithelial tumor	Neurofibroma
Gangliocytoma	Plexiform
Ganglioglioma	Haemangiopericytoma
Anaplastic ganglioglioma	Anaplastic haemangiopericytoma
Central neurocytoma	Angiosarcoma

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Table 1. Continued

Extraventricular neurocytoma Cerebellar liponeurocytoma Papillary glioneuronal tumor Rosette-forming glioneuronal tumor of the 4th ventricle Paraganglioma **Tumors of the pineal region** Pineocytoma Pineal parenchymal tumor of intermediate differentiation Pineoblastoma Papillary tumor of the pineal region **Embryonal tumors** Medulloblastoma Desmoplastic/nodular medulloblastoma Medulloblastoma with extensit nodularity Anaplastic medulloblastoma Large cell medulloblastoma CNS primitive neuroectodermal tumor CNS Neuroblastoma CNS Ganglioneuroblastoma Medulloepithelioma Ependymoblastoma Atypical teratoid / rhabdoid tumor Perineurioma Malignant perineurioma Malignant peripheral nerve sheath tumor (MPNST) Epithelioid MPNST MPNST with mesenchymal differentiation Melanotic MPNST with glandular differentiation	Kaposi sarcoma Ewing sarcoma - PNET **Primary melanocytic lesions** Diffuse melanocytosis Melanocytoma Malignant melanoma Meningeal melanomatosis **Other neoplasms related to the meninges** Haemangioblastoma **LYMPHOMAS AND HAEMATOPOIETIC NEOPLASMS** Malignant lymphomas Plasmacytoma Granulocytic sarcoma **GERM CELL TUMORS** Germinoma Embryonal carcinoma Yolk sac tumor Choriocarcinoma Teratoma Mature Immature Teratoma with malignant transformation Mixed germ cell tumor **TUMORS OF THE SELLAR REGION** Craniopharyngioma Adamantinomatous Papillary Granular cell tumor Pituicytoma Spindle cell oncocytoma of the adenohypophysis **METASTATIC TUMORS**

Table 2. Frequency of brain and CNS tumors according to age. Adapted from [2, 3]

Children <15 y	Adolescents 15-19	Adults > 19
Pilocytic astrocytoma	Pituitary tumors	*Metastatic tumors*
Embryonal tumors	Pilocytic astrocytoma	Meningiomas
Malignant glioma	Other astrocytomas	Glioblastoma
Other astrocytomas	Neuronal and mixed-neuronal glial tumors	Pituitary tumors
Neuronal and mixed-neuronal glial tumors	Neuronal sheath tumors	Neuronal sheath tumors
		Astrocytomas

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The most frequent brain tumors in all age groups are gliomas that represent a wide spectrum of tumors ranging from slow growing to highly aggressive tumors. WHO classifies gliomas into four grades: grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma), and grade IV (glioblastoma multiforme). The grade III and IV are considered high-grade gliomas (malignant gliomas) and are associated with very bad prognosis. In particular, 5 year survival rate of glioblastoma multiforme accounting for half of primary brain tumors is less than 10% [4].

Brain metastases are the most common intracranial tumors in adults, with more than 150,000 cases in the USA, i.e. in approximately 8–10% of adults with cancer developed brain metastases, even though their incidence varies significantly among different primary tumor types. The origin of cerebral metastasis are lung, breast, melanoma, colorectal, or renal cell cancers, 70% brain metastases are consisting of lung and breast cancer [5, 6].

The location of brain metastases are concurrent with cerebral blood flow, with 80% of metastases located in the cerebral hemispheres, 15% in the cerebellum, and 5% in the brainstem [7]. Their incidence is increasing with a longer overall survival of patients with those cancers [5].

Various metal ions, both in their free forms and in the complexes, play a significant role in the development of nervous systems and brain tumors. Moreover, a lot of drugs based on metals are used for treatment of brain tumors. Therefore, the goal of this chapter is to review the knowledge on the brain tumor metallomics.

Metals and Their Biological Significance of Role in the Development of the Numerous Systems

Metals and their ions are ubiquitously present in the environment and many of them can bioaccumulate. Human exposure to metal ions has risen due to use of these metal elements in industrial products, use of deeper sources of water and erosion. Additionally, many occupations involve exposure to metals and metal conjugates. Metal ions are systemic toxins that interact with specific systems to produce neurotoxic, cardiotoxic, teratogenic, and/or nephrotoxic effects. Metals ions are taken into the body *via* ingestion, inhalation, and dermal routes, can accumulate and can be stored in both soft tissues and bones. These ions disrupt a variety of metabolic processes by altering a number of homeostatic processes including antioxidant balance, binding to free sulfhydryl groups, and competing for binding sites on enzymes, receptors and transport proteins. The biological half-lives for metal ions can be decades and many are readily transferred across the placental and blood-brain barriers and are known to have serious damaging effects on the developing nervous system. In adults, chronic symptoms frequently associated with accumulation of metal ions include fatigue, allergic hypersensitivity, and neurological disorders. The actual concentrations of metal ions in cells are crucial for their role in health and diseases. Therefore, their transport across the membranes of the cells and their compartments is essential for their physiological action. Entry of the metal ions into the cell and their transport through the cell is summarized in Figure 1.

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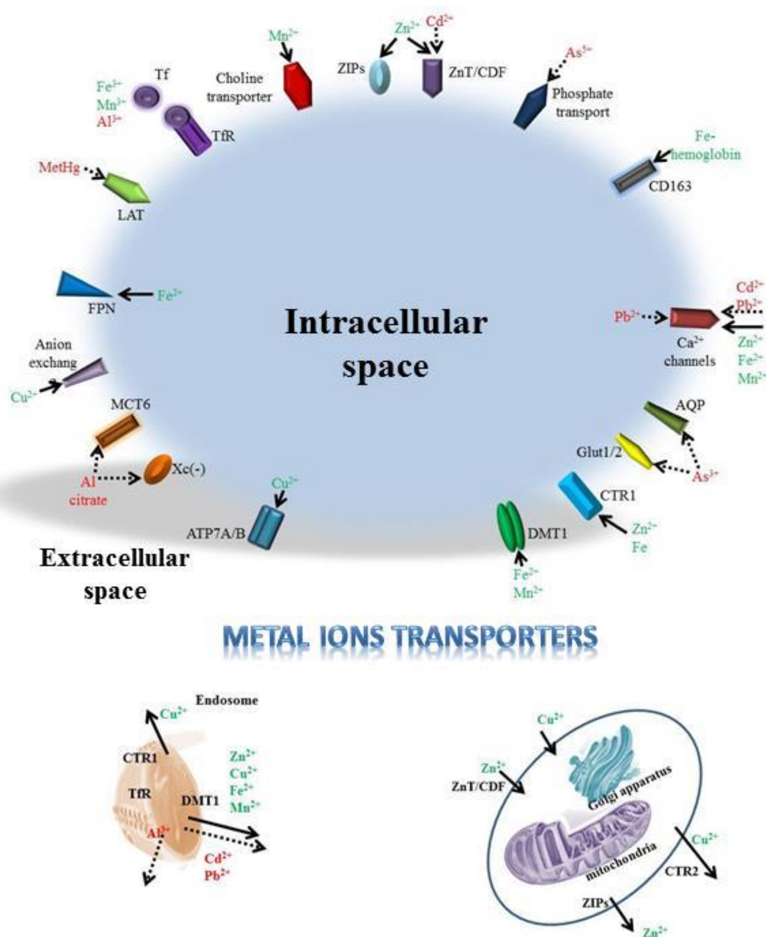

Figure 1. Cellular transport of metal ions. The mechanisms of cellular handling of both essential and non-essential metals. Transporters whose primary functions are to transport metal ions are bolded, while those whose primary function is not the transport of metal ions are italicized. Essential metal ions are represented in green and non-essential metal ions in red. (Adapted from [10]).

Almost one-third of proteins require metal ions, with approximately 47% of enzymes requiring metal ions, and 41% requiring metal ions at their catalytic centers [8]. The enzymes containing metal ions (metalloenzymes) make up about 44% of oxidoreductases, 40% of transferases, 39% of hydrolases, 36% of lyases, 36% of isomerases and 59% of ligases [9].

From the physiological point of view, metal ions are usually divided into two groups: (i) the essential metal ions and (ii) the non-essential metal ions. Ions of iron (Fe), copper (Cu), manganese (Mn), and zinc (Zn) are nutrients or essential metal ions and because of this cells have mechanisms to acquire them from their extracellular environment. There are no known transporters for non-essential metals however; many of the transporters available for essential metal ion transport can be hijacked by non-essential metal ions such as for example ions of cadmium (Cd), lead (Pb), and mercury (Hg). Many metal ions can pass through the cell membrane alone or in a complex with other proteins, a process referred to as molecular mimicry. Oftentimes the metal ions in complexes can enter the cell more readily than the metal ion alone, especially if the latter is charged. Ion pumps can be commandeered by non-

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essential metal ions, leaving essential metal ions to compete for entry. Calcium channels and anion transporters make up another mechanism of entry as well as amino acid and organic anion transporters when the metal ions are bound to amino acids or organic ions. Intracellular transport mechanisms are also present. Increased body burden may occur when metal ions enter and accumulate in body tissues faster than the body's detoxification pathways can dispose of them and the balance between uptake and efflux is tipped [8].

Significance of Metals and Metalloproteins in Physiology and Pathology

The ions of sodium, potassium and calcium metals are the most important in the organism. They provide many important functions e.g. cellular polarization, regulation of blood and body fluids, transmission of nerve impulses, heart activity, and several metabolic functions moreover calcium is important component of bones. The organism of adult human contains approximately 175 g potassium, 50 g of sodium and 40 g of calcium [11]. These metals are usually not subject of interest of metallomics, which deals mainly with metalloproteins.

Metal ions help to stabilize protein structure and to induce a conformational change upon binding, and/or participate in catalysis [12]. The structure of a protein determines its function and its interactions with other components e.g. proteins and cofactors, including metal ions. As it was mentioned above approximately one-third of all proteins bind at least one metal ion, and many different types of metal ion binding proteins are essential for life [9]. As mentioned above, the majority of them are enzymes (e.g., proteins containing Fe, Cu, Zn ions) or transcriptional factors (particularly Zn) (see Table 3). The ability of many these metal ions to exist in multiple oxidation states and different geometries allow them to promote complex biochemical reactions and participate in highly specialized biological functions [13]. Therefore metals deficiencies can cause a variety of faults.

Iron deficiency symptoms are microcytic anemia, atrophy of the mucous membrane, hair loss, restless legs syndrome, brittle or grooved nails, impaired immune functions, and irritability. A copper deficiency, which is rare in humans, causes anemia, neutropenia and a wide variety of neurological problems including myelopathy, peripheral neuropathy, and optic neuropathy. A zinc deficiency may manifest by skin or mucous lesions (acne, eczema, xerosis, seborrheic dermatitis, alopecia, oral ulceration, stomatitis, or white tongue coating), reduced circulating testosterone and hypogonadism, delayed growth and impaired cognitive and motor functions in children. Moreover it compromises immunity. Animal studies and clinical observations show that zinc deficiency induce thymic atrophy, lymphopenia, and compromised both cell- and antibody-mediated immune responses. A zinc deficiency may also affect immune responses against tumors so it may have indirect effects on cancerogenesis [14]. Symptoms of magnesium deficiency include hyperexcitability, dizziness, muscle cramps, muscle weakness and fatigue. It may decrease the calcium and potassium serum levels, the levels of parathyroid hormone and causes sodium retention and worsens insulin resistance. Main symptoms are neurological and muscular (tremor, fasciculations, muscle spasms, tetany), loss of appetite, nausea, vomiting, personality changes, and severe deficiency may cause death from heart failure [15]. A manganese deficiency results in joint pain and arthritis, bursitis, dermatitis, impaired glucose tolerance,

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osteoporosis, epilepsy, infertility, weakness, nausea or vomiting, dizziness, hearing loss, iron-deficiency anemia, weak hair and nails and blindness or paralysis in infants [16]. Symptoms of a nickel deficiency can range from urinary tract infections to severe allergic reactions, most often seen in the form of skin rashes. In very severe cases, those that suffer from a nickel deficiency may experience paralysis alongside inflammation of the liver and lungs. It can disturb the incorporation of calcium into skeleton; reduce iron levels and leads to anaemia. In addition, nickel ions may induce allergies and cancer. Carcinogenic actions of its compounds involve oxidative stress, genomic DNA damage, epigenetic effects, and the regulation of genes expression by activation of some transcription factors [17].

Moreover, ions of metals such as arsenic, nickel, cadmium and chromium have been reported to be carcinogenic. Nickel subsulfide enhances infidelity of a gene replication *in vitro* and alters bacterial DNA repair. Chromium is carcinogenic as a chromate ion, which is in cells reduced to Cr(III) and binds to DNA. Cadmium affects cell proliferation, differentiation, apoptosis and induces mutations by inducing oxidative DNA damage. Arsenic induces reactive oxygen species that cause DNA damage. However, molecular mechanisms of metal-induced carcinogenesis have not yet been fully elucidated [18].

It is clear from the above data that metal ions and metalloproteins play an important role in many physiological functions of organisms and in pathogenesis of many diseases including carcinogenesis and tumor development.

Physiological Significance of Metals and Metalloproteins in Neural Tissue

Metal ion homeostasis is essential for the proper brain function [19]. When homeostatic controls fails, due to aging or diseases, alterations can result in pathological changes, as the brain is very vulnerable to metal-induced oxidative stress [20]. Among the most fundamental biometals, playing a key role in diverse physiological processes in brain, belong copper, iron, calcium and zinc [21]. As was reported previously, many neurodegenerative (e.g. copper and zinc sequestration in senile plaques) and neuropsychiatric disorders (e.g. zinc deficiency in autistic children) coincide with the dysregulation of the above mentioned divalent metal ions, leading to a binding to metalloproteins in a competitive manner [22-25].

Copper (Cu) is an essential element for life as it plays key roles in a number of important biological processes [26, 27]. In brain, this metal is unequally distributed with an average content of about 80 μM, while higher concentrations can be found in *locus coeruleus* (1.3 mM) and in the *substantia nigra* (0.4 mM) [28]. Only little is known about the mechanisms, which neural cells employ for a maintenance of Cu homeostasis and about the consequence of elevated Cu in environment [29]. However, it was observed that the mutations in genes, encoding intracellular Cu transporting ATP-ases, *ATP7A* and *ATP7B,* result in disruption of Cu homeostasis [30]. The loss of the Cu-ATPases function is thus associated with severe metabolic disorders as Menkes disease and Wilson disease [31]. It has also been hypothesized that copper can act as the “the unknown metal ion” important for a proper function of the ubiquitin proteasome system, while the absence of these ions stops the proteins ubiquitination with undesired health effects [32]. Other detrimental effects of Cu ions are attributed to an ability to bind to inappropriate ligands, thus triggering redox transformation that generates cytotoxic reactive oxygen species (ROS) and neural damage [33].

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Iron (Fe) is an micronutrient important not only for hemoglobin and myoglobin synthesis but also for neuronal functions and survival, since it plays an essential role in DNA and protein synthesis, neurotransmission and an electron transport chain [34]. Although it is not entirely clear how Fe ions cross the blood-brain barrier, suggested routes include a divalent metal transporter 1 (DMT1) and transferrin or lactoferrin receptors (TfR/LfR) that facilitate clathrin-mediated endocytosis [35]. When compared to other brain areas, Fe ions are present in the highest concentrations in the basal ganglia, and more specifically in the subthalamic nucleus (STN) [36, 37]. Iron deficits induce a decrease in the expression and function of important proteins like Insulin-like growth factors I and II (IGF-I/II) or the Brain-derived neurothrophic factor (BDNF), which have an essential role in processes involving learning and memory [38]. In addition, the iron overload is suggested to be a major cause of oxidative stress in neurons [39].

Important iron containing metalloprotein - transferrin (Tf) a 78 kDa-monomeric glycoprotein is synthesized in the brain by choroid plexus and oligodendrocytes [40]. Transferrin is a major iron delivery protein to the brain and a major source of iron delivery to neurons [41]. It has a capacity to reversibly bind two atoms of ferric iron (Fe^{3+}). To enter brain, Tfs with iron must enter the endothelial cells to cross the blood-brain barrier (BBB), through transferrin receptors (TfR1/TfR2), expressed on their luminal surface [42]. Endothelial cells use iron from Tf in an endocytic pathway through a divalent metal transporter 1 (DMT1), and iron is delivered across the BBB to the parenchyma *via* a Tf bound mechanism and through a non-Tf bound mechanism [43, 44]. In that case, the proper role of DMT1 in brain iron uptake is not uniformly accepted, but its requirement for the brain microvasculature is corroborated [45]. The clathrin-mediated endocytosis of drugs through TfR1 and/or TfR2 is currently considered as a promising way, which can be suitable to overcome the protection of brain by BBB [46, 47]. Similarly, lactoferrin (Lf), belonging to the transferrin proteins family was reported to bind iron and cross the BBB *via* receptor-mediated transcytosis [48], provided by Lf receptors (LfR), expressed on the surface of dopaminergic neurons and endothelial cells of mesencephalic microvessels [49]. However, regarding the physiological roles of Lf in the brain, it is still not clear whether or how it contributes to the transport of iron into the brain.

Since Tf and Lf are able to deliver iron into neural cells, ferritin forms a crucial part of a chemical machinery, which cells developed for iron storage, in a nontoxic form, which is not required for immediate metabolic purposes [50]. From this point of view, its main role is to prevent a harmful accumulation of iron inside the organism by collecting free iron in the form of ferrihydrite phosphate $[(FeOOH)_8(FeOPO_3H_2)]$ in its core for further using these ions as the enzymatic cofactors [51]. Although ferritin is considered cytoplasmic iron storage, novel functions for ferritin have recently been discovered, including ability to deliver iron into brain across the BBB *via* clathrin peptides, using the same receptor system as transferrin [52].

Disorders in ferritin functions have been related with typical iron-related diseases, such as hemochromatosis or sideropenic anemia, but ferritin is also increasingly being recognized as a crucial molecule in some neurological pathology, as Parkinson [53] or Alzheimer's diseases [54].

Calcium (Ca) ions are versatile biological signaling factors that regulate numerous cellular processes from cell fertilization, to neuronal synaptic plasticity [55]. Ca^{2+} ions are tightly regulated within the intracellular and extracellular compartments of the central nervous system involving processes that include transport mechanisms across the blood-brain

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barrier and cellular membranes, extensive binding by proteins and sequestration within a variety of intracellular organelles [56]. These events are directly dependent on the entry of extracellular Ca^{2+} ions into the cells through specific voltage-sensitive Ca^{2+} channels (VSCC), found mostly in the membrane of excitable cells (glial cells, neurons, etc.) [57]. VSCC comprise various subunits: α_1, $\alpha_2\delta$, β_{1-4}, and γ, however only α_1 forms the pore and associated subunits mostly modulate the gating [58]. The disruption of proper calcium channeling can result in disorders, collectively termed "calciopathies", covering a broad category of pathological states as paralysis, bipolar disorder, autism or neurodegenerative diseases [59, 60].

Brain zinc (Zn) homeostasis is strictly controlled under healthy conditions, indicating the essentiality of zinc for its physiological functions [61]. Approximately 80 % of total brain zinc exists in form of the zinc metalloproteins. The chemical form of residual zinc in the synaptic vesicles is still unknown, however it was found that vesicular Zn^{2+} composes a major pool for intracellular Zn^{2+} signaling (concentration exceeding 1 $mmol.L^{-1}$) [62, 63]. Generally, Zn^{2+} levels, except for vesicular Zn^{2+}, are estimated to be less than 5 % of the total Zn^{2+} in the hippocampus and cerebral cortex [64].

Zinc regulates ion channels and transmitter receptors such as α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and γ-aminobutyric acid A ($GABA_A$) receptors which are implicated in a synaptic plasticity and a memory consolidation [65]. Thus, disruption of homeostasis directly impacts learning and/or memory skills. Moreover, previously, it was shown that zinc can interact with amyloid-beta (Aβ) to form aggregates [66] and only a small elevation in brain zinc concentration (> 3 μM) can significantly increase the adhesiveness of Aβ and changes the Aβ metabolism [67].

Metallothioneins (MTs) - a family of cysteine-rich, low molecular weight (500 - 14 000 Da) proteins, were documented to bind a wide range of mono- and divalent metal ions [68, 69]. In various types of cancer, MTs have been extensively investigated as molecular prognostic/diagnostic markers [70]. MTs are divided into four isoforms: MT-1, MT-2, MT-3, and MT-4, which are encoded by a single gene and the MT-1 with many subtypes (MT-1A, -B, -E, -F, -G, -H, -M, -X) [71]. The naturally occurring MTs has Zn^{2+} in both binding sites, that may be substituted for another metal ion that has a higher affinity for thiolate (Pb, Cu, Cd, Hg, Ag, Fe, Pt and Pd).

The central nervous system (CNS) comprises mainly the MT-3 isoform that was detected in neurons, astrocytes, in the cortex and hippocampus, but regarding the cellular source of MT-3 in CNS there is still significant uncertainty [72]. MT-3 appears to be involved in the prevention of neurodegenerative disorders caused by insoluble Cu-peptide aggregates, as it triggers a Zn-Cu swap that may counteract the deleterious presence of copper in neural tissues [73]. Due to the zinc-binding sites, highly sensitive to redox states, MT-3 may be further employed as a zinc-donor, raising free zinc levels in neurons and astrocytes during signaling events, where elevation in zinc concentration may stimulate diverse cellular-response signals [74]. Previously, it was also demonstrated that MT-3 has more complex functions that extend well beyond a role as a simple buffer for zinc. It was shown that MT-3 may regulate the levels of lysosomal proteins, thus regulating the functions of lysosomes [75]. The absence of MT-3 in astrocytes leads to changes in the levels of the lysosomal membrane permeabilization (LMP) and glycosylation patterns. Thus, it can be concluded that the absence of MT-3 results in a drastic reduction of a lysosomal degradative capacity, however, the accurate mechanisms is still not satisfactory elucidated.

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Modern Methods in Metallomics

There are few main approaches that are being developed in metallomics and metalloproteomics. The first are the widely used analytical techniques of mass spectrometry, in particular electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) or imaging (MALDI-MSI), surface enhanced laser desorption/ionization-time of flight (SELDI-TOF) or imaging (SELDI-MSI) and inductively coupled plasma mass spectrometry with laser ablation (LA-ICP-MS). These techniques are ideal partners in the structural and functional characterization of metalloproteins and their distribution in the neural tissue. Since for MALDI/SELDI-TOF, sequential chromatographic or 2-D gel separations are required prior to protein identification [76, 77], using an MSI mode, tissue slices are analyzed directly. MALDI/SELDI-MSI offers comprehensive insight into a function of the important metalloproteins and their interaction with fundamental trace metals, such as Cu, Zn, Fe or Ca, whose biodistribution - elemental mapping, can be determined by using LA-ICP-MS [78-80]. Together, these data can provide important pieces into the brain tumor-metal-metabolism puzzle in terms of spatial resolution down to 1 μm, and thus may be employed as an alternative strategy for microanalysis of immunohistochemical sections [81].

The second approach in metallomics is high-throughput X-ray absorption spectroscopy (HT-XAS) to provide a direct metal analysis of proteins and a proteomic metal distribution in tissues and cells [82]. HT-XAS utilized an appropriate energy, produced by a synchrotron source, which ejects the electrons from the first electron shell, surrounding the nucleus of a metal atom. This leads to stabilization of atom through a passage of another electron from higher-energy shell, which fills the “hole”. The differences in the energy between two orbitals are monitored by the subsequent X-ray fluorescence emission [83, 84]. HT-XAS can thus be used to detect and quantify metals bound to proteins based on the energy and intensity of emission signals; however determination of the binding sites is possible only when combining with labor-intensive techniques, such as X-ray crystallography or Nuclear Magnetic Resonance (NMR) [85-87].

The third fundamental approach is computational bioinformatics analysis of the results obtained. Compared to genomics and proteomics, metallomics and metalloproteomics employing modern nanotechnologies are relatively new fields that require the design and development of completely new approaches to analysis of the data. It has to be acknowledged that genomics and proteomics have already collected a large amount of data that can be reused in metallomics and metalloproteomics studies to speed up advancement of these new disciplines.

This is certainly a considerable advantage. But these available data provides only a part of the complete picture – it has to be complemented by numerous additional measurements of a different nature and processed by modern information technologies.

Taken together, modern metallomic methods can provide a large bulk of data which have to be processed by using bioinformatical tools to evaluate the data properly. A combination of the above mentioned methods offers comprehensive insight into molecular processes, which are involved in the development of pathological states as brain tumors.

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Metallomics of Brain Tumors

Introduction to Tumor Metallomics

Further we demonstrated on examples of several metalloproteins their importance for the development and behavior of tumors (tumor supressor gene, oncogene, metalloenzymes that support invasiveness and metastasis, group of metalloproteins that control metal metabolism and oxidative stress, and enzymes modifying cancerogens and anticancer drugs) (see Table 3).

The *TP53* gene, which encodes protein p53, is probably the most frequently mutated gene in human cancers, approximately half of all cancers have mutated (inactivated) p53 [88] (see Tab. 4). The p53 protein is involved in different cellular processes that are important for the cell fate (gene transcription, DNA repair, cell cycling, genomic stability, chromosomal segregation, senescence, and apoptosis). A wild-type p53 protein can transcriptionally transactivate genes involved in the cell cycle arrest and interact with the DNA repair proteins, proteins modulating apoptosis; moreover it can inhibit DNA synthesis by a transcription independent mechanism and avert initiation or early replication fork unwinding. The p53 forms protein-protein complexes with cellular proteins involved in DNA synthesis, DNA repair, and apoptosis [89].

Binding of the zinc ion is necessary for a correct conformation of a wild-type p53 protein. Cells with some types of mutant p53 that are cultivated in medium with a high content of zinc may restore wild-type functions [90].

Apo-metallothionein (MT that does not contain metal) can regulate the conformation of zinc containing metalloproteins including p53 by competing for this metal. Moreover, apo-MT1 may forms complex with p53 that inhibits binding of p53 to DNA thus p53 is not able to act as the transcription factor [91]. On the other hand active p53 is necessary for metal-dependent expression of MREs that induces MT expression [92].

Table 3. Overview of some metalloproteins important for cancerogenesis and cancer development

Protein	Metal	Function
p53	Zn	regulates cell cycle, initiate apoptosis, activate DNA repair
p63	Zn	sequence specific DNA binding transcriptional activator or repressor
p73	Zn	apoptotic response to DNA damage
Ras subfamily	Mg	controls actin cytoskeletal integrity, proliferation, differentiation, cell adhesion, apoptosis, and cell migration
BRCA1	Zn	repairs double-strand breaks in DNA
Matrix metalloproteinases	Zn	proteases (play also role in metastasis)
Cytochrome P450 superfamily	Fe	metabolize endogenous and exogenous chemicals including drugs and cancerogens, some metabolize only very few substrates, while others may metabolize multiple substrates
WT1	Zn	transcription factor that plays role in cellular development and survival
Metallothioneins	Zn	metal storage, transport and detoxification, control of oxidative stress

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Abl	Mg/ Mn	non-receptor tyrosine-kinase protein, Abl/Bcr fusion protein is important for development of chronic myeloid leukemia
RAF1	Zn	serine/threonine-protein kinase- regulation between Ras GTPases and MAPK/ERK cascade
TGFBR2	Mg/ Mn	serine/threonine-protein kinase- forming with TGF-βserine/threonine kinase receptor
ST18	Zn	repressors of transcription activity from target promoters
MDM2	Zn	negative regulator of p53
MDM4	Zn	negative regulator of p53
FOG-1	Zn	transcription regulator
CREB	Zn	histone acetylation
PRKCA	Zn	serine/threonine-protein kinase that is involved in positive and negative regulation of cell proliferation, apoptosis, differentiation, migration and adhesion
MGMT	Zn	repairs alkylated guanine in DNA (resistence to alkylating agents)
MAPK1, 3,8-14	Mg	serine/threonine-protein kinases that mediate intracellular signaling
MAPK 4, 6	Mg	phosphorylates microtubule-associated protein
MAPK7	Mg	extracellular signal-regulated kinases
Chromodulin	Cr	interacts with insulin receptor
COX2	Cu	aerobic metabolism
Metalloexopeptidases	Zn	catalysis of hydrolysis of peptide bond (carboxypeptidase 4 SNP increases risk of prostate cancer)
Carbonic anhydrase IX	Zn	catalyze the reversible hydration of carbon dioxide
Chek2	Mg	serine/threonine-protein kinase which is required for checkpoint-mediated cell cycle arrest
ATR	Mn	serine/threonine protein kinase which activates checkpoint signaling upon genotoxic stresses
JMJD1C	Fe	histone demethylase
Histone deacetylase class I, II and IV	Zn	remove acetyl groups from lysine on histones
Topoisomerases	Mg	releases the supercoiling and torsional tension of DNA introduced during the DNA replication and transcription
IDH1and 2	Mg or Mn	catalyze oxidative decarboxylation of isocitrate to 2-oxoglutarate
PTEN	Mg	tumor suppressor- dual-specificity protein phosphatase
STK11	Mg or Mn	tumor suppressor serine/threonine-protein kinase that controls AMPK family members
BMPR1A	Mg or Mn	forms receptor complex consisting of two type II and two type I transmembrane serine/threonine kinases
BRP1	Fe	DNA-dependent ATPase and 5' to 3' DNA helicase required for the maintenance of chromosomal stability
DICER1	Mg or Mn	endoribonuclease playing a central role in short dsRNA-mediated post-transcriptional gene silencing
DIS3L2	Mg or Mn	3'-5'-exoribonuclease
ERCC2	Mg	transcription-coupled nucleotide excision repair
ERCC4	Mg	incision made during nucleotide excision repair
ERCC5	Mg	3' incision in DNA excision repair
MUTYH	Fe	DNA glycosylase involved in oxidative DNA damage repair
SDHA, B, C and D	Fe	oxidation of succinate, carries electrons from FADH to CoQ
WRN	Mg	DNA-helicase activity and 3'->5'exonuclease activity towards double-stranded DNA

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Table 4. Frequency of *TP53* mutations in different tumors and frequency of tumors in Li-Fraumeni syndrome (germline *TP53* mutation). Adapted from [93]. n. k. = not known

Tumor	% of *TP53* mutations	% of tumors in Li-Fraumeni syndrome
Ovary	48	7
Colorectum	43	4
Esophagus	43	n. k.
Head and neck	41	<1
Lung	39	4
Pancreas	33	n. k.
Stomach	32	2
Liver	32	<1
Brain	27	13
Bladder	26	n. k.
Breast	25	25
Uterus	21	n. k.
Soft tissue	20	15
Prostate	18	<1
Bones	15	13
Cervix	6	n. k.

The Ras proteins, H-Ras, K-Ras and N-Ras, are GTPases that regulate signal transduction. They control signal pathways affecting cell proliferation, survival, growth, migration, differentiation or cytoskeletal integrity. Ras proteins convert extracellular stimuli into intracellular signaling cascades. The two major structural components in Ras proteins are the catalytic domain, called the G domain, and the C-terminal hypervariable region (HVR). The catalytic G domain, which is highly homologous among the three isoforms, contains five G motifs that bind GDP/GTP. The motif G2 binds magnesium ion that is necessary for correct GTPase activity [94].

Conversely, uncontrolled activity of the Ras proteins, or the molecular components of their downstream pathways, can result in serious consequences, including cancers and other diseases. Mutations that activate Ras include missense substitutions at codons 12, 13, and 61. Other, so called non-classical, mutations can also activate Ras e.g. codon 146 K-Ras mutations are common in colorectal carcinoma. Approximately 30% of human tumors have mutations in one of the three Ras isoforms, (see Tab. 5). Each Ras family member has unique functions. That is evident from the mutation selection seen in human cancers (Table 5), but also from the fact that the mutant forms different changes in genes expression [95]. Detection of K-Ras and N-Ras mutation in patients suffering from colorectal and squamous cell head and neck carcinoma is very important because patients with those mutations do not respond to cetuximab and panitumumab (monoclonal antibodies against epidermal growth factor receptor) [96].

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Table 5. Approximate incidence of mutation of Ras isoforms in different tumors. Adapted from Atlas of Genetics and Cytogenetics in Oncology and Haematology (AtlasGeneticsOncology.org) and [95] n. k. = not known

Tumor	% H-Ras mutation	% K-Ras mutation	% N-Ras mutation
Stomach	20	6	25
Urinary bladder	30	3	3
Prostate	8	15	2
Thyroid	0-60	0-60	0-60
Breast	0-10	10	n.k.
Head and neck	0-30	10	n.k.
Endometrium	5	25	n.k.
Pancreas	0	80	1
Tumor	**% H-Ras mutation**	**% K-Ras mutation**	**% N-Ras mutation**
Colorectal	0	40	3
Lung	0	25	4
Liver	0	10	5
Ovary	n.k.	30	n.k.
Kidney	n.k.	30	n.k.
Brain	n.k.	8	8
Testis (germ cell)	0	10	10
AML and MDS	0	10	20
CML, ALL	0	2	10
Neuroblastoma	0	3	7

Other groups of metalloproteins that are important for cancer behavior are matrix metalloproteinases (MMPs). The role of the microenvironment in the progression and metastasis of cancer is important [97]. Extracellular matrix (ECM) degradation is an important step in tissue invasion and metastasis, enabling cancer cells to invade surrounding tissues. Stroma is degraded by proteases secreted by both cancer and stroma cells. They may degrade all components of ECM, and their overexpression is sign of worse prognosis. MMPs are a family of zinc-dependent endopeptidases secreted as zymogens by cancer and stromal cells. They play a major role in the cancer cells invasion and metastasis. They degrade matrix proteins and are also involved in early steps carcinogenesis [98]. The overview of MMPs subfamilies is shown in Table 6.

Metallothioneins (MTs) binding to zinc and copper ions serve as reservoirs of those metals for synthesis of apoenzymes and zinc finger transcription regulators (Zn is a cofactor for approximately 300 enzymes and Cu is essential for metalloenzymes that are important in the electron transfer, oxygen transport and oxygenation reactions). Zn cellular levels are regulated by specific transport proteins of which there are two families, the ZnT family and ZIP family. The ZnT family proteins transport Zn out of cells and into intracellular compartments from the cytoplasm, whereas ZIP proteins transport Zn into the cytoplasm from either outside the cell or from intracellular compartments. Cu has three carriers - ceruloplasmin, albumin and transcuprein. MTs sequester metal ions when they are present in excess [100].

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Table 6. Overview of MMPs subfamilies. Adapted from [98, 99]

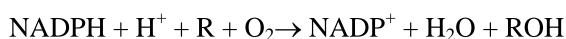
Subfamily	MMP No.	Substrate
Mytrilysins	7, 26	fibronectin, laminin, nidogen, type IV collagen, proteoglycans, β4-integrin
Collagenases	1, 8, 13	Triple helical fibrilar collagen
Gelatinases	2, 9	gelatin, collagen
Stromelysins	3, 10, 11	ECM components except for triple helical fibrilar collagen
Membrane type	14, 15, 16, 17, 24, 25	activation of other MMPs, collagen
Other	12, 19, 20, 21, 23A, 23B, 27, 28	

As mentioned above, MTs interact with NF-κB, PKCl, and GTPase Rab3A, and they can modulate the biological activity of p53. MT-1 and MT-2 regulate the level, activity and cellular location of the transcription factor NF-κB that protects cells from apoptosis induced by TNF and other stimuli. MTs also control the redox pool. The metal binding sites have a low redox potential and are easily oxidized by intracellular oxidants. Biological disulfides such as glutathione disulfide (GSSG) oxidize MTs with release of Zn, while glutathione (GSH) reduces the oxidized protein to thionein, which then binds to available Zn.

This coupling of GSH/GSSG with MTs is effective redox modulator [101]. Nuclear MTs protect cells against UV and ionic radiation as well as against some cytotoxic alkylating agents. The most important role of MTs in cancer cell chemoresistance is their ability to bind platinum in therapy by platinum cytostatics (see below) [100]. Moreover, they stabilize lysosomes and decrease apoptosis following the oxidative stress by inhibition of the Fenton-type reactions and by ensuing peroxidation of lysosomal membranes. MTs are also released to the extracellular matrix where they have modulatory effects on cellular specific, non-specific and humoral specific immunity. However, mechanisms causing this effect are still unclear [102].

MTs are overexpressed in cancers that progress and/or are resistant to drugs in a variety of malignancies e.g. leukemia, melanoma, breast, ovarian, renal, lung, pancreatic, gall bladder, esophageal, and basal cell carcinomas. There is supposed that MTs protect cancer cells from apoptosis and support their metastatic behaviour and/or proliferation. On the other hand in other studies significant correlation between MT expression and prognosis was not found [103].

Cytochrome P450 (CYP) gene superfamily comprises at least 57 active genes and 58 pseudogenes and the majority of genes are polymorphic [104]. CYPs contain a heme cofactor and catalyze hydroxylation of a many different compounds. They performs a transfer of electrons by transition between the states of Fe^{2+} / Fe^{3+}.



$$NADPH + H^+ + R + O_2 \rightarrow NADP^+ + H_2O + ROH$$

The CYP enzymes (overview shown in Table 7) are expressed mainly in the liver, but extrahepatic expression also occurs. In the cells, they are located either in the membrane of endoplasmic reticulum or in the inner membrane of mitochondria. CYPs are the most important enzymes participating in drug metabolism. They activate a variety of carcinogens

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(see Table 8) or metabolize a broad spectra of anticancer drugs (tamoxifen, taxanes, oxazaphosphinans, imatinib, gefitinib, irinotecan, thalidomide, podophyllotoxin and vinca alkaloids) (see Table 8). In addition, many drugs may influence activity of various CYPs by induction of their expression or by inhibition of their activity [104].

Metallomics of Brain Tumors

In addition to the role of p53 in other tumors, this protein plays an important role also in brain tumors. Over 60% of diffuse astrocytomas have loss of alleles on 17p, including the *TP53* locus, and the retained *TP53* allele is mutated in the majority of cases [106, 107]. The absence of a wild type p53 is therefore the most common abnormal finding in astrocytomas grade II. Mutation of only one allele was found in a small percentage of tumours because the p53 protein functions as a tetramer that does not function with one abnormal p53 [108].

Table 7. Human CYPs modified according to [105]

Families	Members	Function
CYP1 /3 subfamilies	1A1, 1A2, 1B1	Xenobiotic metabolism
CYP2 /13 subfamilies	2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C81, 2C91, 2D6, 2E1, 2F1, 2J2, 2S1, 2U1, 2W1	Xenobiotic and steroid metabolism
CYP3 /1 subfamily	3A4, 3A5, 3A7, 3A43	Xenobiotic metabolism
CYP4 / 6 subfamilies	4A11, 4A22, 4B1, 4F2, 4F3, 4F8, 4F11, 4F12, 4F22, 4V2, 4X1, 4Z1	Arachidonic acid and fatty acid metabolism
CYP5 /1 subfamily	5A1	ThromboxaneA_2 synthesis
CYP7 /2 subfamilies	7A1, 7B1	Cholesterol elimination
CYP8 /2 subfamilies	8A1, 8B1	Prostacyclin and bile acid synthesis
CYP11 /2 subfamilies	11A1, 11B1, 11B2	Steroid synthesis
CYP17 /1 subfamily	17A1	Testosterone and estrogen synthesis
CYP19 /1 subfamily	19 A1	Estrogen synthesis
CYP20 /1 subfamily	20 A1	Not known
CYP21 /1 subfamily	21A2	Steroid synthesis
CYP24 /1 subfamily	24 A1	Vitamin D metabolism
CYP26 /3 subfamilies	26A1, 26B1, 26C1	Retinoic acid metabolism
CYP27 /3 subfamilies	27A1, 27B1, 27C1	Bile acid synthesis, vitamin D activation
CYP39 /1 subfamily	39A1	Cholesterol metabolism
CYP46 /1 subfamily	46A1	Cholesterol metabolism
CYP51 /1 subfamily	51A1	Cholesterol synthesis

Mutations of the *TP53* gene in anaplastic astrocytomas also occur at approximately the same frequency as is found in the diffuse astrocytomas [109]. Glioblastomas are divided into primary (*de novo*) and secondary (develop from low grade astrocytoma). Both have impaired p53 and Rb1 pathways.

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Table 8. Procarcinogens that are activated by CYPs

CYP	Activation of carcinogen
1A1	Benzo(a)pyrene, dimethylbenzanthracene, 2-amino-1-methyl1-6phenylimidazo-[4,5-b]pyridine
1A2	Aryl and heterocyclic amines, aflatoxin B1
1B1	Polycyclic aromatic hydrocarbons
2A6	N-nitrosamines
2B6	4-(methylnitrosamino-1(3-pyridyl)-1- butanone, aflatoxin B1
2E1	Benzene, carbon tetrachloride, chloroform, styrene, vinyl chloride, vinyl bromide, N-nitrosodimethylamine
3A4,5,7	Aflatoxin B1andG1, estradiol, benzo(a)pyrene, naphthalene, senecionine, sterigmatocystin

Primary glioblastomas are characterized by 12q14 amplification involving the *CDK4* and *MDM2* genes but in rare cases they show loss of one allele of each of *TP53* and RB1, with mutation of the retained alleles, requiring four genetic mutational events. Secondary glioblastomas usually have loss of one *TP53* allele and mutation of the second one [109].

Overexpression of MTs has been described in many malignancies [100] and their expression in cancer cells is induced by the tumor necrosis factor (TNF), interferon α, interleukin-1, interleukin-2 [110]. The synthesis and intracellular distribution of MTs seem to be important for cancer cell properties. Florianczyk et al. detected MTs in different cellular fractions of glial tumors. They found the highest MTs content in the cytosolic fraction both of the grade II astrocytomas and glioblastomas, a slightly lower level in the nuclear and mitochondrial fractions and the lowest levels in the microsomal fraction, while the increase was greater in glioblastoma multiforme [111]. Some other researchers have also found large differences in the levels and the cell distribution of MTs in different malignant tumors. Among 53 cell lines of the National Cancer Institute tumor panel there have been found a 400-fold range in MT levels and a tenfold range in the ratio of the nuclear to cytoplasmic MT immunostaining [112].

Expression of zinc transporters (14 ZIPs and10 ZnTs) was examined in samples of gliomas. Most of the ZIPs were upregulated in grade IV patients, compared with grade II ones, and ZIP4 was the most significantly associated with grade and shorter survival and its levels correlated with the key genes for cell growth and angiogenesis in glioma, such as MMP-9,VEGF-A, PDGF-A, IL-6, IL-8, and IGFBP-2. ZnTs showed diverse patterns of expression. Most of the other zinc transporters ZIP3, ZIP4, ZIP8, ZIP14, ZnT5, ZnT6, and ZnT7, showed positive associations with tumor grade, and only ZnT10 was negatively associated with tumor grade. ZIP3, ZIP4, ZIP8, and ZIP14 are membrane transporters that provide the cellular zinc uptake, while ZnT5, ZnT6, and ZnT7 transport cytoplasmic zinc into endoplasmic reticulum and Golgi apparatus. These findings indicate that gliomas may have an elevated cellular zinc turnover. Lin et al. speculated that ZIP4 may be used as a novel diagnostic and/or prognostic marker for gliomas and for the development of new targeted therapies for gliomas. They supposed that ZIP4 may promote cancer proliferation through a regulation of the activity of zinc finger transcription factors [113].

Two zinc containing proteins AGBL2 and ZSWIM5 have been identified as highly expressed in glioma. However, further research is necessary to investigate the ZSWIM5 protein whose function is unknown and that is selectively and strongly expressed within

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normal brain and malignant gliomas. Whereas, the linkage of metallocarboxypeptidase AGBL2 to tubulin dynamics may be important for glioma cell motility, Meyer speculated about therapeutic suppression of this gene [114].

The expression of the zinc finger transcription factor Zfx was significantly higher in glioma samples independently on grade compared to the normal brain tissue and it was overexpressed in glioblastoma derived cell lines (U251, U87, U373, and A172). Zfx knockdown in U251 cells by siRNA inhibited proliferation, arrested cells in S phase, and induced apoptosis. Zfx knockdown induced Akt hypophosphorylation but the phosphorylation of mTOR and GSK-3b, downstream effectors of Akt, remained unchanged. Akt signaling is a survival pathway that regulates the expression of the oncogene c-Myc, the pro-survival factor survivin and the CDK inhibitor p27. This pathway is supposed to be the mechanism of the effect of Zfx knockdown on glioblastoma cells. The group of Zhu speculated that Zfx plays important role in glioma proliferation and cell survival and its targeting may serve as a promising strategy for glioma therapy [115, 116].

According to molecular profiling, the medulloblastomas are currently classified into four main subgroups (WNT, SHH, group 3, and group 4) [117]. Approximately 30% of medulloblastomas were found to have SHH pathway activation, which may be detected by the positivity of GLI1 and/or GLI2 proteins. Those proteins belong to the family of zinc finger transcription factors, GLI1 is transcription activator, GLI3 is a repressor, and GLI2 can play both activating and repressing roles. Expressions of GLI1 and GLI2, but not of GLI3 in tumors were associated with a shorter survival of medulloblastoma patients. Knockdown of GLI2 by siRNA in medulloblastoma-derived cell lines caused a G0 phase cell cycle arrest while knockdown of GLI1 had no effect [118]. GLI1expression was detected mainly in the nodular/desmoplastic medulloblastoma types than classic or anaplastic ones. Tumors with high GLI1 overexpress an apoptosis inhibitor Bcl-2 [119].

These results suggest that targeting the Sonic hedgehog pathway in positive patients may be a therapeutic strategy for medulloblastomas.

Analysis of the X-Ray Absorption Spectroscopy of iron in the samples of gliomas showed that the Fe(II) / Fe(III) ratio in the glioma tissues correlated with tumor grade. This relationship cannot be explained by tissue hypoxia in highly malignant tumors because no correlation was found between the average oxidation state of iron and hypoxia evaluated as expression of carbonic anhydrase IX [120].

As it has already shown above, MMPs play an important role in aggressiveness and metastasis of cancers including brain tumors both primary and particularly brain metastasis. Kachra et al. described that equilibrium between MMPs and tissue inhibitors of MMPs (TIMPs) play an important role in human brain tumors. Moreover, expression of MMPs and TIMP may be a marker for tumor malignancy. MMP-9 and MMP-12 expression was higher in high grade gliomas than in low grade ones and expression of TIMPs was highest in tumors of grade I [121]. MMP-9 but not MMP-2 expression also correlated with invasiveness of meningiomas [122]. Because MMPs are necessary for metastasis they were intensively studied also in brain metastases. Significance of MMP-1, 2, 3 and -9 overexpression for brain metastasis in melanoma, breast and lung cancer was proved in *in-vitro* models in following studies [123-127]. Blocking of ERK1/2 phosphorylation by treatment with MEK inhibitors inhibited the expression of MMP2. This phenomenon seems to be important for a design of target therapy [128].

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Table 9. Anticancer drugs that are metabolized by CYPs.

Drug	CYP
Docetaxel	3A4, 1B1
Etoposide	3A4, 2E1
Exemestane	3A4
Flutamide	1A2
Fulvestrant	3A
Gefitinib	3A4, 2D6
Idarubicin	2D6, 2C9
Imatinib	3A
Irinotecan	3A
Letrozole	3A, 2A6
Mitoxantrone	1B1, 3A
Paclitaxel	2C8, 3A
Tamoxifen	3A, 2D6, 1B1, 2C9, 2C19
Vinca alkaloids	3A
Cyclophosphamide *	2B6, 2C19,
Ifosfamide *	3A, 2B6
Dacarbazine *	1A1, 1A2, 2E1
Procarbazine *	2B6, 1A
Tegafur *	2A6, 2C8, 1A2
Thiotepa *	3A, 2B6

*prodrugs that are activated by CYPs

On the other hand Basset et al. [129] reported that MMP-11 is produced mainly by the stromal fibroblasts surrounding tumor cells and not by the breast cancer cells themselves.

The studies utilizing the in situ hybridization demonstrated that the main localization of MMP-1, MMP-2, MMP-3 and MT1-MMP mRNA is in stromal fibroblasts particularly adjacent to invading breast, colorectal, lung, prostate, and ovarian cancer cells, but not in the carcinoma cells [130]. Moreover MMP-2 is important for angiogenesis because it destroys the basement membrane, while other MMPs are involved in the generation of angiostatin that is an inhibitor of angiogenesis [131].

There were conducted clinical studies with MMTs inhibitors (marimastat, prinomastat, and BAY 12-9566 alone or in a combination with chemotherapy) in patients with advanced cancers without any positive effect [131]. These data show that the importance of MMPs is not entirely clear, and their potential use as therapeutic targets requires further experimental and clinical studies.

In conclusion, some metalloproteins may be in future used as diagnostic, prognostic and/or predictive markers and moreover some of them may be the therapeutic target.

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Metallomics and Brain Tumors Chemoresistance

Chemoresistance is a complex system with multiple and heterogeneous mechanisms of action which are orchestrated not only by the biology of the tumor but also by the tumor microenvironments [132]. Metallomic studies showed that metallothioneins (MTs), sulfhydryl proteins, has been overexpressed in tumor cell lines resistance to platinum coordination complexes (cisplatin, carboplatin, oxaliplatin) [133-135]. Their expression may be induced not only by metals but by other stimuli e.g. glucocorticoids, catecholamines, free radicals, tumour necrosis factor α, interleukins-1, -2 and -6 [100, 102]. Resistance has been postulated to be mainly the result of protection against ROS damage, anti-apoptotic properties and by the direct sequestering of alkylating agents by MT cysteines [136, 137]. Recent evidence supports also the interactions with other important thiol compounds, involved in chemoresistance - glutathiones [138, 139]; however, the direct mechanism of interactions is still not properly elucidated. Previously, it has been shown that neuroblastoma cell line resistant to cisplatin but not sensitive ones significantly increases its intracellular MT levels after incubation with cis- or carboplatin [100]. Upregulated MT was identified also in medulloblastoma cells with induced resistance to the alkylating drug BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea] [140].

In the view of these findings and its obvious relation to proliferative activity in human brain tumors, the levels of MT expression may explain the poor response of a subset of these lesions to antineoplastic drugs. In glial tumors, high-grade glioma patients with MT positivity demonstrated a shorter survival compared to MT-negative subjects, as was shown in [141]. This phenomenon can be linked with the ability of MT to decrease treatment effects (ROS scavenging, anti-apoptotic ability). Contrary to this study, Korshunov and coworkers revealed that low MT expression (<50% MT-positive cells) can serve as an indicator of a disadvantageous outcome in patients suffering from glioblastoma [142]. Interestingly, the treatment protocol did not include platinum-based chemotherapeutics, and thus it was hypothesized that favorable prognosis of MT overexpression can resulted from an enhanced MT-based detoxification capacity of tumors. Hence, the presence of high MT concentrations in glioblastoma cells may be advantageous for the binding of a broad spectrum of intracellular endotoxins released during chemotherapy.

Investigation of the expression levels of chemoresistance-related metalloprotein metallothionein in human brain tumors can be exploited to choose the optimal anticancer drug for patients suffering from these neo-plasms. Furthermore, by combining approaches to downregulate the expression by specific agents as antibodies, aptamers or antisense nucleotides, it is possible to overcome drug resistance and improve the treatment success in brain tumor chemotherapy.

Metal Containing Cytostatics

Cancer chemotherapy is the treatment of cancer with an antineoplastic drug or usually with a combination of such drugs. Most commonly, chemotherapy acts by killing rapidly dividing cells, which is the property of most cancer cells. Therefore, chemotherapy also harms the normal cells that divide rapidly [143]. There are usually used drug combination

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regimens that provide maximal cancer cell killing with tolerable side effects and prevent the development of secondary chemoresistance [144, 145]. However, the blood brain barrier (BBB) penetration of cytostatics is specific problem of brain tumor chemotherapy [143].

Platinum drugs (cisplatin, carboplatin, oxaliplatin and some new derivatives tested in clinical studies) have antitumor activity caused particularly by their binding to cellular DNA that leads to cell death [146, 147]. Cisplatin, carboplatin, and oxaliplatin are used in a combination with other cytostatics for therapy of non–small and small cell lung cancers, lower gastrointestinal malignancies, breast, head and neck and bladder cancers, gynecologic and genitourinary malignancies, neuroblastoma, osteosarcoma, hepatoblastoma, some lymphoma and also some brain tumors [148].

For anticancer activity of platinum cytostatics, the *cis* coordination by bidentate amine ligands or two amines (at least one NH_2 group on the amine) and two leaving groups with an intermediate binding strength (e.g. Cl^-, SO_4^{2-}, citrate or oxalate) to platinum are necessary. Investigation of new platinum drugs is focused on reduction of toxicity of cisplatin, overcoming of the acquired drug resistance and increasing spectrum of anticancer activity [149]. The main mechanism of anticancer action of platinum cytostatics is formation of DNA adducts [150, 151]. Platinum compounds in cells form a reactive monoaquamonochloro complex by dissociation of one chloride ligand. The complex reacts with the N(7) of DNA guanines and form monoadducts. The major adducts are N7-d(GpG)-intrastrand adducts (60%), N7-d(ApG)-intrastrand adducts (30%), N7-d(GpXpG)-intrastrand adducts (~10%), and N7-d((X)-(X))-intrastrand adducts < 2% [143]. Dissociation of the second chloro ligand converts monoadducts to a variety of stable diadducts, from which most are formed by intrastrand binding of a guanine residue [150]. Intrastrand adducts block DNA replication and transcription. Other types of adducts (e.g. interstrand crosslinks, and DNA-protein crosslinks) have less importance because they cause less than 1% of platinum adducts in cell [152]. Covalent binding of platinum cytostatics to cellular proteins, lipids and RNA in their anticancer effects is also supposed [153].

Cisplatin, the first platinum cytostatic, has two amine groups in the *cis*-configuration, which are opposite the two chloride leaving groups also in the *cis*- configuration. It is effective against broad spectrum of cancers including brain, but it has significant toxicity that include nephrotoxicity, electrolyte imbalance, severe nausea and vomiting, peripheral neuropathy, myelosuppression and cytotoxicity. **Carboplatin** is similarly effective as cisplatin but it is not so nephrotoxic and emetogenic but it is more myelotoxic. It has two amine groups in the *cis*- configuration, which are opposite the cyclobutane- dicarboxylato leaving group. The difference between the cisplatin and carboplatin molecules lies in the leaving groups. **Oxaliplatin** is registered for therapy of colorectal cancer. Because of high toxicity and resistance to platinum cytostatics, development of its new derivatives aims to find less toxic and/or more efficient analogues. **Satraplatin** is active in prostate, breast and lung cancer [154][154][154]. **LA-12** (OC-6-43)-bis(acetato) (1-adamantylamine) amminedichloroplatinum (IV) is a new promising derivative of Pt(IV) that exhibits higher anti-tumor activity than cisplatin and satraplatin [155]. The clinical studies with this drug have been started.

Arsenic trioxide has been evaluated for the treatment of various cancers. Nowadays, arsenic trioxide is approved by FDA for therapy of all-trans retinoic acids resistant acute promyelocytic leukemia [156]. In addition, As_2O_3 inhibits the proliferation of multiple

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myeloma cells [157]. Indeed, clinical trials showed As_2O_3 efficacy against AML, HTLV-I-associated leukemia and some solid tumors. The mechanisms of its action are based on the reaction between arsenic and thiol groups of proteins, accumulation of ROS and down regulation of Bcl-2 [158].

Complexes containing non platinum metals - ruthenium, titanium, gallium, iron, cobalt, gold, paladium were tested for their anticancer effect in preclinical experiments and in clinical studies [159]. Promising results were found with various non-platinum metal complexes in preclinical research. Some compounds have also been investigated in clinical trials. Nevertheless, there is long way to have a registered drug from these compounds.

Metal Nanoparticles Used in the Brain Cancer Thermal Therapy

Nanoparticles are likely to play a key role in the future diagnosis and treatment of CNS malignancies, because they have the potential to revolutionize both preoperative and intraoperative brain tumor detection. In addition, nanoparticles may also serve as novel, targeted delivery devices for chemotherapy, gene therapy, photodynamic therapy and thermotherapy [160]. Approaches to nanoparticle-mediated thermal therapy include absorption of infrared light, radio frequency ablation, and magnetically-induced heating. These approaches have demonstrated high efficacy in animal models, and early clinical testing is currently underway [161-163]. Among the metal nanoparticles that are used for thermal cancer therapy belong mainly silver, gold and magnetic nanoparticles [161, 164].

A most famous nanoproduct is nanosilver. Nanosilver particles are smaller than 100 nm and contain 20-15,000 silver atoms [165]. Silver nanoparticles (AgNPs) have strong antimicrobial and oxidative activity. [166]. These factors cause negative effects on the structures and functions of cells, which finally induce cytotoxicity, genotoxicity, immunological responses, and even cell death [165, 167]. AgNPs have a potential to induce genes associated with chromosomal aberrations [168], cell cycle progression, DNA damage and apoptosis in human cells at non-cytotoxic doses [169] and enhance radio-sensitivity on cancer cells [170]. AgNPs are more cytotoxic than gold nanoparticles [171]. Liu et al. have shown that AgNPs could enhance thermo-sensitivity of glioma cells and this effect was size dependent [172]. AgNPs could induce cell cycles arrested in G2/M phase and enhanced the apoptosis rate of cancer cells after magnetic nanoparticles hyperthermia [172, 173]. It was found that the combination of AgNPs and radiotherapy resulted in marked enhancement in the mean survival time, and a near 40% cure rate in glioma bearing rats without apparent systemic toxicity, which may be due to its potent antiproliferative activity [173].

Simple gold nanoparticles (AuNPs) are solid gold nanospheres that can range in diameter from 2 nm to several 100 nm. They contain a large number of gold atoms (about 70 000 gold atoms for 15 nm AuNPs) and possible adjustments of their size and coating make them promising for photoactivation. Gold nanoparticles can be also prepared in different shapes and structures, such as nanospheres, nanoshells, nanorods, and nanocages [174]. Intravenously administered small gold nanoparticles can improve x-ray therapy [175]. The combination of gold nanoparticles and radiation therapy increased survival of mice with orthotopicaly transplanted glioblastoma multiforme. Potential benefits resulting from

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increased tumor cell radiosensitization to preferential targeting of tumor-associated vasculature [176]. Bodyk et al. reported that radiosensitization efficacy of gold nanoparticles (AuNPs) with low energy radiations (88 keV) was evaluated *in vitro* and *in vivo* on rats bearing glioma. The positive effect for rats had combination of treatments (AuNPs: 50 mg/mL, 15 Gy) [177].

Recent advances have shown that gold-silica nanoshells - composite nanoparticles composed of an inner silica (glassy) core ~ 100 nm diameter and a thin outer layer of gold (10-15 nm thick) [163, 178] show a red shift of the gold's characteristic plasmon absorption spectrum into the near-infrared (NIR) region (650-950 nm). Nanoshell photothermal cancer therapy works through preferential accumulation of nanoshells in a tumor and absorption of NIR light by those particles to locally generate heat at the tumor site [161].

Day et al. have developed a novel therapeutic approach for high-grade gliomas using near infrared-absorbing silica-gold nanoshells that are thermally activated upon exposure to a near infrared laser, thereby irreversibly damaging cancer cells. Tumors were induced in male mice by subcutaneous implantation of Firefly Luciferase-labeled U373 human glioma cells and biodistribution and survival studies were performed. There was a significant improvement in survival for the nanoshell treatment group versus the control and 57% of the mice in the nanoshell treatment group remained tumor free at the end of the 90-day study period [179].

Magnetic nanoparticles (NPs) are iron oxide NPs with a diameter less than 10 nm. They have been used as both diagnostic and therapeutic nanoscale materials to treat deep tissue tumors [163]. Many groups have tested these molecules as contrasting agents for magnetic resonance imaging MRI, through conjugation of iron oxide NPs with hydrophilic polymer coatings of dextran or polyethylene glycol [180]. A new strategy to achieve selective drug delivery to the tumor tissue is magnetic targeting. This approach has the advantage of enhancing the attraction of drug-loaded magnetic NPs in cancer cells by using an externally applied magnetic field [181].

Recently, aminosilane-coated iron oxides NPs have been utilized in thermotherapy to treat brain tumors. Using magnetic field-induced excitation of iron oxide superparamagnetic NPs, thermotherapy in the rat model can prolong the survival time 4.5-fold over controls [182]. In another work Yi et al. established a rat model of brain glioma by injecting C6 glioma cells into the right nuclei caudatae of rats. Fixed doses of magnetic nano-iron were then injected into the tumors. The survival time of tumor bearing rats was subsequently observed, and imaging studies were conducted. After injection of different doses of magnetic nano-iron, the survival times of the different dose groups of tumor bearing rats were not significantly different. However, the tumor size exhibited a significant decrease with magnetic nano-iron hyperthermia therapy [183].

Nanoparticles have the potential for advancing the diagnosis, operative management and adjuvant therapy brain tumors in the future. Because the field of nanotechnology is young, the long-term health effects of nanoparticles are unknown. For a successful translation of nanoparticles to the clinics, characteristics of nanoparticle therapeutics need to be strictly and rigorously defined. From the scientific studies it can be concluded that biodistribution and pharmacokinetics is largely dependent on the nanomaterial and therefore necessary measures need to be done to examine possible toxic effects of fabricated nanoparticles.

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Future Directions

Metallomics is focused on the qualitative identification, quantitative analysis and structural characterization of metal-binding proteins. In this area, the understanding of interactions of metals with nucleic acids, proteins, and other biomolecules depends on the knowledge of metals fate in cells and tissues and their cellular localization, which is mainly provided by interactions with proteins. These questions can be answered by a combination of various analytical, bio-analytical and molecular-biological assays, where development of these is of great interest.

From the point of view of application, disease-related proteomes and metalloproteomes attract attention of numerous scientists worldwide. Metalloproteomes of majority of tumors including brain ones are still not fully understood, where combination of techniques and methods from multiple disciplines, including biochemistry, biophysics, engineering and computer sciences, are being used for full identification and understanding of cancer metalloproteomes.

From the point of view of new treatment strategies, knowledge of the fate of metal ions can be used for designing and developing of new drugs containing a metal including new platinum cytostatics. Moreover, metal ions can also mediate the assembly of peptides to form nanoparticles for various applications in medicine. Besides these, some metal based nanoparticles designed for tumor targeted therapies are also considered as an important field of the interest.

Conclusion

Metallomics as a branch of metabolomics can be defined as comprehensive analysis of the entirety of metal and metals containing proteins within cells and tissues. From a physiological point of view, biologically active metals can be divided into two groups: i) essential metals, which are crucial for numerous biochemical processes; ii) toxic metals, which are harmful to the body. Approximately 1/3 of proteins are associated with some metal. They are diverse classes of proteins, in which the metal atoms play different roles as catalytic, regulatory, or structural. The Zn ions, the most abundant metal ions in cells, play significant roles in the function of more than 300 enzymes, in stabilizing of the DNA double helix and in control of gene expression. On the cellular level, the key cellular processes such as proliferation, differentiation and apoptosis have been connected with its signaling. Zinc dysregulation, deficiency and over-supply are connected with various cancers. Imbalance of zinc transporters regulation is described in cancers, too. To date, the data on the role and the influence of zinc ions on a tumor development are very limited. From these, the decreased serum zinc ion levels have been found in patients with lung, head and neck, breast, prostate, liver and lung tumors. On the contrary, the increase in tissue zinc ion levels has been found in breast cancer. In the case of brain tumors, the situation is not clear, but the number of studies on this topic steadily increases.

From the point of view of metalloproteins, the greatest knowledge can be found on zinc-containing ones because these have been shown to be not only structural components, but also signaling substances in a number of cascades and cofactors of numerous important enzymes.

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It is clear that some of these proteins are associated with the tumor development. Zinc finger proteins ZFX and WT1 are upregulated in malignant gliomas as compared with healthy tissues. The expression of a zinc transporter ZIP4 correlates with glioma grade and its overexpression is a sign of worse prognosis. Cellular levels of zinc finger transcription factors GLI1 and GLI2, but not of GLI3 are associated with a shorter survival of medulloblastoma patients. Matrix metalloproteinases are family of zinc-dependent endopeptidases secreted by tumor and/or stromal cells that degrade extracellular matrix and therefore increase invasiveness and metastasis including into brain and therefore play a key role in brain metastasing.

Moreover some drugs contain a metal in their structure and are referred to as metallodrugs. Metallomics studies the metabolism of these drugs, their transport and interactions with biomolecules. The most important are platinum containing cytostatics (cisplatin, carboplatin, oxaliplatin and some others that are in clinical studies). The approved platinum cytostatics are used in childhood and adult brain tumors e.g. cisplatin in medulloblastoma and chorioid plexus tumors, and carboplatin in ependymoma, intracranial germ cell tumors, low and high grade gliomas including glioblastoma. The cytotoxic effect of platinum drugs consists in DNA adducts formation, especially to guanine and adenine, which causes DNA strand crossing and subsequent interference with normal transcription, and/or replication. The damage of DNA activates the apoptotic pathway, which is the main type of cell death induced by platinum cytostatics. However, before cisplatin enters the cell, it may bind to several phospholipids such as phosphatidylserine in the cell membrane. In addition, numerous potential platinum-binding molecules are available, including RNA and sulfur-containing biomolecules, such as glutathione and metallothioneins in cytoplasm. Metallothioneins are low molecular mass cysteine-rich proteins having naturally-occurring zinc that may be substituted for another metal ion with higher affinity for thiolate. Binding of platinum cytostatics to metallothioneins results in their inactivation and is one of the mechanisms of chemoresistance to those cytostatics. Recently published evidences suggest that MT may cause also resistance to several “non-metal cytostatic drugs“ e.g. etoposide, irrinotecan and BCNU. The exact mechanism, by which metallothioneins prevent “non-platinum” cytostatics induced cell death, has not been found yet, however, an application of various types of nanocarriers could be a way how to overcome these barriers.

From the above it is clear that the metals and metalloproteins play an important role in physiological functions and in the pathophysiology of many diseases, including tumors, where detection of metalloproteins would play a major role in diagnosis, prognostics and prediction. Moreover, some of them will serve as therapeutic targets.

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Chapter 5

Hepatitis B Virus and Hepatitis C Virus Infections and Risk of Pancreatic Ductal Adenocarcinoma

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Abstract

Pancreatic ductal adenocarcinoma (PADC) represents a highly lethal cancer with a very dismal prognosis. Absence of early symptoms, advanced stage at diagnosis, aggressive biological behaviour and lack of effective systemic treatment are the most

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important factors, explaining its elevated mortality rate and its low overall five-year survival (< 5%).

Until now, the causes of this malignancy remain still largely unknown and further efforts are underway to reach a better knowledge of PADC aetiology and to improve our understanding of mechanisms involved in carcinogenesis of this organ. In the last years it has progressively emerged that viruses play a key role in human carcinogenesis. Unfortunately, some host and viral factors have contributed to make the study of the pancreas extremely difficult and to hamper the identification of pathogenetic processes involved in cancer development, including its retroperitoneal localization as well as the small size of precursor cancer lesions. However, in the past and more recently, some histological investigations suggested that both antigens and genome of hepatitis B (HBV) and hepatitis C (HCV) viruses, two pathogens with well-known high liver tropism and pro-oncogenic properties may be detected also in extra-hepatic tissues, such as pancreas. In addition, some epidemiological articles have suggested that HBV and HCV might be involved even in pancreatic carcinogenesis. Here we review the results of available reports, evaluating the possible association between HBV or/HCV infections and risk of pancreatic cancer development as well as to discuss the limiting factors of these researches.

Introduction

Pancreatic ductal adenocarcinoma (PADC) represents an extremely aggressive and lethal malignancy. In 2012, it was the 5th most common cause for cancer-related mortality in Europe, with about 100,000 deaths as well as the 7th worldwide, with about 330,000 demises [1]. The absence of early symptoms, a generally advanced stage at diagnosis, an aggressive biological behaviour and the substantial inefficacy of the current systemic treatment make its prognosis poor. Up to now, cigarette smoking [2] and family history [3] for this cancer are the main risk factors for PADC.

However, an increased incidence of this malignancy has been also observed in subjects with history of chronic pancreatitis [4, 5], alcohol abuse [6], diabetes mellitus [7, 8] and elevated dietary fat consumption [9]. On the whole, these risk factors only account for about half of all pancreatic cancer cases. Therefore, further investigations are required to obtain a better knowledge of PADC causes. In the last years, a growing number of epidemiological and experimental observations suggest that infectious agents, both bacteria and viruses, may be involved in the development of human tumours. Collectively these pathogens may be responsible for about 15-20% of all malignancies worldwide [10]. In particular, in the past it has been reported that several viruses are able to infect the pancreas and to induce acute pancreatitis [11-18]. However, despite these evidences, only recently some authors have explored the possible role of viruses in pancreatic carcinogenesis. Some host and viral factors have contributed to made the study of the pancreas extremely difficult and to hamper the identification of pathogenetic processes involved in cancer development, even if modern imaging techniques and biopsy procedures are now available, such as its localization in retroperitoneum, the small size of precursor cancer lesions or the difficulty to detect viral antigens and/or genome in normal and/or malignant tissue of this organ. As a consequence, the possible association between HBV/HCV infection and risk of PADC has not been investigated for a long period. Nevertheless, since 2008 a growing interest has been devoted

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to explore this topic and a large number of studies with different design have been performed with this purpose.

To date, some epidemiological reports have shown that the infections caused by different viruses, including Human Immunodeficiency Virus [19-21], poultry viruses [22] as well as the Hepatitis B (HBV) [23] and Hepatitis C (HCV) [24] viruses may be associated with an increased risk of PADC. In particular, the majority of these studies have been focused on the possible link between the exposure to HBV and HCV and the development of this pancreatic malignancy. Nevertheless, to date, the results of these works are not univocal and further investigations are required to definitively confirm or exclude the existence of this association. In addition to epidemiological studies, only few articles both in the past and more recently have detected the presence of HBV/ HCV antigens, genes and/or replicative intermediate forms in extrahepatic organs, including pancreas [25-28]. The results and conclusions of these investigations will be widely discussed below.

However, the effective role of both viruses in pancreatic carcinogenesis as well as the pathogenetic mechanisms potentially responsible of this process in subjects with serum markers of persistent or past exposure to HBV and HCV remain to be elucidated.

The present study is aimed at:

a) reviewing and synthesizing available investigations, to assess the possible association between pancreatic cancer and HBV or HCV infection as well as the related meta-analyses, evaluating this topic;
b) discussing possible pathogenetic mechanisms involved in pancreatic carcinogenesis in patients with past or present exposure to HBV or HCV infection.

Data Sources

With the aim to evaluate this association, we performed a comprehensive systematic research of scientific literature in order to identify relevant studies on the potential association between HBV or HCV infection and PAC risk as well as to detect the published meta-analyses, concerning this topic. The literature search was performed in October 2014. We considered the following electronic databases: MEDLINE (1950 to September 30th, 2014), the Cochrane Library (until the first quarter of 2014) and EMBASE (1980 to September 30th, 2014) for all potential relevant articles. The used keywords were the following: “HBV and pancreatic cancer”, “HBV and pancreatic adenocarcinoma”, “HBV and pancreatic carcinoma”, “HBV infection and pancreatic cancer”, “HBV infection and pancreatic carcinoma”, “HBV infection and pancreatic adenocarcinoma”, “pancreatic cancer risk”, “pancreatic cancer and chronic hepatitis”, “pancreatic carcinoma and chronic hepatitis”, “pancreatic adenocarcinoma and chronic hepatitis”. The PubMed “related manuscripts” and the reference lists of retrieved studies were also searched to identify additional pertinent studies. The literature search was independently performed by two authors (A.F., M.M.), who detected and screened relevant articles, on the basis of title and abstract. The text of the potentially relevant articles was retrieved for further evaluation. The full-text of studies, which were considered potentially eligible by either of the two reviewers, was further assessed. We extracted from each study the following data: first author’s name, study design,

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inclusion and exclusion criteria, year of publication, country of origin, ethnicity, matching criteria, number of cases and controls, diagnostic methods of PAC, HBV/HCV detection assays used. A third author (S.F.) checked the accuracy of obtained results.

Our systematic search, concerning HBV infection and PADC risk, identified 14640 articles. According to a preliminary evaluation of titles and/or abstracts, we excluded 14602 citations. A more detailed analysis was performed on the full-text of the remaining 38 articles. Of these, 23 were excluded, because they were conference abstracts, case reports, editorials, articles not written in English or included incomplete data. Eligible publications were 15: 6 case-control and 2 cohort-studies as well as 7 meta-analyses. On the other hand, our search, evaluating HCV infection and risk of PAC, identified 14503 citations. Following, the preliminary assessment of the title and/or abstract, we excluded 14478 articles. Then, of 25 potentially relevant publications, 16 were not considered, for reasons similar to those reported above. Eligible papers were 9. Among them, 4 case-control-, 2 cohort-studies and 3 meta-analyses. The flow-chart underlying search and selection, evaluating HBV and HCV infection and risk of PADC is reported in Figure 1 (section 1a and 1b respectively).

Results and Discussion

The present systematic review represents an update of a previous paper from our research, published in 2013, focusing on the same topic [29]. Since then additional trials, evaluating the association between HBV/HCV infection and risk of PADC, have been performed [23, 24, 30-38] as well as further meta-analyses have been carried out [39-45].

The growing number of epidemiological findings on this subject, which have been published in a very short period of time, is the hallmark of the increased interest for this emerging field of research. Table 1 and 2 synthesize the major characteristics of reports, currently available, that have been designed to assess this potential link. Although some of these articles support the hypothesis that HBV and HCV may be involved in pancreatic carcinogenesis, other papers do not confirm this assumption. Several reasons may explain these discrepancies. Heterogeneity in study design, sample-size of enrolled patients, end-points and distinct HBV and HCV prevalence rates, depending on the countries where the trials have been carried out, are the most important factors, explaining the lack of univocal conclusions.

Most of studies, assessing the possible relationship between HBV/HCV infection and development of carcinoma in the pancreas, have taken place in peoples of Asian ethnicity, in particular in Chinese [23, 30-34] or Korean population [35].

Up to now, only five trials have been performed outside of China with the purpose to assess PADC risk in HBV infection: one case-control- and one cohort-trial in United States of America (US) [23, 38] one in South Korea [35] and two cohort studies in Sweden [36-37]. Both Swedish reports have been considered in our article, although they utilize similar population-based nationwide registers to generate cohorts of HBV- and HCV-persistently infected subjects, because they have included different periods of surveillance and have investigated distinct end-points. On the other hand, four trials have investigated the risk of this malignancy in HCV infection: two studies in United States of America (US), one in South Korea [35] and one in Sweden [36]. It is well-known that prevalence of these two

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pathogens varies widely worldwide. Both Sweden and US are low endemic countries for HBV and HCV.

In the European Country, the reported prevalence of Hepatitis B surface antigen (HBsAg) positivity in the general population is <1% with overall markers <5% [46], whereas the estimated rate of the peoples infected with HCV is 0.5% [47]. Likewise, in US the percentages of subjects with chronic HBV infection and anti-HCV antibodies are lower than 1% [48] and about 1% [49] respectively. On the other hand, in China, the prevalence of HBV persistent infection is still high (nearly 6-7%), although long-term vaccination programs, have reduced the HBsAg-positivity rate in the general population [50], whereas the proportion of anti-HCV positive subjects varies considerably, ranging from 1.0% to 3.2% in most areas [51].

Taking advantage from the studies, concerning prevalence of both viruses worldwide as well as epidemiological reports designed to assess the possible association between these pathogens and PADC, some considerations may be drawn. Some studies, concerning patients with HBV infection, have suggested that HBsAg persistence is associated with a significant higher risk to develop PADC [23, 30-33] in comparison with its absence. On the other hand, other trials have not confirmed this relationship [35, 37]. Furthermore, no univocal conclusions have been obtained from the small number of available studies, evaluating the probability of this malignancy in individuals with signs of previous exposure to HBV [23, 31, 32, 37, 38]. In particular, two reports have shown an enhanced probability to develop PAC in subjects with serum HBsAg negative/Hepatitis B core antibody (HBcAb) positive/ Hepatitis B surface antibody (HBsAb) negative pattern [23, 32] whereas two did not confirm this assumption [31, 37] as well as two trials have correlated serum HBsAg-/HBcAb +/HBsAb + profile with an increased risk of this malignancy [23, 31], whereas two did not [32, 37]. Once more, three of these studies have been carried out in Chinese patients [31, 32, 37].

It should be also considered that HBsAg-/HBcAb +/HBsAb - as well as HBsAg-/HBcAb +/HBsAb + serum patterns are generally considered as signs of past HBV exposure, with subsequent HBV clearance. In the last years, following the introduction in clinical practice of highly sensitive techniques of molecular biology, HBV-DNA has been detected in liver specimens of some HBsAg negative subjects and irrespective of serum HBcAb and/or HBsAb. Therefore, these observations demonstrate that this pathogen is able to persist and replicate, although at a low rate, even in a percentage of individuals with serum markers, regarded as indicative of complete HBV recovery. This complex biological entity has been defined as "occult infection" [52], but its clinical impact in carcinogenesis both in pancreas and in different organs has not been yet clearly established, although some studies have suggested an its involvement in several pathologies, including cryptogenic chronic hepatic diseases as well as liver carcinoma [53, 54].

In this scenario, several meta-analyses have been published, with different end-points (Table 3). In particular:

1 some have assessed the association between the probability to develop PADC and HBsAg positivity [39, 40, 41, 42, 43, 44, 45],
2 some have evaluated the relationship between PADC risk and HBsAg positivity as well as between PADC risk and serum HBV antigens/antibody patterns [39, 42, 43, 44];

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3 some have assessed the correlation between PADC risk and HBsAg positivity, or between PADC risk and serum HBV antigens/antibody patterns or between PADC risk and HCV infection [42, 43, 44].

The number of studies considered in each of these works varies widely, according to different inclusion criteria used by the authors. Table 4 synthesizes main findings of studies not published as English full-text or published as incomplete data [55-60]. On the basis of the results, HBsAg has emerged as risk factor for PADC in all the published meta-analyses, whereas serum patterns indicative of "past exposure to HBV" resulted to be associated with a higher probability to develop this malignancy in some of these analyses, but not in others. However, it has to be underlined that none of meta-analyses available to date includes the two Swedish reports [35, 36] as well as the Korean research [34] and the last Chinese case-control study [37], because these studies have been published only recently, in particular, between the end of 2013 and the beginning of 2014.

Furthermore, some of available cohort- and case-control studies have suggested that HCV infection represents a risk factor for PADC, whereas others have not confirmed this association [23, 32, 37]. Two trials have carried out in Chinese population [32, 37], one in Korea [34], two in US [23, 24] and one in Sweden [35]. It has to be underlined that the largest-sized research has been performed in US [24]. To date, three meta-analyses have been published on this topic and have shown that HCV is associated with an increased probability to develop this malignancy [42, 43, 44].

Once more, studies included in these works differs widely, on the basis of various selection criteria used by each author.

Most of available meta-analyses, concerning HBV infection and PADC risk, have adjusted it for some pathological conditions, including diabetes, smoking status and alcohol intake and have suggested that it is independent of all these potential confounding factors in some works or of smoking status and alcohol in others [40-44]. Reasons of these discrepancies may be various, depending on different number and type of studies included. On the other hand, only a few studies assessed the possible association between HCV and PADC [23, 24, 32, 34, 35]. Therefore, the adjustment for diabetes, alcohol use or smoking habits was not possible in the meta-analyses. In addition, no studies have been performed with the purpose to understand whether some HBV or HCV genotypes have a major oncogenic role for pancreas in comparison with others as well as up to now it is unclear if a synergism exists between the above mentioned variables in development of this malignancy in subjects with coexisting markers of persistent or "past" HBV- or HCV-related infection [32].

To our knowledge, to date, only one case control-study has assessed this point. In particular, from this research it has emerged that HBsAg positive patients with a history of diabetes present a relative excess risk for this cancer, exceeding the sum of each risk factor alone [32]. In addition, only one meta-analysis has suggested that PADC incidence is characterized by a peculiar distribution worldwide, it is higher in the more economically developed nations worldwide as well as in the countries more distant from equator [42].

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Table 1. Characteristics of available studies, reported in English, designed to assess the association between HBV infection and PAC risk

Author/ Journal/ Publication Year	Country/ Ethnicity	Study Design/ StudyPeriod	PAC diagnosis	Control source	Sample size (Cases/ controls)	Matching criteria	Matching factors			HBV status assessed								
							Diabetes	Alcohol	Cigarette	HBsAg	HBeAg	HBsAb	HBcAb	HBeAb	HBsAg+/ HBcAb+	HBsAg-/ HBcAb+	HBsAb+/ HBcAb+/	HBsAb-/ HBcAb+
Hassan MM J Clin Oncol. 2008 (23)	USA/ Caucasian	-Hospital-based case-control study - 2000-2007	Histological	Community-based (healthy genetically unrelated family members of patients with cancer other than pancreatic, GI, lung or head cancers)	474/872	age (± 5 years), sex and race	Y	Y	Y	Y	N	Y	Y	N	Y	Y	Y	Y
Iloeji UH Liver Int 2010 (30)	Taiwan/ Asian	-Population-based prospective cohort study -1991-2007	Histological or radiological/ endoscopic (retrograde cholangio-pancreatography)	Residents (30-65 years aged), living in seven townships in Taiwan	22471 participants 48 PAC detected	Not available	N	Y	Y	Y	Y	N	N	N	N	N	N	N
De-Shen W Int. J Cancer. 2011 (31)	China/ Asian	-Hospital-based case-control study -1999-2010	Histological	Hospital (randomly selected patients after surgical management for non-neoplastic diseases)	605/711	age and sex	Y	Y	Y	Y	Y	Y	Y	Y	Y	NR	Y	NR
Ben Q Pancreas 2012 (32)	China/ Asian	-Double-Centre Ongoing hospital-based case-control study .-2003-2009	Histological or citological	Hospital (patients admitted to the same Hospitals for any acute conditions)	943/1128	age (within 3 years) and sex	Y	Y	Y	Y	N	Y	Y	N	NR	Y	Y	Y
Zhu F Asian Pacific J Cancer Prev. 2011 (33)	China/ Asian	-Multi-centre hospital- based case-control study. -1997-2008	Histological or clinical/ radiological (symptoms, signs and more of two types of imaging tools)	Hospital (randomly selected patients from Orthopaedics and Neurology Departments of the same Hospitals)	533/533	age (± 5 years) and sex	Y	N	N	Y	Y	Y	Y	Y	NR	NR	NR	NR
Woo SM J Korean Med Sci 2013 (34)	Korea/ Asian	-Retro-spective case-control study -2001-2011	Histological or radiological/ clinical	Individuals subjected to routine health examination in the Cancer Screening Cohort	753/3,012	age (within 5 years) and sex	Y	N	Y	Y	N	N	N	N	N	N	N	N

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Table 1. (Continued)

Author/ Journal/ Publication Year	Country/ Ethnicity	Study Design/ StudyPeriod	PAC diagnosis	Control source	Sample size (Cases/ controls)	Matching criteria	Matching factors			HBV status assessed								
							Diabetes	Alcohol	Cigarette	HBsAg	HBeAg	HBsAb	HBcAb	HBeAb	HBsAg+/ HBcAb+	HBsAg-/ HBcAb+	HBsAb+/ HBcAb+/	HBsAb-/ HBcAb+
Huang J Br J Cancer 2013 (35)	Sweden/ Swedish population (Country of origin: -Nordic countries, -non-Nordic European countries; -Other)	-Retrospective Nationwide cohort study -1990-2006	Identification of PAC cases from the Swedish Cancer Register (International Classification of Disease ICD-7: 157) and from the Cause of Death Register (ICD-9: 157; ICD-10: C25)	Control population obtained from the national surveillance database at the Swedish Institute for Infectious Disease Control	- Individuals with HBV infection: 11511, -HBV reference cohort: 57554 participants. 5 PAC detected	age (year of birth), sex and country of residence	Y	Y	N	Y	N	N	N	N	N	N	N	N
Sundquist K J Med Virol 2014 (36)	Sweden/ Swedish population	-Retrospective Population cohort study -1987-2008	Identification of PAC cases from the Swedish Cancer Register (International Classification of Disease ICD-7: 157 and histological codes) and from the Cause of Death Register (ICD-9: 157)	Control population obtained from the Swedish Hospital Discharge Register and Outpatient Register	-10,197 Participants, -10 PAC detected	age (within 5 years), sex, occupation, region of residence and time period	Y	Y	N	Y	Y	Y	Y	Y	Y	N	N	N
Chang MC World J Gastroenterology 2014 (37)	China/ Asian	-Case-control study -2000-2013	Histological or citological	Controls were individuals recruited from a free screening program in a community located in Northern Taiwan	585/1716	age (within 5 years) sex and race	Y	Y	Y	Y	N	Y	N	Y	Y	Y	Y	N
Tang J J Clin Gastroent 2014 (38)	USA/ American population: (-White -African American -Hispanic -Asian population)	-Retrospective cohortl study -1995-2008	Administrative data from an integrated health care delivery system (Henry Ford Health System)	Patients were subdivided into 3 cohorts on the basis of HBV status: -Negative infection, -Previous exposure -Active infection	Subjects:: -28,719 without signs of previous or active HBV exposure; -5141 with previous HBV exposure; -404 with active infection	age (within 5 years) sex and race	Y	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y

Y=determined, N=not determined, NR=not reported.

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Table 2. Characteristics of available studies, reported in English, designed to assess the association between HCV infection and PAC risk

First author/Journal/ Publication Year	Country Origin/ Ethnicity	Study design	PAC diagnosis	Study period	Control source	Sample size (Cases/controls)	Matching criteria	Matching factors		
								Diabetes	Alcohol	Cigarette
Hassan MM J Clin Oncol. 2008 [23]	US/Caucasian	Hospital-based case-control study	Histological confirmation	2000-2007	Community-based (healthy genetically unrelated family members of patients with cancer other than pancreatic, GI, lung or head cancers)	474/872	age (± 5 years), sex and race	Y	Y	Y
Qiwen Ben Pancreas 2012 [32]	China/Asian	Double-centre ongoing hospital-based case-control study.	Histological or citological confirmation	2003-2009	Hospital (patients admitted to the same Hospitals for any acute conditions)	943/1128	age (within 3 years) and sex	Y	Y	Y
El Serag Hepatology 2009 [24]	US/Caucasian	Retrospective cohort study	Identification of PAC cases by means of ICD-9-CM diagnosis codes (157.0, 157.1, 157.2, 157.3, 157.8, 157.9) Identification of HCV infected subjects by means of ICD-9-CM diagnosis codes (070.41, 070.44, 070.51, 070.54 and V02.62)	1988-2004	Sources included inpatients records from more than 150 of US Veterans Affairs (VA) hospitals in the Patients treatment file and outpatients records from any VA facility in the Output Clinic File	718,687 patients (146,394 HCV-infected cohort, 572,293 HCV-uninfected cohort) 617 PAC detected (140 in HCV infected patients, 477 in HCV uninfected subjects)	HCV-uninfected and HCV-infected subjects matched by age (± 1 year) and sex	Y	Y	N
Woo SM J Korean Med Sci 2013 [34]	Korea/Asian	Retro-spective case-control study	Histological or radiological/clinical confirmation	2001-2011	Individuals subjected to routine health examination in the Cancer Screening Cohort	753/3,012	age (within 5 years) and sex	Y	N	Y
Huang J Br J Cancer 2013 [35]	Sweden/ Swedish population	- Retrospective Nationwide cohort study	Identification of PAC cases from the Swedish Cancer Register (International Classification of Disease ICD-7: 157) and from the Cause of Death Register (ICD-9: 157; ICD-10: C25)	1990-2006	Control population obtained from the national surveillance database at the Swedish Institute for Infectious Disease Control	- Individuals with HCV infection: 39,442 - HCV reference cohort: 197,208 participants: 34 PAC detected	age (within 5 years) and sex	Y	Y	N
Chang MC World J Gastroenterology 2014 [37]	China/ Asian	- Case-control study	Histological or citological	2000-2013	Controls were individuals recruited from a free screening program in a community located in Northern Taiwan	585/1,716	age (within 5 years) and sex	Y	Y	Y

Y=determined, N=not determined, NR=not reported.

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Table 3. Characteristics of available meta-analyses, reported in English, assessing the association between HBV/HCV infection and PAC risk

First Author/ Country	Journal/ Publication Year	Title	Number of studies considered	Main conclusion	Adjustment for		
					Diabetes	Alcohol	Cigarette
Wang Y. China [39]	Eur J Cancer Prev 2013	Hepatitis B virus status and the risk of pancreatic cancer: a meta-analysis	9 studies	- Increase in PAC risk in chronic or inactive HBsAg carriers in comparison with subjects without previous exposure to HBV infection: OR=1.60 (95% CI: 1.26-2.05). - Increase in PAC risk in HBcAb positive, but HBsAb negative patients in comparison with controls: OR= 1.76 (95% CI: 1.05-2.93)	N	N	
Luo G. China [40]	Cancer Causes Control 2013	HBV infection increases the risk of pancreatic cancer: a meta-analysis	9 studies	- Increase in PAC risk in subgroup of HBV chronic carriers versus subgroup of subjects never exposed to HBV: RR 3.83 (95%CI: 1.76-8.36). - Increase in PAC risk in subgroup of patients with active HBV infection versus subgroup of subjects never exposed to HBV: RR 1.92 (95%CI: 1.06-1.87). - Increase in PAC risk in subgroup of patients with past exposure to HBV versus subgroup of subjects never exposed to HBV: RR 1.41 (95%CI: 1.06-1.87).	Y	Y	Y
Li L. China [41]	Asian Pacific Journal of Cancer Prevention 2013	Chronic Hepatitis B virus infection and risk of pancreatic cancer: a meta-analysis	8 studies	- Increase in PAC risk in patients with chronic HBV infection versus individuals without HBV infection: OR=1.403 (95% CI: 1.139-1.729)	Y	Y	Y
Fiorino S. Italy [42]	Pancreatology 2013	Association between hepatitis B or hepatitis C virus infection and risk of pancreatic adenocarcinoma development: a systematic review and meta-analysis	- 5 studies included for assessment of HBV infection and PAC risk - 3 studies available for assessment of HCV infection and PAC risk	- Increase in PAC risk in HBsAg positive patients in comparison with HBsAg negative individuals: RR 1.18 (95%CI: 1.04-1.33). - No association between HBsAg negative/HBcAb positive/HBsAb negative pattern (RR=1.12 (95%CI: 0.78-1.59) as well as HBsAg negative/HBcAb positive/HBsAb positive profile (RR=1.30 (95%CI: 0.93-1.84) and risk of PAC was observed. - No statistically significant relationship between anti-HCV positivity and PAC risk, although a borderline value was detected in this comparison (RR=1.16 (95%CI: 0.99-1.3).	Y	Y	Y

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Table 3. (Continued)

First Author/ Country	Journal/ Publication Year	Title	Number of studies considered	Main conclusion	Adjustment for		
					Diabetes	Alcohol	Cigarette
Xing S China [43]	Hepatobiliary Pancreat Dis Int 2013	Chronic hepatitis virus infection increases the risk of pancreatic cancer: a meta-analysis	- 10 studies included for assessment of HBV infection and PAC risk - 7 studies included for assessment of HCV infection and PAC risk	- Increase in PAC risk in HBsAg positive patients in comparison with controls: RR 1.28 (95%CI: 1.11-1.48). - No association between HBsAg negative/HBcAb positive/HBsAb negative as well as HBsAg negative/HBcAb positive/HBsAb positive profile patterns and risk of PAC was observed (RR=1.57 (95%CI: 0.83-2.98 and RR=1.24 (95%CI: 0.72-2.14, respectively). - Higher PAC risk in anti-HCV positive patients: RR=1.21 (95%CI: 1.02-1.44).	Y	Y	Y
Xu JH. China [44]	World J Gastroenterology 2013	Hepatitis B or hepatitis C virus infection and risk of pancreatic cancer: a meta-analysis of observational studies	- 8 studies included for assessment of HBV infection and PAC risk - 5 studies available for assessment of HCV infection and PAC risk	- Increase in PAC risk in HBsAg positive patients in comparison with individuals without a history of chronic hepatitis B individuals: RR 1.20 (95%CI: 1.01-1.39), especially in the Chinese population: RR 1.30 (95%CI: 1.05-1.56), - Higher risk of pancreatic cancer in patients with past exposure to HBV: RR=1.24 (95%CI: 1.05-1.42), especially in HBcAb positive/HBsAb positive: RR=1.67 (95%CI: 1.13-2.22. No association between/HBcAb positive/HBsAb positive pattern and pancreatic cancer risk was observed: RR=0.98 (95%CI: 0.80-1.16). - Higher risk of pancreatic cancer in subjects with past-exposure to HCV: RR=1.26 (95%CI: 1.03-1.5).	Y	Y	Y
Majumder S US [45]	J Gastrointest Cancer. 2014	Association between HBsAg positivity and pancreatic cancer: a meta-analysis.	- 3 studies included for assessment of HBV infection and PAC risk	- Increased PAC risk in HBsAg positive patients in comparison controls: RR 1.50 (95%CI: 1.21-1.87). - No significant higher risk of pancreatic cnacer in patients withpositive /HBcAb positive status (RR=1.23 (95%CI: 0.95-1.59)	Y	Y	Y

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Table 4. (a) Main findings of available studies, not reported in English, evaluating HBV and/or HCV infection and PAC risk. (b) Characteristics of studies, reported in English, assessing HBV and/or HCV infection and PAC risk, with no complete data or not reported as full-text. (c) Meta-analysis not reported in English, evaluating HBV infection and PAC risk

STUDIES (First Author/Journal/ Year of publication)	Study Title	Main findings	Study conclusion
(a)			
Ma W, 2009 Chinese Journal of Clinical Oncology 2009; 36:24 (1388-90) [55]	Association between hepatitis B virus infection and pancreatic cancer	Full-text in Chinese	Possible association between HBV infection and pancreatic cancer.
Hong SG, 2010 The Korean Journal of Hepatology 2010; 16:1 [56]	The relationship between hepatitis B virus infection and the incidence of pancreatic cancer: a retrospective case-control study	Full-text in Korean	Absence of significant association between HBsAg or anti-HCV positivity and pancreatic cancer.
Xu P, 2011 Cancer (Chinese J) 2011; 31: 653-7 [57]	Risk factors for pancreatic cancer: a case-control study	Full-text in Chinese	Absence of significant relationship between HBsAg or anti-HCV positivity and pancreatic cancer.
(b)			
Fang Zhu Asian Pacific J Cancer Prev. 2011 [33]	Chronic hepatitis virus infection and pancreatic cancer: a case-control study in southern China	No detailed description of number of patients with HCV-infection in case- and control group	Increased prevalence of anti-HCV antibodies in patients with pancreatic cancer
Tang J, 2009. Hepatology 2009; 50: 988A [53]	Is previous exposure to hepatitis B a risk factor for pancreatic cancer?	Abstract	No higher risk of pancreatic cancer in patients with markers of "past" exposure to HBV.
Chang YT, 2009. Pancreatology 2009; 9: 518A [58]	Chronic hepatitis B virus and hepatitis C virus infection are not associated with pancreatic cancer in Taiwan-A high endemic area.	Abstract	Absence of a significant association between HBsAg or anti-HCV positivity and pancreatic cancer.
Berrington de Gonzales, 2008. Cancer Epidemiol Biomarkers Prev 2008; 17:359-64 [59]	Pancreatic cancer and factors associated with the insulin resistance syndrome in the Korean cancer prevention study.	Data on HBsAg status was reported only in a subgroup (32%) of study population	Absence of significant association between HBsAg status and pancreatic cancer risk.
c)			
Zhuang H, 2014 Zhonghua Gan Zang Bing Za Zhi. 2014; 22: 416-9 [60]	Association between hepatitis B virus infection and risk of pancreatic cancer: a meta-analysis	Full-text in Chinese	Significant association between HBsAg positivity and increased pancreatic cancer. risk

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According to this report, no correlation has emerged between areas of HBV/HCV prevalence and PADC incidence. Reasons of this discrepancy may be different, depending on accuracy of data collection as well as of diagnostic and methodological procedures, used to detect this cancer. Therefore, these factors might have an impact on the final conclusions. Further studies are required to clarify definitively these points in a more adequate way.

Up to now, it is not understood what mechanisms might be involved in the process of pancreatic carcinogenesis in patients with past or present HBV/ HCV exposure. However, according to the available evidences, some elements may be taken into account to explain the possible role of both viruses in the promotion and development of this malignancy. In subjects with pancreatic cancer and serum markers of persistent or previous HBV/HCV infection, the development of this malignancy might share some of mechanisms involved in liver carcinogenesis. This topic has been widely discussed in a recent systematic review [61].

On the basis of this report, the following points have to be considered:

1 Current studies have shown that both liver and pancreas originate from common multipotent cells, derived from endoderm layer; in particular, upon different standardized conditions of culture, lines of hepatoma cells and pancreatic cancer cells have been induced to transdifferentiate into hepatocyte-like cells [62, 63]; a further research has reported that pancreatic cells, upon a treatment with dexamethasone and oncostatin M and following permanent transfection with HBV genome, have been able to transdifferentiate into permissive hepatocyte-like cells and have supported replication of this pathogen, generating its antigens and DNA [64].
2 HBV and HCV present distinct genomic organization and transcription/translation pathways, nevertheless they are able to perturb cellular homeostasis, by targeting common intracellular signals and functions. In particular, several in “vitro” and in “vivo” studies have investigated mechanisms by which both viruses are able to promote liver carcinogenesis [65]. Some viral proteins, such as hepatitis B virus X protein (HBx), HCV-NS3A as well as HCV-NS5A non-structural proteins and core protein, are able to affect distinct cellular signalling cascades and to perturbate their activities [66-69].
3 Some cellular paths which are altered in HBV- and HCV-related hepatic inflammation and cancer development are very similar to those deregulated during pancreatic carcinogenesis [70, 71].
4 Antigens and distinct forms of HBV and HCV genome have been observed in pancreatic tissue, as well as several reports have described the relationship between fulminant and non-fulminant variants of viral hepatitis and acute pancreatitis [25, 27, 28, 72, 25, 27, 28, 72].

Based on results of these experimental investigations and clinical reports, it may be hypothesized that these viruses might infect pancreas not only acutely, but even persistently, causing a long-lasting inflammatory process and a chronic damage in this organ [29]. These events might represent important pro-oncogenic stimuli, leading, as a consequence, to the development of pancreatic carcinoma. To date, only a small number of studies have evaluated in pancreas the possible association among presence of HBV and HCV and histological findings of inflammatory response, eventually induced by both viruses. Concerning HBV, in 1981 [73] a research has demonstrated the existence of HBsAg and HBcAg in the cytoplasm

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of pancreatic acinar cells as well as a chronic inflammatory injury and fatty necrosis in pancreatic specimens, obtained from 30 HBsAg positive individuals, who underwent an autoptic procedure after their death. Hepatic histological findings, which were observed in these individuals, included cases of fatty liver disease, chronic viral hepatitis, fibrosis, massive and submassive cellular necrosis, cirrhosis and hepatocellular carcinoma (HCC). In 1984 a study has reported detection of HBV-DNA sequences, integrated in pancreatic tissue of two subjects: one subject died because of cirrhosis/ HCC, the other individual died because of severe acute hepatitis after pancreatectomy for pancreatic cancer [74]. A further research was carried out in 1985 in a series of individuals, undergoing a surgical procedure, in pancreas. HBsAg was detected in pancreatic tissues of two patients with chronic hepatitis and of five individuals with pancreatic carcinoma [75]. Recently a study has evaluated for the first time the possible impact of HBV genome and antigens in the development of these pathological conditions, assessing the possible presence of these viral markers in pancreatic malignant- as well as in adjacent non-cancerous tissues of a large number of patients with chronic pancreatitis and/or PADC [76]. An elevated percentage of patients with PADC and HBV-DNA positivity resulted to be HBsAg negative. Authors concluded that in their research the subjects with this cancer had a high prevalence of occult HBV infection. Concerning HCV, as previously reported, only few studies have evaluated the presence of antigens and/or genome of this virus in normal pancreas [25]. On the other hand, to date, no research has evaluated the possible presence and role of this pathogen in pancreatic cancerous and/or non-cancerous tissue of HCV positive patients with PADC. According to available reports, a multistep process characterizes the development of this malignancy [77]. Although the histogenesis and cytogenesis of this cancer is not completely understood, some recent reports suggested that pancreatic carcinoma might derive from acinar or centro-acinar cells as well as from the gland-like mucinous outpounches of major ducts [78, 79]. The existence of a persistent inflammatory process in pancreatic tissue represents a very important predisposing condition for development of acinar to ductal-mucinous metaplasia [80, 81].

This event consists in the replacement of a cellular type with another and it is characterized by an increased risk of cancer, because it promotes a permissive environment, where pro-oncogenic agents may act [82]. In the last years, several studies have been carried out with the attempt to define the possible pathways, by which chronic inflammation is able to induce substantial changes in normal structure of pancreatic parenchyma, causing its malignant transformation [83]. In the early stages, this multistep process is characterized by a mutual and dynamic crosstalk among epithelial cells, which are progressively undergoing a switch towards a neoplastic phenotype, and supporting connective tissue [84, 85]. A wide range of elements are considered to contribute to the initiation and progression of cancer in pancreas, including different cell types, such as malignant-, immune- (effector and regulatory lymphocytes, neutrophils, macrophages, dendritic-cells) [86, 87] stellate-, endothelial- and bone-marrow-stem-cells as well as modulating-factors, such as prostaglandins, leukotriens, cytokines, interleukins and growth factors.

As a consequence of this complex interplay, deposition of an abnormal connective tissue occurs [88]. It consists of several components, in different quantitative and qualitative ratio in comparison to stroma in normal pancreatic tissue. The modified connective structure includes distinct types of collagens (mainly I, III, V) [89] and increased amounts of non-collagenous proteins, such as periostin [90, 91], fibronectin [92], tenascin [93, 94], fibrinogen [95]. This series of events is the progressive perturbation of normal cellular activities, with subsequent

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progressive remodelling of pancreatic parenchyma and alteration of its tissue architecture and stiffness. The final effect of this process is represented by the development of genetic alterations and by the rising of malignant transformation.

Therefore, available epidemiological, histological and pathogenetic studies support the conclusion that HBV and HCV may exert a key-role in the promotion of carcinogenesis in this organ.

Conclusion

Although the published studies and meta-analyses are providing new promising findings on the possible risk factors for PADC and are suggesting that these hepatotropic viruses may be involved in the development of this malignancy, the results of these reports should be considered with caution, because of several limiting factors. They include: the relatively small number of available epidemiological reports, the geographical location of the studies (nearly all carried out in Asian populations), the different trials and meta-analyses designs and end-points, the shortage of histological works assessing the presence of antigens/genomes of both these pathogens in pancreatic normal and/or cancerous tissues.

Therefore, further studies are absolutely required to improve our knowledge on this topic. These works should be focused on peoples of different ethnicity and geographical regions and should be also aimed to explain pathogenetic mechanisms, possibly involved in the process of pancreatic carcinogenesis.

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Conflict of Interest

None declared

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Chapter 6

The Risk and Prognostic Factors Associated with Undifferentiated Nasopharyngeal Carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) is a unique head and neck cancer with characteristic geographic distribution. In endemic area, keratinizing undifferentiated carcinoma is the major histological type and the cancer is closely associated with Epstein-Barr virus infection. NPC is a rare disease in Western countries. The incidence of undifferentiated NPC is very much higher in Southern China in comparison with other parts of the world. Oncogenic gene mutations are rarely found in NPC suggesting other risk factors are associated with the disease. Genetic predisposition is a major risk factor at the high incidence areas. Other risk factors including environmental exposure and viral infection could also contribute to the disease. The mainstay treatment for early NPC is radiation therapy as the tumor is very sensitive to radiation. Functional imaging represents a noninvasive method to provide metabolic and clinical information for the guideline of NPC management. Tumor stage and tumor histology have major influence on the treatment outcomes and are significant prognostic factors for local or regional control of NPC. EBV, HPV and a number of oncogenic proteins could serve as critical molecular biomarkers for the monitoring of patients. Comprehensive evaluation on the latest findings on risk factors and prognostic factor may help to identify high-risk community earlier and predict treatment outcome.

Keywords: screening, prognosis, EBV, EBV DNA, genetic predisposition, survival, HPV, EGFR

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1. Introduction

Nasopharyngeal cancer (NPC) is an epithelial cancer arising from the mucosal epithelium of the posterior nasopharynx (behind the nose toward skull base). From the global perspective, NPC is a rare disease with incidence rate below 1 per 100,000 people annually [1]. According to the histological characteristic, there are 3 major subtypes including keratinizing squamous cell carcinoma, non-keratinizing carcinoma and undifferentiated NPC. NPC has higher prevalence in the male group. Epidemiological data suggest that it is a disease associated with lower social class and occupation exposure. Urbanization however is not a major risk factor. In terms of geographic distribution, NPC is remarkably biased to particular regions including southern China, Greenland, Alaska, Hanoi, Singapore, Manila, and Setif [1]. The Chinese community in the central of Guangdong province (China) has the highest NPC incidence around the world. In Southern China (including Hong Kong) the incidence is elevated to 20-50/100,000 per years making it a significant disease to the area [2]. NPC in the endemic areas is mainly undifferentiated carcinoma which is closely associated with the oncogenic activity of the Epstein-Barr virus. Luckily, the undifferentiated NPC is highly susceptible to radiation in comparison with their keratinizing counterparts.

Functional imaging, such as MRI, CT and 18F-FDG PET or PET/CT, are noninvasive means which provide metabolic information on the entire tumor as well as useful clinical information to guide treatment. The value of these techniques in predicting prognosis in patients with NPC becomes increasingly critical. MRI is a common practice to evaluate pretreatment condition of patients with NPC. It has great efficacy for T staging and is better than CT in determining the skull base invasion status [3]. Because of its superior capability in demonstrating primary soft tissue invasion and excellent spatial, MRI has become an integral part in the management of NPC. The main treatment is standard radiotherapy or use in combination with chemotherapy. About 30-40% of patients would experience distant or local metastasis despite treatment, and the 5-year survival rate is about ~40-50% in general [4].

2. Risk Factor of Nasopharyngeal Carcinoma

2.1. EBV Infection

Epstein-Barr virus (EBV) is a ubiquitous lymphotropic virus infecting more than 95% individuals in the human population [5]. EBV is a herpes virus with double-stranded linear DNA genome. The causal role of EBV with human malignancies is recognized and is listed as group 1 carcinogen under the International Agency for Research for Cancer [6]. The virus could be found in the oropharyngeal secretion and infect the epithelial cells. The dynamics of virus shedding is affected by multiple factors including cell-mediated immunity, pregnancy, aging and tonsillitis [6]. In the infected cells, EBV exists as nuclear minichromosome in form of circular DNA. In latency period, the virus will express a unique collection of genes. In latency III period, EBV will express all the latently infected genes including EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNALP, latency membrane proteins 1, latency membrane protein 2, and the non-coding RNAs including EBERS, BART and microRNA (mainly located in the BART regions) [7]. In nasopharyngeal carcinoma, EBV is in type II

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latency with a more restricted latent gene expression pattern [8]. The viral genes expression in type II latency period is evident to promote the malignant transformation process in the mucosal epithelium [9]. At present, how EBV infects the oropharyngeal epithelial cells remain unresolved as it remains difficult to repeat the immortalization process in the in vitro conditions [10]. Detection of the viral gene products in nearly all the undifferentiated NPC cells suggested that EBV is a close mediator in the transformation process of nasopharyngeal epithelium.

2.2. Ethnic Background

Nasopharyngeal carcinoma is strongly associated with the Cantonese ethnicity. In addition, NPC demonstrated strong familial aggregation. The risk remains even if the susceptible offspring are migrated to other parts of the world. Further, it is established that individuals with first-degree relative with NPC will have high risk (up to 10-fold) to develop NPC in comparison with the sporadic cases [1]. For familial NPC in the Guangdong province, NPC onset is usually early with average ages around 36 years [11]. Thus, families with members affected with NPC in our locality will perceive multiple life-long stress resulting from the unpredictability and uncertainty of the cancer onset. Genome-wide screening on the familial cases revealed that the gene predisposed to the high-risk families (with > 2 members having the disease) is located on chromosome 4 [12]. The precise genetic predisposition however remains to be resolved. Whole-exome sequencing revealed that genes are involved in ERBB-PI3K signaling pathway, autophagy machinery and chromatin modification [13]. Routine screening of the high risk group with enzyme-linked immunosorbent assay against EBV nuclear antigen and viral capsid antigen is suggested to be an effective approach to enhance early cancer discovery in the first degree family members and siblings [14].

2.3. Consumption of Preserved Food

In the high incidence areas, consumptions of particular preserved foods are linked with NPC development. In southern China and Southeast Asia, salt-preserved fish consumption (especially to infants during weaning) has long been implicated as risk factor of NPC development [15]. The volatile nitrosamines (dimethylnitrosamine and diethylnitrosamine) in the salted fish are potent carcinogens which could induce development of cancer in the nasal cavity in the rat model [16]. In North Africa, special preserved foods including quaddid (dried meat in oil), harissa (a spicy condiment) and toklia are contributing factors to the disease [17]. There might be common constituents in the preserved food, which promote cancer development in the early ages of the high-risk communities. In addition, the carcinogenic constituents may activate the ubiquitous infected EBV in the head and neck regions, which promotes early cancerous changes in the nasal epithelium [18].

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3. Prognostic Factors of Nasopharyngeal Carcinoma

3.1. The Prognostic Significance of EBV

Circulating EBV is a potential diagnostic marker in NPC patients. In addition, pre-treatment plasma EBV DNA level is a prognostic indicator to the NPC patients. Early-staged NPC patients with low plasma EBV DNA concentration have better survival than those with high plasma EBV DNA concentration [19]. Serial monitoring plasma EBV DNA concentration after treatment is a useful way to evaluate treatment outcome and detect disease recurrence. Once radiotherapy is initiated, there will be a surge of EBV DNA level in the first 2 weeks followed by a gradually decrease [20]. Patients who detected a higher efficiency on clearance of EBV DNA from the circulation reflected more sensitivity to treatment and better outcomes after therapies compared with their counterpart [20]. The dynamics of circulating EBV DNA could serve as an evaluation mean to determine individual response to radiation. Clinician might change and adjust the auxiliary treatment plan accordingly. For example, in patients with low responsiveness to radiotherapy, adjuvant chemotherapy may consider necessary in the therapeutic regime. The plasma EBV DNA level would remain constantly low during continuous clinic therapy [21]. Sudden increase in circulating EBV DNA levels is a sign of loco-regional recurrence or development of distant metastasis.

3.2. Human Papillomavirus (HPV)

HPV is suggested to be a causative agent in head and neck cancers especially in the carcinoma developed in the oropharynx. Due to the close proximity of nasopharynx with oropharynx, involvement of HPV in the NPC development is suspected. HPV positive NPC in general has a better prognosis. In NPC patients with HPV-16 and/or HPV-18 detected in the primary tumor, the overall recurrence rate and mortality rate is lower in comparison with the HPV-negative counterparts [22]. Comparing the HPV infection status in the endemic regions and non-endemic regions suggested that HPV infection is more prevalent in low incidence areas and are detected in the EBV-negative cases [23]. HPV could be detected in the both keratinizing and non-keratinizing NPC, which is usually EBV negative [24]. The HPV-positive NPC could possibly originated from the oropharynx [24]. Although there are studies suggesting that co-infection of HPV and EBV might have additive advantages in NPC transformation, co-infection is not always found [25, 26]. Hence, whether HPV or particular HPV subtypes is involved in NPC remains speculative.

3.3. Latent Membrane Protein 1 (LMP1)

The metastatic potential of NPC will directly affect the prognosis of NPC patients. This is in part controlled by the EBV-encoded viral protein LMP1. LMP1 is a transmembrane protein containing six-transmembrane domains [27]. Although its transforming or oncogenic properties have been demonstrated in the cell line models, LMP1 expression is not universal

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in all the NPC cases. LMP1 expressing NPC cell line C666-1 shows a significant reduction in cell mobility and invasion ability in the presence of LMP-1 specific small hairpin RNA [28]. The cytoplasmic domain of LMP1 can activate protein c-Myc and activate the metastasis related pathways subsequently [29]. Further, LMP1 could modulate genes involved in invasion and metastasis such as E-cadherin, MMP and EGFR in the nasopharyngeal carcinoma cells [30]. Using systemic review and meta-analysis, it was shown that LMP1 expression is associated with local lymph node metastasis and distant metastasis. The cumulative metastasis rate was significantly higher (67%) in the LMP1 positive cases in the case-control studies reviewed [30]. Apparently, LMP1 expressing cases tend to be more aggressive resulting to the poor prognosis. In addition, LMP1 expressing NPC showed differential response to the standard therapy and 24-months survival rate [31]. The G2 checkpoint is essential for the cancer cells to recover from radiation-induced damages as it controls the time for correcting or repairing the damaging DNA. LMP1 can impair the G2 checkpoint by hindering Chk1 activation leading to the genomic instability [32].

3.4. Epidermal Growth Factor Receptor (EGFR)

EGFR is a tyrosine kinase receptor expressed on the cell surface of the NPC cells. The extracellular ligand-binding domain can convey the growth and proliferative signals upon ligand binding. The NPC tumor rested in the lymphoid tissues contains a lot of infiltrating lymphocytes. It is suggested that special granulocyte named tumor-associated tissue eosinophilia (TATE) in non-keratinizing carcinoma is activated by EGFR [33]. The association between EGFR overexpression and epithelial cancer progression is well established. In a prospective study in Chinese with undifferentiated NPC, it was shown that EGFR expression is significantly correlated with the overall survival, disease-free survival and advanced T-stages [34]. The latest meta-analysis supported the idea that high EGFR expression will lead to a poor overall survival with prognostic implications [35]. In addition, EGFR extent 25% showed as an independent prognostic factor to predict poor prognosis in advanced stage (Stage III-IV) NPC who was post-treated by induction chemotherapy and RT [36]. Antibodies targeting EGFR could bind to the extracellular domain which subsequently shut down the downstream signaling cascade including P13K/PTEN/AKT and RAS/RAF/MAPK pathways [37].

3.5. Hypoxia-Inducible Factor (HIF)

Tumor hypoxia is linked with the therapeutic resistance. Hence, the hypoxic-related genes have close relationship with the treatment outcome. Hypoxia inducible factor (HIF) is a responsive gene. HIF can induce microvessel development in the tumor bulk. There is a significant high level of HIF-1alpha in the NPC cases with lymph node metastasis. Further, using immunostaining, positive correlation between HIF-1alpha expression and microvessel density is found in the nasopharyngeal carcinoma tissues [38]. Nuclear expression of the transcriptional complex HIF-1 is associated with the poor prognosis of undifferentiated NPC patients [39]. In comparison with HIF-2α, the prognostic role of HIF-1α in undifferentiated is

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more prevalent. HIF-1alpha expression is correlated with autophagy-related Beclin 1 protein in NPC tissues [40].

Given the clear correlation between hypoxia and cancer progression, increasing clinical and preclinical trials have been conducted to evaluate the clinical efficacy to target hypoxia-related genes in NPC treatment. For instance, carbogen (mixed by 95% oxygen gas) and Nicotinamide (an amide of vitamin B3) are employed to increase oxygen delivery to tumor cells, resulting in high local and regional control rates [41]. Berberine, a benzyl-tetra isoquinoline alkaloid isolated from herbal medicine could sensitize NPC cancer cells to radiotherapy by targeting HIF-1a [42]. Other HIF-1a inhibitor such as PX-478 and AC1-004 is also effective in reducing HIF-1a level together with the downstream target genes (such as VEGF and CA IX).

3.6. Carbonic Anhydrase IX (CA IX)

Acidic microenvironment will promote the aggressiveness and metastasis of malignant cells [43]. CA IX (Carbonic anhydrase IX) is suggested to be the one controlling the acidity in the nasopharynx. CA IX (Carbonic anhydrase IX) is the downstream gene controlled by HIF-1α. CA IX regulates pH of tumor microenvironment through catalyzing extracellular CO_2 to H+ and HCO_3-. CA IX activates the mTOR pathway in NPC cell in the cancer cell lines and animal models [44]. Patients with high level of CA IX have poor overall survival and progression free survival. In view of the association of CA IX with poor outcome, the use of specific molecule such as BAY 79-4620 is warranted as a second line treatment for NPC [45, 46].

3.7. Vascular Endothelial Growth Factor (VEGF)

In response to the hypoxic condition, the cancer cells will secret VEGF (vascular epidermal growth factor) to stimulate microvessel generation in order to bring in oxygen and nutrients. Multiple reports suggested that high VEGF is a strong independent predictor of survival and is associated with poor prognosis of NPC [47, 48]. In advanced stage NPC, circulating plasma VEGF level indicates poor survival [49]. There is a report suggesting that a polymorphic site in the promoter region of VEGF (-2578 C/A) is a predisposing factor to the NPC patients with large tumor size and tumor stages [50].

3.8. Metastasis-Associated Protein 1 (MTA1)

Metastasis-associated protein 1 (MTA1) is an independent prognostic marker of worse overall survival in NPC patients [51]. MTA1 is a component of the nucleosome remodelling and histone deacetylase (NuRD) complex [52]. It functions as transcription repressor via histone deacetylation leading to the changes in chromatin conformation. In addition MTA1 could possibly function as GATA-element transcription factor [53]. In NPC, MTA1 could re-organize the actin cytoskeleton via modulating Rho GTPases and Hedgehog signaling leading to the development of aggressive phenotype [54]. Overexpression of MTA1 in NPC is

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associated with the latently expressed viral gene products and latent membrane protein. LMP2A could enhance MTA1 expression via mTOR signaling cascade and induce epithelial-to- mesenchymal transition [55].

3.9. S-Phase Kinase-Associated Protein 2 (Skp2)

Skp2 (S-phase kinase-associated protein 2) belongs to F-box protein family and functions as a cell-cycle regulator. Skp2 expression is not significantly correlated with the EBV IgA VCA titer [56]. However, high Skp2 expression in the primary tissues could predict poor survival and progression free survival [56, 57]. In NPC, however, knock-down Skp2 inhibits cell proliferation, decreases colony forming efficiency and triggers senescence [56, 57]. Although cell cycle arrest is common in the Skp2 knock-down cancer cells, the impact on NPC cell cycle arrest is not obvious [57]. Skp2 was highly expressed in the NPC tissues and was negative in the control [57]. In the Skp2 knock down NPC cell lines, the stem cell population (using ALDH1 as cancer stem cell marker) showed a significant reduction [57]. Hence, Skp2 expressing cells may become more resistant to the conventional treatment regime for NPC leading to the poor post-treatment outcome.

Conclusion

NPC is a multifactorial disease. At present, there is no effective preventive measure to prevent the development of cancer. Early discovery could only be achieved by routine endoscopic examination or serological EBV DNA/ antibody titer screening. By clarifying the risk factor and prognostic factors of NPC, high-risk community could be identified earlier and treatment outcome could also be predicted. Realizing the risk factors may also help to prevent cancer.

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Chapter 7

Widespread Expressions of TCRs in Cancer Cells and the Implications in Cancer Immunology

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Abstract

RP215 is a monoclonal antibody which specifically recognizes a carbohydrate-associated epitope located predominantly on the heavy chains of immunoglobulins, as well as other immunoglobulin superfamily (IgSF) proteins, including TCRs (TCR) and cell adhesion molecules, all of which are expressed among most cancer cells but not among normal immune cells. These cancerous glycoproteins are designated, in general, as CA215. Molecular biological analysis, including RT-PCR and cDNA sequencing of mRNA isolated from cancer cells, revealed that as many as 80% of cancer cell lines express significant levels of TCR-α and/or TCR-β genes. In contrast, co-receptors and co-stimulators of TCR, such as CD3, CD4 and CD8, were rarely expressed in these cancer cells, suggesting undefined roles for these receptors. Consistent with TCR expression as detected by RT-PCR, both immunohistochemical staining and Western blot assay also indicated significant levels of TCR expressions in various cancer cells of different tissue origins. Similar to anti-human IgG and RP215, anti-TCR monoclonal or polyclonal antibodies were also shown to induce apoptosis and complement-dependent cytotoxicity (CDC) to OC-3-VGH ovarian cancer cells, as well as several other cancer cell lines. Upon treatments with anti-IgG and anti-TCRs, the gene expression patterns of cancer cells were compared and found to be highly correlated. Based on the results of cell-based functional assays, these RP215 epitope-specific antigen receptors on the cancer cell surface may act as potential targets for RP215-based anti-cancer drugs for future therapeutic applications in humans.

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Abbreviations

CDC	Complement-dependent cytotoxicity
cDNA	Complementary deoxyribonucleic acid
c-fos	Cellular proto-oncogene
Cyclin D1	G1/S phase regulator protein
EGFR	Epidermal growth factor receptor
IgG	Immunoglobulin G
IgSF	Immunoglobulin superfamily
IHC	Immunohistochemistry
Mab	Monoclonal antibody
MALDI-TOF MS	Matrix adsorption laser desorption ionization-time of flight mass spectrometry
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NFκB-1	Nuclear factor of kappa-B P105 subunit 1
P_o, P_1	Ribosomal proteins for protein synthesis
P_{21}	Cyclin-dependent kinase inhibitor 1
RT-PCR	Reverse transcription polymerase chain reaction
TCR	T cell receptor
TLR	Toll-like receptor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

1. Background Information

Gene expressions of antigen receptors, including immunoglobulins and T cell receptors (TCRs), among cancer cells have been reported almost two decades ago with initial observations explained as a phenomenon of spontaneous gene expressions [1-18]. In the conventional human immune system, the respective expressions and functions of immunoglobulins and TCRs in B and T lymphocytes have been fully elucidated several decades ago [1-19]. In contrast, the expression and function of antigen receptors in cancer cells are not fully understood and their functions may vastly differ from antigen receptors originating from B and T cells [20, 21]. Nevertheless, the expression and function of these antigen receptors in cancer cells may play a crucial role in cancer immunology and immunotherapy.

In 1987, a major breakthrough occurred which would allow one to study these cancerous antigen receptors more closely. RP215 is a monoclonal antibody (Mab), which was generated and characterized against the cell extract of OC-3-VGH ovarian cancer cell line [22, 23]. This Mab was later shown to react specifically with a carbohydrate-associated epitope located mainly on the heavy chains of cancer cell-expressed immunoglobulins, but not on normal B-cell derived ones [22, 23]. Therefore, RP215 has been used as a unique probe to investigate

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the expressions and functions of antigen receptors in cancer cells through many biological and immunological studies [20, 24-27].

Besides cancerous immunoglobulins, TCRs were also detected among RP215-recognized CA215 glycoproteins, when subject to peptide analysis by matrix adsorption laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) [11, 23]. In contrast to immunoglobulins, which interact with pathogens and/or produce their toxic products in the extracellular space of the body, T cells recognize only foreign antigens that are presented on the cell surface as peptide fragments by major histocompatibility (MHC) glycoproteins from self or immune cells. Structurally, TCRs resemble surface bound Fab of immunoglobulins [19]. Subsequently, one would expect similar functional expressions or structural patterns for both TCRs and immunoglobulins on the cancer cell surface. Additional molecular and immunological studies were performed to investigate the expression of TCRs on the cancer cell surface [20]. Gene regulation analysis was also performed to study the correlated effects between RP215 and different anti-antigen receptors on the growth/proliferation, as well as innate immunity, of cancer cells in general [21]. Results generated from these various studies have been summarized in this review to hypothesize that cancerous immunoglobulins and TCRs play similar biological functional roles in cancer cells and to document the significance of these antigen receptors in the immunology of cancer cells.

2. Detection of Cancerous TCRs and Other IgSF-Related Genes from Cancer Cells

2.1. Molecular Biological Studies

CA215 consists of cancerous glycoproteins, each of which contains the RP215-specific "sugar" epitope [11, 27]. Initially, CA215 was isolated by affinity chromatography from shed media of cultured cancer cells using RP215 as the affinity ligand [11, 23]. Purified CA215 was then subjected to tryptic digestion and analysis by MALDI-TOF MS [11, 23]. Among the numerous (>100) tryptic peptides found, only a few were revealed to exhibit sequence homology to TCRs listed in the gene bank [20]. This existence of TCRs in cancer cells is consistent with the initial observations made by Kimoto when cancer cell lines were analyzed with single cell reverse transcription polymerase chain reaction (RT-PCR) [1].

To confirm this observation, RT-PCR was also performed with twenty other established cancer cell lines. About 80% of these cancer cell lines demonstrated relatively high expression levels of TCR, including MDA-MB231 (breast), C33A (cervix), HCT115 (colon), Hep 3B (liver), Calu-6 (lung), H441 (lung), WI38 (lung), A549 (lung), HEL1 (lymphocyte), Raji (lymphoblast), SKOV-3 (ovary), OVCAR-3 (ovary), OC-3-VGH (ovary), Du145 (prostate), Jurkt (lymphoblast) and K562 (B lymphocyte). Only four cell lines, SW-48 (colon), HT-29 (colon), MRC-5 (lung) and MMRU (melanoma), revealed little or no expression of TCR genes [20, 28].

To further verify the expression of TCR genes in cancer cells, the PCR product of the TCR-α gene derived from the Calu cell line (lung) was subjected to sequence analysis in the constant region of the TCR-α subunit [20].

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As expected, the cDNA sequence in the constant region of TCR-α molecules of Calu-6 lung cancer cell line was determined to be identical to *homo sapiens* TCR-α constant region (from 1-220 amino acid residues) [20]. In addition, relative gene expressions of TCR-related and cell adhesion molecules were analyzed from different cancer cell lines by RT-PCR. Unexpectedly, among all the cancer cell lines tested, little or no gene expression of the co-stimulators or co-receptors of TCR, CD3, CD4 and CD8, which are required for normal T cell activation, was observed [29]. Positive expressions of these genes in peripheral blood cells served as the positive control [29].

Semi-quantitation of TCR-α gene expression was determined by RT-PCR method by using primers corresponding to the constant region of TCR-α gene. With the expression level of TCR-α gene in the T cell leukemia cell line, Jurket, adjusted as 100%, the relative levels of TCR-α gene were found to range from 56% in Raji cells to 16% in Hep3B cells. Results are presented in Figure 1.

In comparison, immunoglobulin G (IgG) and all cell adhesion molecules, including CD47, CD54, CD58 and CD147, were found to be highly expressed in greater than 90% of the twenty cancer cell lines employed in this study [20].

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Figure 1. Semi-quantitative RT-PCR products using TCRα constant region-specific primers to reveal the relative expression of the human TCRα gene in various cancer cell lines listed as followed: Lane 1: Jurkat, Lane 2: SKOV-3, Lane 3: MMRU, Lane 4: MDA-MB-231, Lane 5: WI-38, Lane 6: HCT115, Lane 7: OC-3-VGH, Lane 8: Hep3B, Lane 9: Raji, Lane 10: Calu-6. Expression of GAPDH is also presented to check the integrity of cDNA from each cancer cell line and to normalize the cDNA levels. The signal intensities of TCR products of different cancer cell lines were adjusted by assuming that the expression level in Jurkat is 100%.

2.2. Western Blot Assays

Western blot assay was employed to detect protein bands which were specifically recognized by rabbit anti-TCR-β monoclonal or polyclonal antibodies. So far the results are consistent with the molecular size of TCR-β subunit [20]. Both OC-3-VGH ovarian cancer cell extract and affinity purified CA215 were employed separately for comparative Western blot assay with results presented in Figure 2. In the case of anti-TCR-β monoclonal or polyclonal antibodies, broad protein bands of 40-55 kDa were detected in either CA215 or crude cancer cell extract. In contrast, when using RP215 as the probe, protein bands ranging from 50-75 kDa were detected in CA215 or in cancer cell extract. Protein bands of 50-60 kDa were also detected when anti-human IgG was used as the probe [20]. Results are presented in Figure 2.

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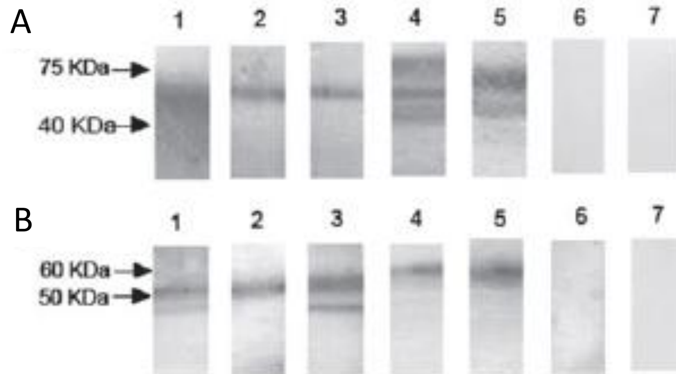

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Figure 2. (A) Western blot assays to reveal the protein bands of 40-55 KDa from OC-3-VGH cancer cell extract recognized by anti-TCRβ Mab, rabbit anti-TCRβ1 and rabbit anti-TCRβ2 (IgG fractions). Normal mouse and rabbit IgG were used as the negative controls. From left to right: Lane 1: anti-TCRβ Mab, Lane 2: rabbit anti-TCRβ1, Lane 3: rabbit anti-TCRβ2, Lane 4: RP215, Lane 5: goat anti-human IgG, Lane 6: normal mouse IgG, and Lane 7: rabbit IgG (molecular weight markers are indicated by arrows on the left); (B) Western blot assay with affinity-purified CA215 in the presence of the same antibody probes for parallel comparison with those in (A). The arrangement from lane 1 to lane 7 is the same as in (A). Molecular marker is indicated by arrows are on the left.

2.3. Immunohistochemical (IHC) Staining Studies

The expressions of immunoglobulins and TCR receptors for cancer cells in tissue sections were also determined with IHC staining studies respectively. Cancerous tissue sections were studied with confirmed cases of stage 1 or stage 2 cancer, from the endometrium, colon, breast, liver, throat, thyroid, lung, ovary, kidney and esophagus. When anti-TCR- β and RP215 Mabs were used as separate probes, results of IHC studies revealed positive rates of 75 and 85%, respectively. In contrast, neither of these Mabs were shown to exhibit positive staining in normal human tissue sections such as the kidney, liver, brain and placenta [24].

3. Cell-Based Functional Studies of TCRs in Cancer Cells

3.1. Induction of Apoptosis of Cancer Cells by RP215 and Anti-Antigen Receptors

Apoptosis of cancer cells can be induced upon treatments of cultured cancer cells with RP215, anti-IgG, or anti-TCRs (anti-TCRs) [20, 27, 30, 31]. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay was performed to assess induced apoptosis with cultured OC-3-VGH cancer cells. It was found that in the presence of either 10μg/mL or 1μg/mL of murine RP215 (mRP215) or anti-human IgG (GαhIgG), or 10μg/ mL of rabbit anti-TCRβ (RαTCRβ) (polyclonal, protein A-purified IgG fraction) for 24

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or 48 hr incubation, significant apoptosis of cultured cancer cells was induced, indicating a similar or parallel growth inhibitory effect of RP215 or anti-antigen receptors on cultured cancer cells (Figure 3A/1B).

Induced apoptosis of several other cultured cancer cells, including DU-145 (prostate), A549 (lung), C-33A (cervix), and MDA-MB-435 (breast) was also found to take place upon 24-48 hr incubation with 10μg/mL of anti-antigen receptors or RP215. Results of this comparative study are presented in Figure 3 A, B, and C.

3.2. Effects of RP215 and Anti-Antigen Receptors on Complement-Dependent Cytotoxicity (CDC) of Cultured Cancer Cells

By use of a typical complement-dependent cytotoxicity (CDC) assay procedure, RP215, goat anti-human IgG, or rabbit anti-TCRβ at 10μg/mL were found to induce significant CDC reactions and specific cell lysis of cultured cancer cells observed in the presence of complement [32]. Results of the CDC reaction assay with OC-3-VGH ovarian cancer cells are presented in Figure 3 for comparisons.

4. Effects of RP215 and Anti-Antigen Receptors on Gene Regulations of Cultured Cancer Cells

Effects of RP215 and anti-antigen receptors, including anti-TCR and anti-IgG, on cultured cancer cells were studied in terms of the regulations of genes involved in the growth/proliferation, as well as innate immunity of cancer cells [21].

By using semi-quantitative RT-PCR, changes in gene regulations upon treatments of cultured OC-3-VGH ovarian cancer cells with RP215 or either of the two anti-antigen receptors were compared and found to be correlated. Among the genes involved in this study are those implicated in proliferation, protein synthesis, cell cycle regulations, and innate immunity, such as IgG, TCR, NFκB-1, cyclin D1, P_{21}, c-fos, P_o, P_1, EGFR, and toll-like receptors (TLRs) (specifically TLR-3, TLR-4, and TLR-9). Results of the semi-quantitative analysis on gene regulations with OC-3-VGH ovarian cancer cells are summarized qualitatively in Figure 4. The correlation analyses between the gene regulation changes upon respective treatments of cancer cells with anti-IgG, anti-T cell receptor, and RP215 β are presented in Figure 5.

Results of such analysis suggest that treatments of cancer cells with anti-IgG and anti-T cell receptor have similar/parallel effects on the regulations of many genes involved in the growth/proliferation and the innate immunity of cancer cells. RP215 also induced similar gene regulation changes to either of these anti-antigen receptors. The correlation efficient of these gene regulation changes was found to range from 0.91 to 0.94.

Among these genes, NFκB-1 and P_{21} were upregulated, whereas cyclin D1 and c-fos were downregulated upon treatments of OC-3-VGH cancer cells with anti-antigen receptors or RP215 [21]. In the case of toll-like receptor genes, significant upregulation of the TLR-3 gene and downregulation of TLR-4 and TLR-9 genes were observed upon treatments of OC-3-VGH ovarian cancer cells with any of these three ligands [21].

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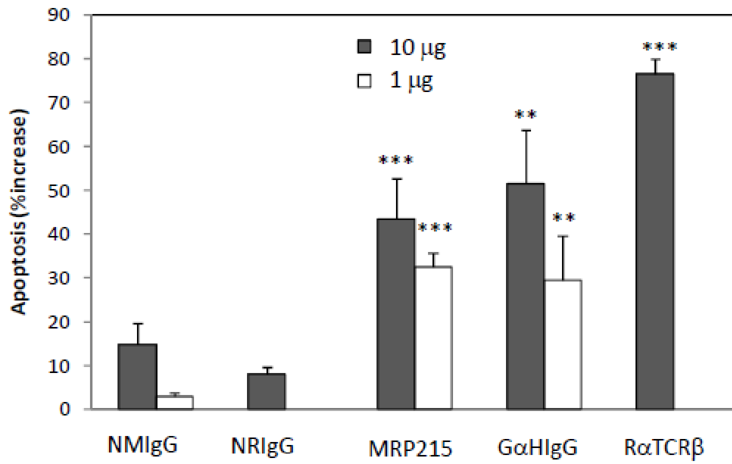

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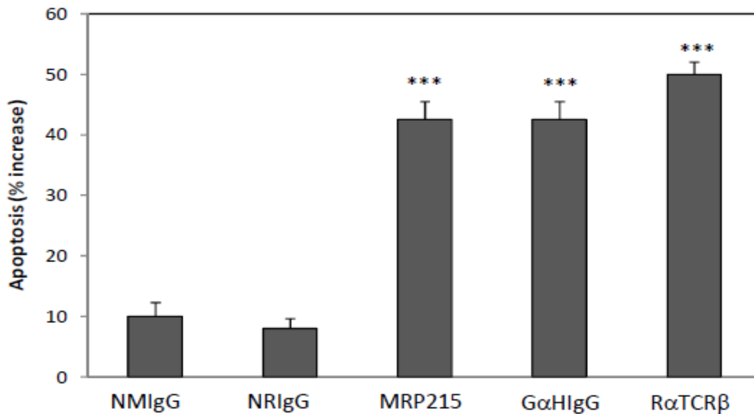

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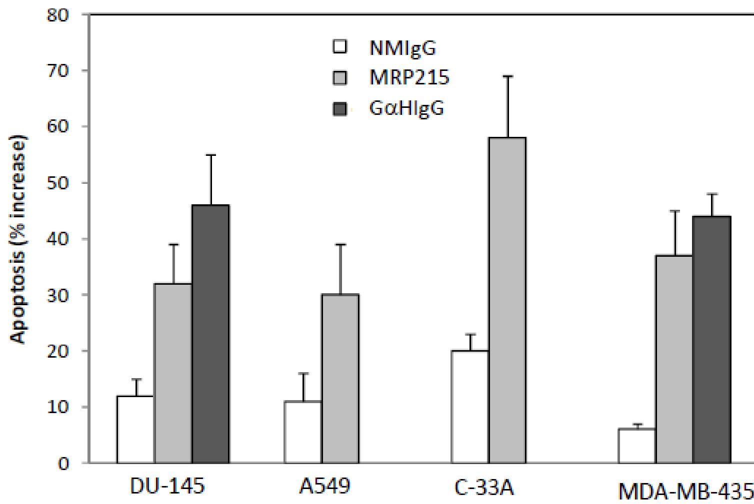

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Figure 3. (Continued).

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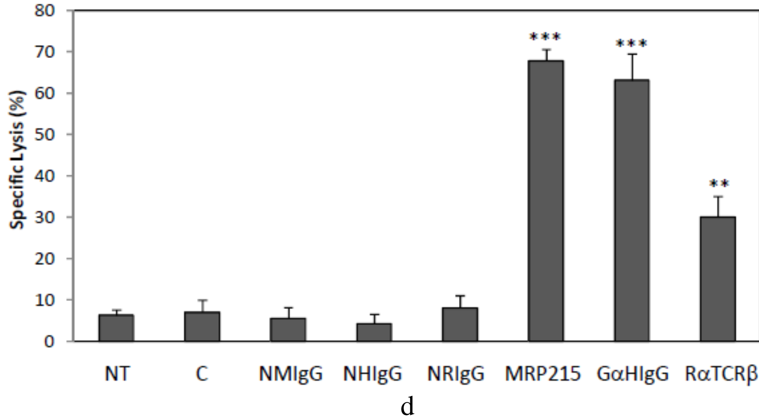

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All data presented are statistically significant at *P<0.05, **P<0.01 and ***P<0.001. Obtained [21] from with permission.

Figure 3. (A), (B) and (C): Effects of RP215, anti-human IgG and anti-TCRs on induced apoptosis of cancer cells; (A): Induced apoptosis to treated cultured OC-3-VGH cancer cells was presented and expressed in % of apoptotic cells when incubated with normal mouse IgG (NMIgG) (Lane 1), normal rabbit IgG (NRIgG) (Lane 2), murine RP215 (MRP215) (Lane 3), goat anti-human IgG (GαHIgG) (Lane 4), and rabbit anti-TCRs β (RαTCRβ) (Lane 5) for 24h incubation. □ and ■ represent 1 µg/ml and 10 µg/ml, respectively of the ligands used for the apoptosis assay; (B): ■ represents the corresponding data obtained for 48h incubation (10 µg/ml); (C): Induced apoptosis of several cultured cancer cells including DU-145 (prostate) (Lane 1), A549 (lung) (Lane 2), C-33A (cervix) (Lane 3) and MDA-MB-435 (breast) (Lane 4). □, ■ ■ and represent the treatments with normal mouse IgG (NMIgG), MRP215 and goat anti-human IgG (GαHIgG) (10 µg/ml), respectively; (D): Complement-dependent cytotoxicity (CDC) reactions in the presence of 10 µg/ml each of MRP215 and antibodies against antigen receptors, as well as normal immunoglobulins used as the negative control. Lane 1: no treatment (NT); Lane 2: 3 µL freshly prepared rabbit baby complement (C); Lane 3: normal mouse IgG plus complement (NMIgG); Lane 4: normal human IgG plus complement (NHIgG); Lane 5: normal rabbit IgG plus complement (NRIgG); Lane 6: MRP215 plus complement (MRP215); Lane 7: goat anti-human IgG plus complement (GαHIgG); Lane 8: rabbit anti-TCRs β plus complement (RαTCRβ).

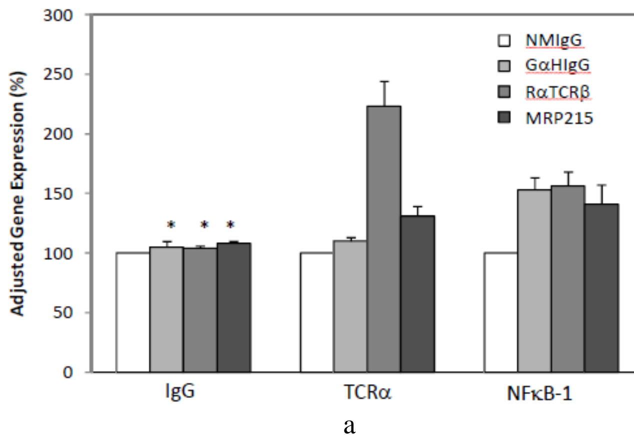

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Figure 4. (Continued).

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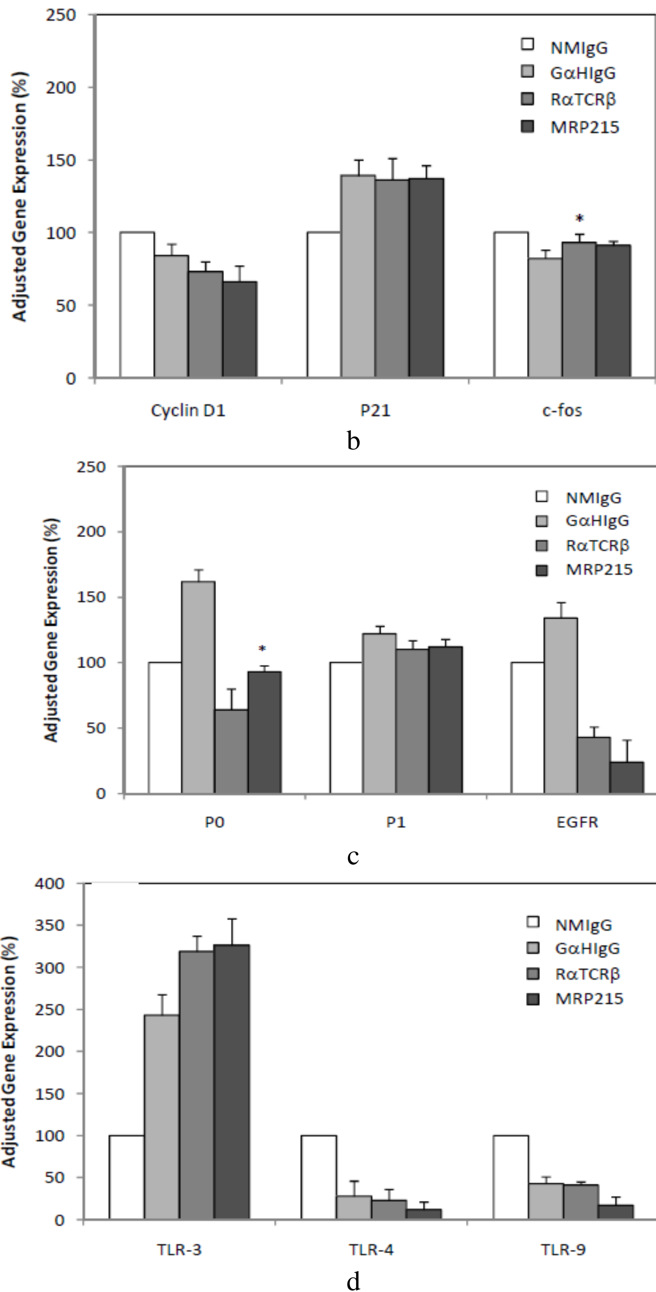

All data presented are statistically significant at P<0.01 except those labeled with * which are not statistically different from the negative control. Obtained [21] from with permission.

Figure 4. (A), (B), (C) and (D): Effects of treatments of cultured OC-3-VGH ovarian cancer cells with goat anti-human IgG (GαHIgG) (▫), rabbit anti-TCRs β (RαTCRβ) (▪), and MRP215 (■), respectively on the expressions of genes involved in cell proliferation, protein synthesis, cell cycle regulations as well as the innate immunity; Expressions of 9 genes involved were adjusted with that of GAPDH. The negative control (□) with normal mouse IgG (NMIgG) was considered 100% in all cases. Antibody concentration of 10 µg/ml was used for all parallel studies of comparisons. List of genes involved: (A): Lane 1: IgG; Lane 2: T-cell receptor α and Lane 3: NFκB-1; (B): Lane 4: Cyclin D1; Lane 5: P21 and Lane 6: c-fos; (C): Lane 7: P_0; Lane 8: P_1 and Lane 9: EGFR. (D): Changes in

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gene expressions of toll-like receptors (TLR-3, TLR-4 and TLR-9) of cultured OC-3-VGH ovarian cancer cells upon treatments with 10 μg/ml each of goat anti-human IgG (GαHIgG) (▫), rabbit anti-TCRs β (RαTCRβ) (▪) and MRP215 (■). The negative control (□) with normal mouse IgG (NMIgG) was adjusted to 100% in all cases. Lane 1: TLR-3; Lane 2: TLR-4 and Lane 3: TLR-9.

Conclusion

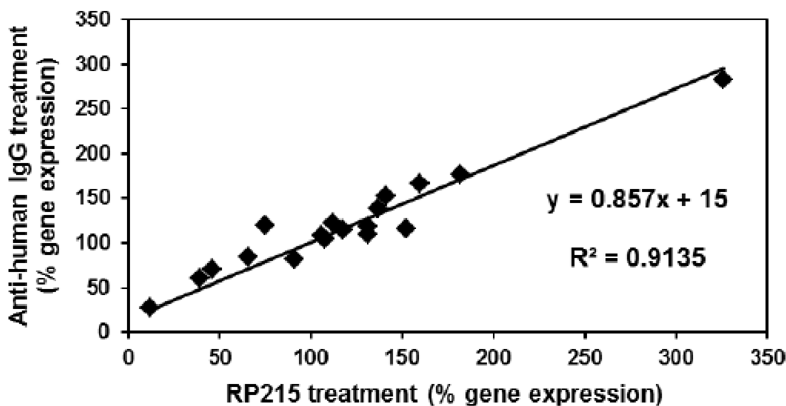
The gene expressions of TCRs among cancer cells were initially documented in 1998 [1]. It was not realized until a decade later that the expressions of TCRs are widespread among most cancer cells of many different tissue origins [1-5]. At the same time, co-receptors or co-stimulators of TCRs, including CD3, CD4, and CD8 were not found to be significantly expressed compared to the high expression level of TCRs [20].

Consequently, TCRs on cancer cells may be different from those on normal T lymphocytes in terms of their mechanisms of action [20, 21].

It remains to be observed whether TCRs on the cancer cell surface serve to recognize any pathogenic peptides or fragments from any cells bearing MHC molecules.

Nevertheless, it can be demonstrated through cell functional-based assays, that any of three anti-antigen receptors behaves similarly in inducing apoptosis and complement-dependent cell lysis to cultured cancer cells, indicating their absolute requirement for proliferation and growth of cancer cells [20, 30, 32].

To further document the similarity in the mechanisms of action between these two anti-antigen receptors and RP215, semi-quantitative RT-PCR was employed to study the changes in gene regulations upon treatments of cancer cells with any of these three anti-antigen ligands [21].



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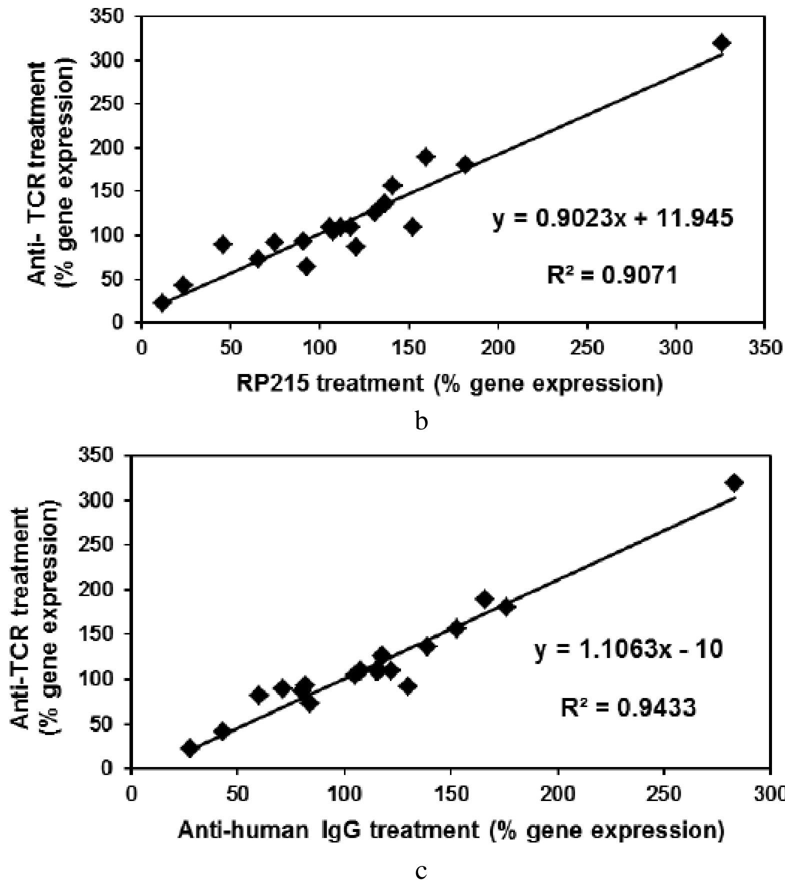

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Figure 5. Correlation analysis of changes in gene expression levels between different pairs of anti-antigen receptor treatments. (A): RP215 and anti-human IgG treatments; (B): RP215 and anti-TCRs (TCR) treatments; (C): anti-human IgG and anti-TCRs (TCR) treatments. The correlation coefficients (R^2) were determined to be 0.9135, 0.9071 and 0.9433, respectively.

As expected, a high degree of correlations were observed for their respective effects on the regulations of genes involved in the growth/ proliferation and innate immunity of cancer cells (Figure 4). Therefore, it can be concluded, in general, that RP215, anti-IgG, and anti-TCR act similarly in causing growth inhibition of cancer cells.

In addition, both cancerous IgG and TCRs may also serve to react with antigen or pathogenic peptides, which may be hostile to cancer cells within the human body. Therefore, these results indicate that cancer cells may have evolved to create a separate immune system, in which both immunoglobulins and TCRs are expressed on the cancer cell surface for immunological functions, which may be different from their traditional roles in the conventional immune system. These antigen receptors may also act as growth factors for the survival of cancer cells *in vitro* and *in vivo* [33-37].

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Chapter 8

Hormone Therapy in Young Cancer Survivors

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Abstract

As the overall survival rates of cancer treatment have increased, the short- and long-term sequelae faced by the growing number of young survivors have become more of a concern. Among these sequelae, hypogonadism is of particular importance and demands specialized medical care. However, a scarcity of evidence leaves no ideal solution. Hypogonadism caused by cancer therapy leads to impairment of pubertal development, hormonal regulation, fertility and sexual function in childhood and adolescent cancer survivors, and produces menopausal symptoms such as vasomotor and urogenital symptoms, and osteoporosis in young adult survivors. In the majority of cases except hormone receptor-positive cancers, hormone therapy is the most effective option for hypogonadism-induced problems and should be age-specific. For the prepubertal survivors, timing of hormone-therapy is crucial to ensure acceptable growth. For the postmenarchal who cease menstruating during or after cancer therapy, monitoring of menstrual resumption for one year is an acceptable management strategy. Those who remain amenorrheic, have symptoms of gonadal failure, or have elevated gonadotropin levels should be offered individualized hormone-therapy options. For the young adult survivors, sparing ovarian reserve and fertility as well as reduction of menopausal symptoms should be the main objectives of hormone therapy. In this review, we focused on effective hormone therapy according to these age groups.

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Introduction

As the advances in cancer treatment have prolonged the survival duration of cancer patients, attention has shifted to the quality of life of cancer survivors. The side effects of multimodal treatment as well as direct involvement of sexual and endocrine organs affect the quality of life in women. Young female cancer survivors need more attention because of their longer life expectancy and of future reproduction potential. Growth and development are especially important for childhood and adolescent survivors. In this regard, all female cancer patients should receive information regarding quality of life before, during and after cancer treatment. Early identification of hypo- or hypergonadism, growth problem, fertility issues, and management of menopausal symptoms should be sufficiently discussed. This review focuses on the hormone treatment of various age-group female cancer survivors, including children, adolescents, and young women.

1. Childhood/Adolescence

1) Hypogonadism and Growth

Hypogonadism is of particular concern in cancer survivors and is a condition that demands specialized medical care. Oophorectomy, radiation and chemotherapy cause primary hypogonadism. Hypothalamic or pituitary damage by tumor, radiation or surgery and emotional stress may cause secondary hypogonadism, in which ovarian stimulating hormones are not released. Disturbance of hormone secretion results in growth impairment [1] which can negatively affect quality of life, and hamper social development [2,3]. Therefore, oncologists, in cooperation with pediatric and reproductive endocrinologists, should counsel women who receive gonadotoxic therapy regarding their risk of primary ovarian failure and help them to optimize growth and pubertal progress.

In prepubertal survivors, onset and tempo of puberty, menstrual history, and Tanner stage should be evaluated annually until sexual maturity. Assessment of baseline luteinizing hormone (LH), follicular stimulating hormone (FSH), and estradiol levels is recommended at 13 years of age. To date, anti-Müllerian hormone (AMH) is of limited usefulness due to limited normative data in pediatric patients. When patients have delayed puberty, irregular menses, primary or secondary amenorrhea, and/or clinical signs and symptoms of estrogen deficiency, they need screening LH, FSH, and estradiol levels. For hypogonadal patients, bone mineral density tests should be considered as well.

The goal of treating hypogonadism is to induce normal secondary sexual characteristics. This process includes pubertal breast development, induction of menses, attainment of appropriate stature, pubic hair development, and normal bone mass acquisition [4]. In addition, an age-appropriate hormonal milieu is necessary for the normal development of other organ systems such as the nervous system and cardiovascular tissues [5-7]. While the optimal regimen remains unclear and data in adolescents are limited, hypogonadism can be treated with estrogen replacement using oral, or transdermal preparations. Progesterone should be added in women with a uterus to maintain endometrial health. Timing and tempo of estrogen replacement differ among the survivors who are prepubertal before cancer therapy

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and those who experience gonadal failure after menarche, therefore the treatment strategy should be individualized.

For pubertal patients, acceptable final height is a crucial factor and, as such a pediatric endocrinologist should be involved. In an adolescent who has no external signs of secondary sex characteristics, low-dose hormone replacement therapy (HRT) is generally begun at approximately 12 years of age. Daily administration of oral estrogen includes conjugated equine estrogen 0.3 mg, estrone sulfate 0.3 mg, or micronized estradiol 0.5 mg. A transdermal estrogen patch 25 mcg can be applied to the skin and changed twice weekly. During the first 6-18 months, low-dose estrogen-only therapy is generally used. When to increase doses of estrogen should be a decision individualized for each patient. Adding progestins in this period is controversial. In case of spontaneous puberty, the majority of early cycles are anovulatory and thus associated with unopposed estrogen secretion. Adding progestin too early results in the development of tuberous breasts [4]. No clear data exist to direct this decision-making. During Tanner stages 3-5, doses of estrogen are generally increased (conjugated equine estrogen 0.625 mg, estrone sulfate 0.625 mg, or micronized estradiol 1 mg, transdermal estrogen patches 50 mcg) [8], as would naturally occur during spontaneous puberty. Progestin therapy is generally added to maintain endometrial health. The starting point of progestin therapy is usually 2-3 months after increasing the estrogen dose. The progesterone administration includes medroxyprogesterone 5-10 mg or micronized progesterone 200 mg; either dose is given daily for the first 5 days of the month. Once breast development is completed, the progestin course is increased to 10 days per month.

Postmenarchal women who cease menstruating during or after cancer therapy should be monitored for the resumption of menses for one year. If the patients remain amenorrheic, have symptoms of gonadal failure, or have elevated gonadotropins, HRT should be offered.

2) Precocious Puberty

Precocious puberty is defined as Tanner stage 2 breast development before 8 years of age. Precocious puberty is caused by premature pulsatile secretion of GnRH after cranial irradiation. The major issues related to precocious puberty are psychological and emotional issues. Universal expectations of "mature" behavior are inevitable, placing a difficult burden on a young child and resulting in significant psychosocial disturbance. Therefore, survivors with cranial irradiation should be evaluated annually for height, growing velocity, weight, and Tanner stage.

For the survivors with Tanner 2 breast development before age 8, serum levels of LH, FSH, and estradiol, bone age and pelvic ultrasound should be assessed. Premature activation of hypothalamic-pituitary-ovary (HPO) axis is indicated by an elevated basal LH, advanced bone age, and ultrasonic evidence of uterine stimulation. GnRH-stimulation test can be performed to identify LH elevation. In addition, an imaging study of the head may be considered for the survivors with neurologic symptoms suggestive of other intracranial pathologies.

Severe hypothyroidism also should be considered. A cross-over effect of thyroid-stimulating hormone and FSH can result in estrogen excess and breast development [9]. If this effect is extreme, very large ovarian cysts can occur, although these usually regress with the treatment of underlying condition.

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GnRH analogs are used to desensitize gonadotrophs and reduce LH release, thus halting ovarian stimulation. This treatment preserves final adult height, delays menarche, and optimizes the development of secondary sex characteristics. Treatment usually continues until the normal age of puberty. When treatment commences after the age of 8 years in a girl, growth potential is not altered.

3) Concerns Regarding HRT in Childhood

The effect of HRT on breast cancer risk in childhood cancer survivors is unknown. There was a report indicating that after early exposure of the breast to radiation as part of childhood cancer protocols, breast cancer risk is estimated to be 100-fold higher than that for other young women [10]. However, the benefits of HRT, such as cardiovascular and bone health, should be stressed to reluctant patients, and factual information provided.

2. Young Adulthood

1) Reduced Fertility

Most young survivors want to be parents sometime in the future [11,12] and are distressed about the possibility of sterility [13,14]. After therapy with radiation, cytotoxic chemotherapy, or hormone antagonist therapy, fertility may be reduced by direct damage to the reproductive organ or disruption of the HPO axis. Cancer treatment may result in loss of primordial follicles from the ovary [15]. Because the number of primordial follicles determines reproductive lifespan, follicular atresia will accelerate menopause. [16-20] Gonadal dysfunction and fertility correlate significantly with age and therapy intensity. [21] It is intriguing that low birth rates were observed in breast cancer survivors after advanced-stage treatment. [21] Many patients misunderstand the connection between that menstrual function and fertility. The ovarian reserve in young adult survivors may be reduced despite normal menstrual function. Moreover, the presence of regular menstrual cycles induced by oral contraceptives may mask the patient's cognition of primary ovarian failure. Therefore, at the initial stage of evaluation of a patient's baseline fertility status, counseling about the possibility of treatment-related infertility, infertility prevention options, preimplantation genetic diagnosis and alternative parenting should be provided prior to treatment.

In recent years, oncologists have sought to preserve fertility before gonadotoxic therapy. Mostly, success of assisted reproductive techniques depends on harvesting and banking the postpubertal patient's oocytes and cryopreserving unfertilized oocytes or embryos before treatment. However, ovarian stimulation and oocyte cryopreservation or IVF take 2-4 weeks and thus may not be appropriate for rapidly progressing tumors. Alternatively, ovarian tissue cryopreservation and later autotransplantation can be offered to prepubertal girls despite this being currently experimental. Lastly, the use of GnRH analogs for ovarian chemoprotection is another option. Chemotherapeutics may destroy follicles engaged in the maturation pathway. FSH secretion is increased as a result due to a loss of negative feedback, which induces additional follicles to enter the maturation pathway. GnRH analogs prevent follicles from

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entering the maturation pathway by suppressing FSH. It is questionable, though, whether GnRH treatment will sufficiently work since primordial follicles are not gonadotropin-sensitive. A recent meta-analysis of nine randomized studies indicated that temporary ovarian suppression with GnRHa significantly reduces the risk of chemotherapy-induced premature ovarian failure in young cancer patients [22].

Although the prediction of reproductive lifespan for young cancer survivor would be of considerable value, current fertility prediction is limited. The impact of chemotherapy on fertility is significantly affected by the patients' baseline ovarian reserve. Therefore, all women concerned about future fertility should undergo an initial fertility work-up, including history of menstruation cycles, sexual function and libido, Tanner stage, physical examination, and laboratory screening including LH, FSH, and estradiol. Imaging studies including ultrasound, uterine blood flow, and tubal patency, should be considered.

Ovarian reserve should be assessed with antral follicle count and AMH, which have been demonstrated to be better predictors than age, basal FSH, estradiol and inhibin B [23-28]. Antral follicle count by transvaginal ultrasonogram is the most established method [29-31]. AMH correlates well with antral follicle count and does not vary with menstrual cycle day or exogenous estrogen or progesterone. Although the normal range is wide, a low AMH level might indicate reduced ovarian reserve and thus increased risk of future premature ovarian failure.

2) Menopausal Symptoms

(1) Vasomotor Symptoms

Vasomotor symptoms include hot flushes, night sweats and sleep disturbances. Although for some women these symptoms are bearable, for others these have a considerable impact on their quality of life. Conventional estrogen therapies alone or those combined with a progesterone are the most effective agents for reducing vasomotor symptoms. Tibolone is a synthetic steroid with an estrogenic action in various tissues, especially in the bones and vagina, and is an effective agent against hot flushes [32].

Women with hormone receptor-positive breast and endometrial cancers are discouraged from using systemic estrogens because of an increased cancer recurrence rate [33-35]. For these patients, safe treatment options are lifestyle modification with avoiding triggers, such as spicy food, alcohol, hairdryers, anxiety, smoking, and weight gain [36]. Non-hormonal pharmacological therapies include selective serotonin reuptake inhibitors, serotonin noradrenalin reuptake inhibitors, the gamma-aminobutyric acid gabapentin, and clonidine [33,37]. Some of these drugs prevent tamoxifen from being metabolized into its active compound [38,39] and thus, should be cautiously prescribed to tamoxifen users. Acupuncture was reported to be an alternative therapy for hot flush [40].

(2) Sexual Dysfunction

Most young cancer survivors want or need to discuss sexual issues with their doctors, but both providers and patients tend to avoid this topic. Cancer treatment may alter the female sexual function both anatomically and hormonally. Pelvic irradiation may induce impairment

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Table 1. Management according to age

		Assessment	Management	
Childhood /Adolescent	Hypogo-nadism	History taking /Physical examination - Onset and tempo of puberty - Menstrual history - Tanner stage Lab - LH, FSH, Estradiol - BMD	Pubertal	Low dose estrogen for first 6-18 months - Conjugated equine estrogen 0.3 mg, daily - Estrone sulfate 0.3 mg, daily - Micronized estradiol 0.5 mg, daily - Transdermal patch 25 mcg twice weekly Estrogen increase during Tanner stages 3-5 - Conjugated equine estrogen 0.625 mg, daily - Estrone sulfate 0.625 mg, daily - Micronized estradiol 1 mg, daily - Transdermal patch 50 mcg twice weekly Progestin 2-3 months after increasing estrogen, daily 5 days per month - Medroxypregesterone 5-10 mg - Micronized progesterone 200 mg After complete breast development, daily 10 days per month
			Postmenarchal	Monitoring resumption of menses for one year If patients are amenorrheic or gonadal failure, HRT is offered
	Precocious puberty	History taking /Physical examination - Onset and tempo of puberty - Menstrual history - Tanner stage Lab - LH, FSH, Estradiol, TFT - Bone age - USG (if possible) - GnRH-stimulation test Brain imaging study		GnRH analogs

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Young adult	Fertility	History taking /Physical examination - Menstrual cycle - sexual function/libido - Tanner stage Lab/Imaging study - LH, FSH, Estradiol, AMH - USG, antral follicle count	Counseling pretreatment fertility preservation - Cryopreserving unfertilized oocytes or embryos - Cryopreserving ovarian tissue GnRH analogs
	Vasomotor symptoms		Hormone replacement therapy - Estrogen and progesterone - Tibolone Non-hormone pharmacological therapy - SSRIs, SNRIs, Clonidine - Gamma-aminobutyric acid gabapentin Avoiding spicy food, alcohol, hairdryers, anxiety, smoking, weight gain.
	Sexual dysfunction	History taking /Physical examination - Genital sensation - Dyspareunia - Vulvar pain - Postcoital bleeding Lab/Imaging study	Physical therapy - HRT / Tibolone - Vaginal estrogen - Lubricants or moisturizers - vaginal dilators Psychoemotional therapy

LH, luteinizing hormone; FSH, follicular stimulating hormone; BMD, bone marrow density; HRT, hormone replacement therapy; TFT, thyroid function test; USG, ultrasonogram; GnRH, gonadotropin-releasing hormone; AMH, anti-müllerian hormone; SSRIs, selective serotonin reuptake inhibitors; SNRIs, serotonin noradrenalin reuptake inhibitors.

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of blood flow to female organs and scarring. These result in vaginal and vulvar dryness and vaginal shortening causing dyspareunia. Low serum estrogen due to ovarian failure also causes vaginal atrophy and increased vaginal pH, resulting in frequent infection, incontinence and sexual dysfunction.

Sexual function can be assessed with history taking, including genital sensation level, dyspareunia, vulvar pain, postcoital bleeding, and difficulty with tampon insertion. Because sexual dysfunction in cancer survivors may not abate without appropriate intervention, early identification and treatment strategies are essential.

Treatment should incorporate both physical and psychosocial aspects. HRT and vaginal estrogen and tibolone are most effective in treating sexual dysfunction. Recent reports showed that intravaginal ultra-low-dose 17-beta estradiol (10 mcg) provided statistically significant improvement in menopausal symptoms and sexual dysfunction [41]. In addition, local estriol, which is not converted into the more potent estradiol, is as effective as vaginal estradiol [36]. Non-hormonal interventions such as lubricants or moisturizers [42-44] can also be used. A hyaluronic acid vaginal tablet decreased atrophy and vaginal pH and relieved vaginal symptoms [45]. Although systemic absorption of local estrogen seems to be minimal, it is unclear whether this minimal absorption will affect outcomes of hormone-dependent cancer. For these women, even the use of intravaginal estrogens, testosterones, and dehydroepiandrosterone are controversial. Intravaginal 25 mcg Vagifem increases serum estradiol [46] and may negatively influence hormone receptor-positive cancers whereas recent reports indicated that intravaginal ultra-low-dose 17-beta estradiol (10 mcg) Vagifem provided statistically significant improvement in menopausal symptoms and sexual dysfunction [41] without a significant change in week 12 serum estradiol. However, further study is needed to decide whether this is a safe and appropriate treatment. At present, non-hormonal modalities are frequently used in this group of patients.

Vaginal dilators can be used to prevent vaginal stenosis and agglutination after pelvic irradiation or graft-versus-host disease. Urinary or bowel incontinence, and pelvic pain should also be addressed according to individual patient’s status.

The main psychosocial or psychoemotional issue is poor body image resulting from psychosexual dysfunction and consequent relationship problems [47]. Couple-based psycho-educational interventions that include an element of sexual therapy are recommended [48]. Counseling on depression should be provided by a psychologist.

Conclusion

Hormone therapy is important for young female cancer survivors to maintain normal growth, preserve fertility and prevent menopausal symptoms. Physicians should be aware of the long-term side effects that may affect the quality of life and should provide regular assessments and optimal and safe treatment modalities to every single patient. To increase our knowledge of treatment options, additional well-designed clinical trials and basic research are needed.

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Chapter 9

Inhibitory Effects of Ribosome Inactivating Proteins and Compounds on Choriocarcinoma Cells

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Abstract

The aim of this article is to review the mechanisms of action of compounds with inhibitory activity toward choriocarcinoma cells. Two isomers of linolenic acid, alpha- and beta-calendic acid, inhibited invasion of human choriocarcinoma JEG-3 cells in vitro, and enhanced oxidative stress in the cells as witnessed by elevated levels of the lipid peroxidation product malondialdehyde and reactive oxygen species. The soybean phytoestrogen genistein stimulated the MTA3/Snail/E-cadherin regulatory pathway by binding with estrogen receptor-β, thus suppressing JAR cell invasion. A translocator protein ligand (initially referred to as a ligand for the peripheral benzodiazepine receptor), 1-(2-chlorophenyl-N-methylpropyl)-3-1-(2-chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide (PK11195), reduced the percentage of cells in the S phase of the cell cycle and raised the proportion of cells in the G0/G1 phases, and triggered apoptosis in choriocarcinoma BeWo cells. Tubeimoside I, a triterpenoid saponin, isolated from *Bolbostemma paniculatum* tubers, induced apoptosis in JEG-3 cell, cell cycle arrest at G2 phase and decline in mitochondrial transmembrane potential, mitochondrial cytochrome c release and augmented caspase-3 expression. Tubeimoside I upregulated Bcl-2 associated X protein (Bax) expression, downregulated Bcl-2 expression, suppressed nuclear factor-κ-B (NF-κB) function and affected phosphorylation of p38 mitogen-activated protein kinase (p38/MAPK), extracellular signal-regulated kinases (ERK)1/2 and protein kinase B (Akt). Tubeimoside I produced its apoptosis-inducing effects, at least partially, by induction of mitochondrial dysfunction and regulation of the p38/MAPK, ERK1/2 and PI3K/Akt signaling pathways. Flax seed fractions exhibited antiproliferative activity toward choriocarcinoma Jeg3 cells. Elm bark extract, mainly

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composed of triterpenes, phytosterols, free fatty acids and suberins with smaller amounts of lipids and dilignols, reduced the viability of Jeg3 and BeWo cells. The quinolinone derivative vesnarinone induced expression of c-Myc gene in choriocarcinoma cells, the product of which may be associated with cell growth suppression and apoptosis induction. All-trans retinoic acid exhibited antiproliferative activity toward four choriocarcinoma cell lines. Co-administration of all-trans retinoic acid and methotrexate or actinomycin-D produced an augmented effect. The aminopeptidase inhibitor Ubenimex (bestatin) suppressed the growth of choriocarcinoma NaUCC-4 cells *in vivo* and *in vitro,* not via potentiation of effector cells, but through its direct cytostatic activity, Paclitaxel, a taxane analog isolated from bark of the western yew (*Taxus brevlfolla*), is highly potent against choriocarcinoma cell lines. The activity of VP16-213 (etoposide) against the three human choriocarcinoma cell lines, SCH BeWo, and HCCM-5, was similar to or superior to those of actinomycin D and methotrexate.

Introduction

Choriocarcinoma is a cancer of the placenta and is rarely found in the gonads. Chemotherapy is the main form of treatment. Gestational choriocarcinomas originating from hydatidiform moles are sensitive to chemotherapy. Monotherapy using methotrexate is used for low-risk disease, while combination therapy employing actinomycin, etoposide, methotrexate, and vincristine are adopted for diseases with intermediate risk or high risk. Choriocarcinomas in the gonads are resistant to chemotherapy. The worst prognosis of germ cell cancers is found in testicular choriocarcinomas. Hysterectomy is rarely used.

The purpose of this review is to discuss proteins and low-molecular-weight compounds which have inhibitory action on the growth of choriocarcinoma cells.

Ribosome Inactivating Proteins

Ribosome inactivating proteins (RIPs) are proteins produced by plants and fungi which can inactivate eukaryotic ribosomes by making their 60 S subunit incapable of binding elongation factor 2 and inhibiting protein synthesis due to their N-glycosidase activity on ribosomal RNA which cleaves adenine at position 4324. The RIP trichosanthin (TCS) has been used in traditional Chinese medicine for centuries. Additionally, it manifests a repertoire of pharmacological activities, such as anti-HIV and anti-tumor activities. TCS inhibited growth of cervical cancer, choriocarcinoma, and leukemia/lymphoma cells (Sha et al., 2013). The ribosome-inactivating proteins (RIPs) trichosanthin and alpha-momorcharin inhibited protein synthesis in choriocarcinoma cells. The drastic reduction in progesterone and human chorionic gonadotropin production by choriocarcinoma cells after treatment with the two RIPs was due to cell loss (Tsao et al., 1990).

A nontoxic concentration of TCS reduced the wound-healing and migration of H5V murine cardiac capillary endothelial cells brought about by human choriocarcinoma (JAR) cells, and JAR-elicited angiogenesis of rat third-order mesenteric arteries. TCS produced an effect on both tumor cells and endothelial cells /arteries. TCS decreased vascular endothelial growth factor transcription and secretion by JAR cells, and consequently inhibited the cancer cell-elicited, extracellular signal-regulated kinase-mediated angiogenic signal in endothelial

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cells and blood vessels. The anticancer action of TCS is partly attributed to its anti-angiogenic activity (He et al., 2012).

Fluorescein isothiocyanate-labeled TCS and gold-conjugated TCS alike entered JAR cells via a specific receptor mediated pathway, and rapidly accumulated inside JAR cells (Chan et al., 2003). Low-density lipoprotein receptor-related protein 1 is a large scavenger receptor which binds and phagocytoses TCS in JAR and BeWo cells and may mediate the anti-choriocarcinoma activity of TCS (Jiao and Liu, 2010).

Trichosanthin triggered the Ca2+ -dependent formation of reactive oxygen species (ROS) in human choriocarcinoma JAR cells .The process was dependent on extracellular Ca2+ ions and was attenuated after chelation of cytosolic Ca2+ ions by BAPTA-AM. ROS production was involved in the apoptosis of JAR cells. The antioxidant alpha-tocopherol antagonized trichosanthin-induced ROS formation and cell death (Zhang et al., 2000, 2001). Catalase and mannitol(ROS scavengers), and diethylenetriaminepentaacetic acid (an inhibitor of metal-facilitated hydroxyl radical generation) suppressed trichosanthin-induced cell death, markedly inhibited trichosanthin induced cell death, suggesting that trichosanthin induced formation ROS and hydroxyl radicals in choriocarcinoma cells leading to cell death (Zhang et al., 2001).

The two mutants of TCS, Y55G TCS (with tyrosine 55 replaced by glycine) and FYY140-142GSA TCS (with tripeptide phenylalanine-tyrosine-tyrosine 140-142 replaced by glycine-serine-alanine) with secondary structures and hydrophobility different from TCS, exhibited weaker cytotoxicity than TCS and induced less ROS production (Zhang et al., 2002). Trichomislin, a ribosome-inactivating protein cloned from the genome of *Trichosanthes kirilowii,* induced apoptosis and suppressed growth in choriocarcinoma cells. Trichomislin bound to and gained entry to choriocarcinoma cells, in which it upregulated caspase-3 activity and cytosolic cytochrome c level and downregulated mitochondrial cytochrome c level, thereby inducing apoptosis. Trichomislin induced apoptosis by stimulating mitochondrial cytochrome c release which then brought about the activation of caspases (Mi et al., 2005).

TYchi, a protein from *Trichosanthes kirilowii* with cell-free protein synthesis inhibiting and RNA N-glycosidase activities characteristic of RIPs but also chitin-hydrolytic activity, demonstrated cytotoxicity to choriocarcinoma JAR cells with an IC50 of 73 microg/ml .Its putative domain of TYchi closely resembles the active domain of TCS (Xu et al., 2008).

Battelli et al., (1992) showed that a host of RIPs including bryodin-R, dianthin 32, gelonin, momordin, pokeweed antiviral protein, and saporin 6, inhibited protein synthesis in BeWo cells. The binding and uptake of saporin 6 and momordin by BeWo cells showed no correlation with cytotoxicity. Two immunologically distinct RIPs isolated from *Luffa cylindrica* (sponge gourd) seeds, designated as luffin-a and luffin-b, which are glycoproteins with a molecular weight of 28 kDa and 28.5 kDa respectively, inhibited [3H]thymidine uptake by human choriocarcinoma cells (Ng et al., 1992).

Momordica charantia (bitter gourd) RIP alpha-momorcharin suppressed uptake of [3H]thymidine, [3H]leucine and [3H]uridine into JAR (human placental choriocarcinoma) cell line (Ng et al., 1994).

PEGylated RIPs α-MMC and, MAP30 from *Momordica charantia* seeds presented moderate activities on JAR choriocarcinoma cells and herpes simplex virus-1. Furthermore, both PEGylated proteins showed about 60%-70% antitumor and antivirus activities, and at the same time decreased immunogenicity when compared with their unmodified counterparts (Meng et al., 2012).

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Alfa-Calendic Acid and β-Calendic Acid

Alfa-calendic acid and β-calendic acid, which are geometric and positional isomers of linolenic acid, triggered apoptosis and inhibited invasion of human choriocarcinoma JEG-3 cells *in vitro*. Exposure of JEG-3 cells to α-calendic acid and β-calendic acid elevated oxidative stress as evidenced by heightened levels of reactive oxygen species and malondialdehyde. As a consequence of the oxidative stress, p38MAPK phosphorylation was activated. The selective p38MAPK inhibitor SB203580 inhibited α-calendic acid- and β-calendic acid-induced apoptosis through raising the Bcl-2/Bax ratio and suppressing caspase-3 and caspase-9 activation.

The cell invasive action of α-calendic acid and β-calendic acid was antagonized by SB20350. Thus α-calendic acid and β-calendic acid activated oxidative stress pathways and phosphorylate P38MAPK to bring about apoptosis and prevent invasion in JEG-3 cells (Li et al., 2013).

All-Trans Retinoic Acid

All-trans retinoic acid exerted a dose- and time-dependent antiproliferative effect on choriocarcinoma cells. Cell growth was inhibited by 68%-82.0% following treatment with 1 microM all-trans retinoic acid for 6 days. Cell growth was suppressed by 67.8%-82% compared to the controls. A more pronounced effect was noted when all-trans retinoic acid was administered in conjunction with methotrexate or actinomycin-D. The secretion of human chorionic gonadotropin could be elevated to a maximum of 9-fold in the presence of 1 microM all-trans retinoic acid (Yamada et al., 1997).

Aminopeptidase Inhibitor Ubenimex (Bestatin)

Bestatin, a biological response modifier as well as an inhibitor of aminopeptidase B (AP-B), leucine aminopeptidase (LAP) and aminopeptidase M (AP-M), suppressed growth in choriocarcinoma cell lines, in particular NaUCC-4 cells.

Both an isomer of bestatin devoid of activity against aminopeptidases, and another isomer with more potent activity against aminopeptidase B than bestatin, had no activity on NaUCC-4 cells. It is likely that its mechanisms involve a direct action on the choriocarcinoma cells via its suppressive activity on leucine aminopeptidase and aminopeptidase M rather than that on aminopeptidase B. A combination of bestatin and actinomycin D exerted higher cytotoxic activity on the choriocarcinoma cells. The findings indicate not only an indirect host-mediated anti-tumor activity, but also a direct growth inhibitory effect of bestatin on choriocarcinoma cells (Inoi et al., 1991).

Two choriocarcinoma cell lines, NaUCC-4 and BeWo, had higher aminopeptidase N activity than other cell lines as did HL-60 human myeloid leukemia cells. These choriocarcinoma and leukemia cell lines with high aminopeptidase N activity were substantially more sensitive to bestatin than other cell lines. The growth of NaUCC-4 cells

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was markedly suppressed by the aminopeptidase N inhibitor actinonin as well as by bestatin, but not by the aminopeptidase B inhibitor arphamenine.

The monoclonal antibody, M15, which is able to inhibit aminopeptidase N activity, suppressed cell growth in a dose-dependent manner. Aminopeptidase N inhibitors suppress growth, probably by inhibiting the enzymatic activity of aminopeptidase N on cancer cells, suggesting that aminopeptidase may play important roles in the growth of certain tumors, such as choriocarcinoma and leukemia (Inoi et al., 1994).

Intraperitoneal bestatin injections to NaUCC-4-bearing nude mice for four weeks at 2 and 20 mg/kg/day brought about a reduction in tumor growth without changes in activity of natural killer cell activity or B cell mitogenic activity in splenocytes, suggesting a direct cytostatic activity on choriocarcinoma cells (Inoi et al., 1995).

1-(2-Chlorophenyl-N-Methylpropyl)-3-Isoquinolinecarboxamide (PK11195)

Treatment of PK11195 with BeWo choriocarcinoma cells reduced the proportion of cells in the S phase of the cell cycle and increased cells in the G0/G1 phases. The cells underwent apoptosis as evidenced by Annexin V staining of externalized phosphatidylserine, loss of mitochondrial transmembrane potential, and by immunochemical identification of histones from fragmented DNA. Genes associated with cell growth, malignant phenotype, and apoptosis underwent changes in expression (Takai et al., 2012).

Etoposide

The antitumor effect of VP16-213 (etoposide) on human choriocarcinoma cell lines, BeWo, HCCM-5 and SCH, as judged by inhibition of tritiated thymidine incorporation, was similar to methotrexate (Matsui et al., 1987).

The use of cisplatin in the first-line treatment of patients with poor-prognosis germ cell cancers is unsatisfactory. Mardiak et al. (2007) reported results of clinical investigations. The initial therapy adopted for 24 patients used cisplatin. Primary mediastinal germ cell tumors was found in 3 patients. Four cycles of cisplatin were administered three weeks apart. Paclitaxel (taxol) was administered on day 1 prior to cisplatin treatment. Complete or partial response with negative tumor markers was observed in 13 patients. Median follow-up was 35.6 months. Median survival was not achieved and median time-to-progression was 9.5 months. The major toxicity was myelosuppression with granulocytopenia in nearly 50% of all courses. Two patients died from sepsis. Patients treated with first-line cisplatin did not exhibit a higher response rate or time to progression. The use of this treatment without G-CSF support was not recommended because of considerable toxicity (Mardiak et al., 2007).

Flax Seed Fractions

Flax seed fractions I, V, VI and VII exerted an antiproliferative activity on Jeg3 cells and fractions III, V, VI and VII suppressed hCG secretion. Fractions V and VI decreased

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progesterone secretion. Matairesinol and biochanin A present in flax-seed fraction VI may be the active principles (Waldschläger et al., 2005).

Genistein

The soybean phytoestrogen genistein suppressed invasion of JAR cells as revealed by a matrigel invasion assay. The mRNA level of metastasis-associated gene MTA3 was downregulated whereas that of the transcriptional suppressor Snail mRNA level was increased as determined by real-time RT-PCR. Protein expression of the cell-cell adhesion molecule E-cadherin was enhanced as witnessed by Western blot analysis. In JAR cells in which estrogen receptor β(ERβ) expression had been knocked down by employing ERβ siRNA, genistein was incapable of preventing JAR cell invasion and affecting the expression levels of MTA3, Snail and E-cadherin Hence. genistein works by binding ERβ, activating the MTA3/Snail/E-cadherin regulatory pathway, and thus suppressing JAR cell invasion (Liu et al., 2011).

Taxol

Taxol (paclitaxel) exerted a dose-dependent antiproliferative activity on human choriocarcinoma cell lines JAR and BeW. A taxol concentration of 1 to 3 nM brought about 50% inhibition of growth. Concomitantly the secretion of human chorionic gonadotropin was markedly increased. This effect on human chorionic gonadotropin secretion was dependent on protein synthesis but not related to increased mRNA expression. In JAR and BeW cells taxol promoted differentiation as evidenced by a rise in the number of syncytiotrophoblastic-like cells. Combination therapy using taxol in conjunction with either etoposide or methotrexate is undesirable since an antagonistic effect on growth inhibition of choriocarcinoma cells was observed.

Tubeimoside I

Tubeimoside I, a triterpenoid saponin isolated from *Bolbostemma paniculatum* tubers induced apoptosis in JEG-3 cells. It brought about cell cycle arrest at G2 phase, fall in mitochondrial transmembrane potential ($\Delta\Psi m$), mitochondrial release of cytochrome c and increase in caspase-3 expression in choriocarcinoma cells. TBMS1 enhanced Bcl-2 associated X protein (Bax) expression, lowered Bcl-2 expression, inhibited nuclear factor-κ-B (NF-κB) function and altered phosphorylation of p38 mitogen-activated protein kinase (p38/MAPK), extracellular signal-regulated kinases (ERK)1/2 and protein kinase B (Akt). Thus TBMS1 elicits apoptosis in choriocarcinoma cells by inducing mitochondrial dysfunction and regulating the p38/MAPK, ERK1/2 and PI3K/Akt signaling pathways (Huang et al., 2011).

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Elm Bark Extracts from *Ulmis laevis*

The elm bark extract consisted predominantly of free fatty acids, phytosterols, suberins and triterpenes, with lesser quantities of dilignols and lipids. The elm bark extract reduced the viability of BeWo cells and Jeg3 cells but increased that of primary trophoblast cells (Hartmann et al., 2011).

Vesnarinone

The inotropic quinolinone derivative Vesnarinone employed for treating congestive heart failure exhibited dose-dependent antiproliferative activity on choriocarcinoma cell lines and elicited DNA fragmentation but did not affect the BM cell line prepared by subcultivation from hydatidiform mole. Vesnarinone suppressed proliferation of SCH cells which have a mutant p53 gene at codon 249. Vesnarinone exhibited antiproliferative activity on all choriocarcinoma cell lines and induced apoptosis, irrespective of the presence of p53 mutation. The quinolinone derivative vesnarinone might upregulate c-Myc gene expression in choriocarcinoma cells, followed by inhibition of cell growth and induction of apoptosis (Isaka et al., 2002).

Discussion

The foregoing account reveals that a variety of ribosome inactivating proteins comprising trichosanthin , alpha-momorcharin, trichomislin, bryodin-R, dianthin 32, gelonin, momordin, pokeweed antiviral protein, saporin 6,luffin-a, luffin-b, trichosanthinm, alpha-momorcharin, trichomislin, bryodin-R, dianthin 32, gelonin, momordin, pokeweed antiviral protein, saporin 6,luffin-a, luffin-b, and MAP30 exerted an inhibitory action on choriocarcinoma cells. A variety of compounds including alfa-calendic acid and β-calendic acid, all-trans retinoic acid, etoposide, taxol, tubeimoside I , vesnarinone, aminopeptidase inhibitor ubenimex (bestatin), 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide (PK11195) and genistein, bark extracts from *Ulmis laevis* and flax seed fractions, also have anti-choriocarcinoma activity. Further investigations on the utility of these proteins and compounds in the clinical setting is worthwhile.

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