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HPV Detection in Leukocyte Samples of Spinocellular Carcinomas Using PCR

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Head and neck cancer are a malignant tumours originating in the upper aerodigestive tract, including the oral cavity, larynx, pharynx and nasopharynx. The vast majority of head and neck cancers are squamous cell carcinomas (HNSCC) arising from the epithelial membranes of these regions. The environmental pollution, high alcohol consumption and smoking are associated with this type of cancer, and also human papilloma virus (HPV) is involved in HNSCC carcinogenesis. Many different methodologies have been developed for detection of HPV virus. This manuscript is dealing with the HPV detection in leukocyte samples of 103 patients with spinocellular carcinomas using PCR method for detection and subtypes identification. 56 positives samples were detected for HPV papillomavirus. Further, the positives samples were tested with HPV type-specific primers and 21.4% were HPV 16 type and 12.5% were HPV 18 type. 7% of these samples were positive for both HPV16 and HPV18 type.

Keywords: Human papillomavirus; head and neck cancer; PCR.

1. Introduction

Head and neck squamous cell carcinomas (HNSCCs) are malignant tumors of epidermal keratinocytes, that affect mainly the lip, oral cavity, nasal cavity, paranasal sinuses, pharynx, and larynx [1]. HNSCCs grow locally and infrequently metastasize [2]. This carcinomas are one of the major forms of skin cancer, which arises from the uncontrolled multiplication of epithelial cells or other cell types such as keratinocytes, tonofilament bundles, desmosomes, or structures involved in cell-to-cell adhesion [3]. At the time of diagnosis, HNSCCs spread to the lymph nodes of the neck, and this is often the first sign of the disease. The surgery and radiation are the common therapy against this cancer [4].

The HNSCCs is the sixth leading cancer by incidence worldwide and eighth by death. There are 0.5 million new cases a year worldwide [5]. The environmental pollution and wrong life style, which are associated with the squamous cell carcinomas, involve following risk factors: tobacco smoking, alcohol consumption, UV light, particular chemicals used in certain workplaces, and certain strains of viruses, such as human papillomavirus (HPV) [6].

Human papillomaviruses (HPVs) are small DNA viruses with a strict tropism for human epithelial cells. In general, HPV infections are asymptomatic and persist for 18–24 months before they are cleared by the immune system of the host [7]. Tumorigenicity of HPVs differs markedly among HPV genotypes [8]. This virus

has been associated with HNSCCs in the areas of the oropharynx, lung, fingers, and anogenital region [1]. Functional loss of p53 tumor suppressor gene, mutations in Ras protooncogenes, and certain chromosomal aberrations are characteristics of cancer caused by HPVs [9].

Many different methods are able to give information about the presence of HPVs in biological samples. The combination of a sensitive test, and a specific test, could allow for the best potential to accurately establish the presence or absence of HPVs [10,11].The mainly utilized tests for HPV identification among others, are polymerase chain reaction (PCR) testing, real time PCR, in situ hybridization analysis (IS), immunohistochemical (IHC) staining for tumor suppressor protein p16, and southern blotting assays [11].

The methodology for polymerase chain reaction (PCR) is an indispensable technique used in molecular biology in research labs to amplify a single copy of DNA generating thousands to millions of copies of chosen DNA sequence [12]. This technique has a big variety of applications like DNA cloning for DNA sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints and the detection and diagnosis of infectious diseases. PCR can be extensively modified to perform a wide array of genetic manipulations [10,13].

Patients	Age	Histological group	Localization	Grade	Stage	Metastasis
92% Men	69	97% Spinocellular	73% Dissem	95%High	49% I-II 51% III-IV	5%
8% Women	64	99% Spinocelullar	81% Dissem	93%High	69% I-II 51% III-IV	6%

Table 1: Clinical characterization of patients. Analysis the medical information obtained from patients with head and neck cancer from St. Anne´s University Hospital in Brno, differentiating between men and women and their clinical histological data.

In HPV detection the PCR represents a highly-sensitive and cost-effective method. It can be used to detect as little as one copy of a DNA sequence from paraffin-embedded tissues or fresh tissues from biopsies [14]. The disadvantages of PCR techniques are that they have lower specificity, they do not allow distinction between all HPV types that are present in the neoplastic cells and non-neoplastic and they cannot distinguish between episomal and integrated HPV DNA[15]. Furthermore, the presence of latent viruses leads to false positive results due to the ability of PCR to detect just a few copies of HPV DNA per cell. Different attempts have been made to resolve this issue through the use of real-time PCR, which provides a quantitative analysis of viral load [16].

The combined results of immunocytochemical detection of p16 protein and the detection of HPV DNA by specific PCR enable better discrimination between latent and carcinogenic HPV infections and thus can provide information on the prognosis of HNSCC patients and facilitate therapeutic decisions[17].

The detection of the viral oncoproteins E6 and E7 requires technique that is restricted to the research laboratory, like RNA extraction and polymerase chain reaction amplification. The development of RNA in situ hybridization (ISH) probes complementary to E6/E7 mRNA permits direct visualization of viral transcripts in routinely processed tissues and has opened the door for accurate HNSCC detection in the clinical care setting [18].

2. Results and Discussion

2.1 Clinical Characterization of Patients

In this study 103 DNA samples were isolated from leukocytes of patients with head and neck cancer from St. Anne´s University Hospital in Brno and purified using QIAamp DNA Mini Kit. The average age of the patients was 69 years for

men and 64 years for women.

92% of the patients were men and 8% women, with major diagnosis for both which was oropharynx cancer with spinocellular histological type, followed with adenocarcinomas and basaliomas. Only a low percentage of the high grade tumors in men and women were found. These men presented a 51% of this cancer in the stage III-IV, with a 5% of metastasis, while the women presented a 69% of this cancer in the stage I-II, with a 6% of metastasis (Table.1).

2.2 PCR detection and subtypes identification

The samples were analyzed using PCR with specific primers for HPV detection and subtypes identification. DNA from leukocytes samples was tested using GP5/GP6 set of primers located within the L1 region of HPV genome. Furthermore, the samples were also tested with type-specific primers for HPV types 16 and 18

Figure 1: Scheme of HPV detection using PCR. First, GP5/GP6 primers for HPV detection were used. Next, the 56 HPV positive samples were analyzed with the type-specific primers for HPV-16 and HPV-18, obtaining 12 samples of HPV16, 7 samples of HPV-18 and 4 samples were HPV-16 and HPV-18 at the same time.

(Fig. 1).The quality of the isolated DNA, was determined with β-actin gene. 85 of the 103 DNA samples were β-actin positive and thus, adequate for further analysis. In this group of samples, 56 positives samples were detected for HPV which were obtained in 65.9% cases. We identified the subtypes with type-specific primers for HPV-16 and HPV-18 obtaining 21.4% of HPV-16 type and 12.5% of HPV-18 type. 7% of these samples were HPV-16 and HPV-18 positives at the same time and were determined as high grade tumors (Fig.2).

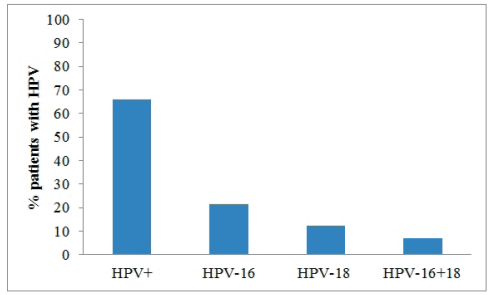

Figure 2: HPV detection and subtypes identification of the patients by PCR method. GP5/GP6 primers were used for HPV detection (65.9%) and HPV16-18 primer for subtyping (21.4% HPV-16, 12.5% HPV-18 and a 7% for HPV16-18).

2.3 Sequencing analysis

The HPV positive samples were also checked by PCR sequencing. After purification and sequencing of these samples, they were studied by in silico analysis using BLAST searches and Clustalw Multiple Sequence Alignment, obtaining 73% of identities with full length sequence of L1 HPV major capsid protein (Fig.3).

2.4 Discussion

We have used in our study the polymerase chain reaction (PCR) method for HNSCCs detection in leukocyte samples, because is a highly-sensitive, fast and cost-effective method. The PCR methods enable the detection of large number of HPV genotypes and also can increase significantly the positivity rate of HPV DNA detection in samples with a low DNA copy number. In conclusion, we preselected the samples using sets of primers located within the L1 region of HPV genome (GP5/GP6 primers) and with an additional amplification to typing of PCR--positive samples with the HPV type-specific primers (HPV16-18 primers). The samples were also tested by PCR sequencing and by in silico analysis, where was obtained a 73% of identities with the sequence of L1 HPV major capsid protein, which confirms the presence of HPV.

The first studies that used the set of GP5/GP6 primers for HPV detection were done in samples of patients with cervical cancer. Jacobs et al, introduced a GP5/GP6 PCR-based procedure in which they included Southern blot

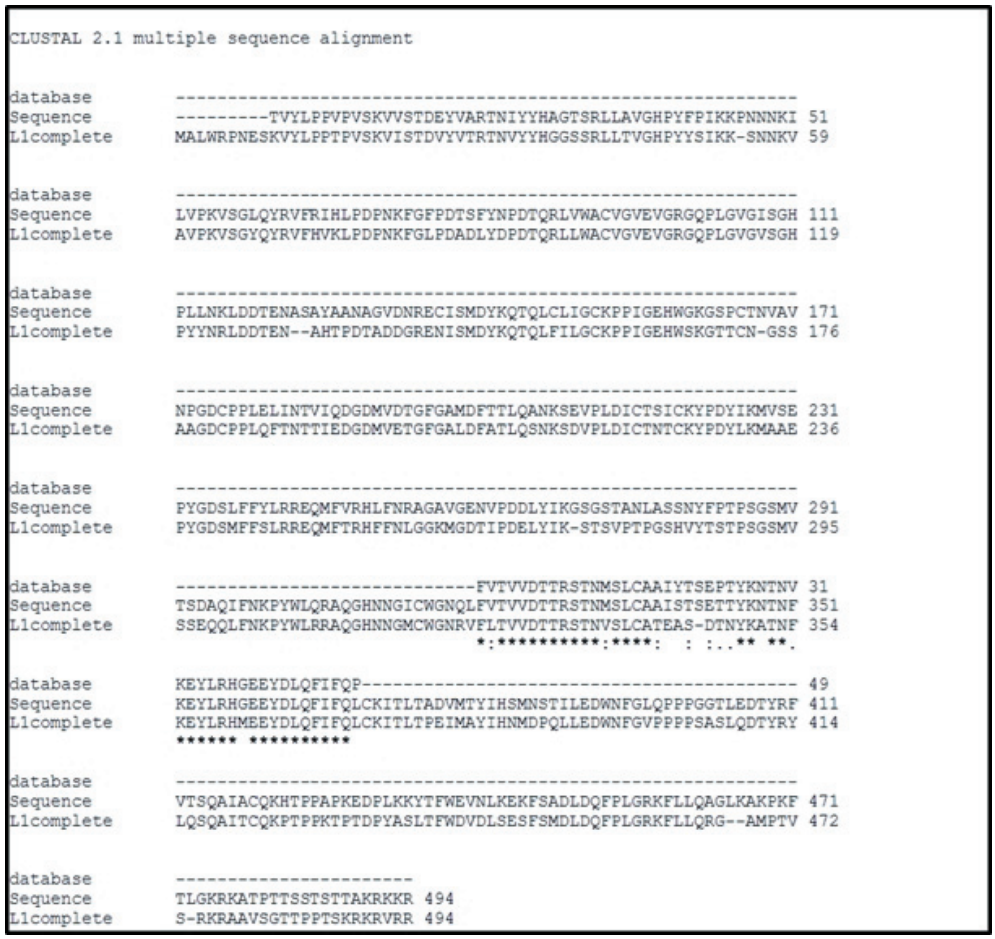

Figure 3: Multiple sequence alignment using ClustalW2. The sequences used in this alignment were, sequence of the amplicon obtained with the GP5/GP6 set of primers (called database), the sequence obtained from analysis of our samples (called Sequence), and a complete sequence of L1 gene (called L1 complete).

hybridization of PCR products with cocktails of radioactively labeled HPV type-specific internal oligonucleotides to detect a broad spectrum of genital HPV genotypes in one PCR[19]. De Roda Husman et al observed that the GP5/GP6 PCR method provided an increased detection of HPV level in poorly cervical scrapes[20]. Baay et al, compared the efficacies of three general primer pairs (GP5/6, CPI/IIG, MY09/11) for the detection of HPV DNA in formaldehyde-fixed paraffin-embedded carcinomas. The efficacy of each primer pair increased in HPV DNA detection in a 87.6%[21].

3. Experimental Section

3.1 Clinical specimens

A total of 103 histopathologically confirmed head and neck cancer subjects were involved in the study. The samples were collected from St. Anne´s University Hospital, Department of Otorhinolaryngology and Head and Neck Surgery. Enrolment of patients into realized clinical study was approved by the Ethic Committee of the Faculty of Medicine, Masaryk University, Brno, Czech Republic.

Primer	Sequence (5'-3')	Reference
GP5 GP6	TTTGTTACTGTGGTAGATAC GAAAAATAAACTGTAAATCA	(Snijders et al., 1990[22])
HPV16fw HPV16rv	CCCAGCTGTAATCATGCATGGAGA GTGTGCCCATTAACAGGTCTTCCA	(Soler et al., 1991[23])
HPV18fw HPV18rv	CGACAGGAACGACTCCAACGA GCTGGTAAATGTTGATGATTAACT	(Soler et al., 1991[23])
β-actin fw β-actin rv	CCTGAACCCTAAGGCCAACC GCAATGCCTGGGTACATGGT	(Ueyama et al.,1987[24])

Table 2: List of primers used in this study [25].

3.2 Extraction and quantification of DNA

DNA was isolated and purified from leukocytes using QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions. The concentration of purified DNA was determined using multimode reader Infinite 200 (Tecan, Männedorf, Switzerland).

3.3 Polymerase chain reaction (PCR)

The 142 base-pair long sequence of L1 gene was amplified using GP5 and GP6 primers. The PCR mixture from New England Biolabs (UK), containing the PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl with 2.5 mM $MgCl_2$ included) 0.05 mM of each dNTP and 0.05 mM of GP5 and GP6 primers (Table 2). The DNA amplification was carried out during 40 cycles that included the denaturation at 94°C for 30 s, the annealing at 45°C for 30 s and the primer extension at 72°C for 30 s.

The positive samples for HPV were analyzed using PCR with the HPV-16 and HPV-18 primers. The PCR amplicons reached length of 202 bp for HPV-16 and 272 bp for HPV-18. The PCR mixture from New England Biolabs (UK), containing the PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl with 2.5 mM $MgCl_2$ included) 0.05 mM of each dNTP and 0.05 mM of each couples of primers (Table 1). The DNA amplification was carried out during 40 cycles that included the denaturation at 94°C for 30 s, the annealing at 58°C for 30 s and the primer extension at 72°C for 30 s.

As the internal quality control of the isolated DNA, β-actin gene (600bp) was used. The PCR mixture from New England Biolabs (UK), contained the PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl with 2.5 mM $MgCl_2$ included) 0.05 mM of each dNTP and 0.05 mM of each couples of primers (Table 2). The DNA amplification was carried out during 40 cycles that included the denaturation at 94°C for 30 s, the annealing at 58°C for 30 s and the primer extension at 72°C for 45 s. Each PCR product was analysed using electrophoresis on 1% agarose gels stained with ethidium bromide.

3.4 Sequencing

GenomeLab DTCS Quick Start kit (Beckman Coulter, USA) with 20 ng of purifed amplified DNA from the PCRs was used for the sequencing reaction. The cycling conditions were as follows: 30 cycles of denaturation at 96 °C for 20 s; annealing at 50 °C for 20 s and 60 °C for DNA synthesis for 4 min. DNA fragments from this reaction were purified using magnetic particles CleanSEQ (Beckman Coulter, USA). DNA sequencing was performed on Genetic Analysis System CEQ 8000 (Beckman Coulter, USA). After denaturation at 90 °C for 2 min, the fluorescence-marked DNA fragments were separated in 33 cm capillary with 75 μm i.d.

(Beckman Coulter, USA), which was filled with a linear polyacrylamide denaturing gel (Beckman Coulter, USA). The separation was performed at capillary temperature of 50 ºC and voltage of 4.2 kV for 85 min.

3.5 Sequence analyses

Sequence analyses were performed using, BLASTX algorithm available from the NCBI (http://blast.ncbi.nlm.nih.gov/;[26]) and ClustalW2 algorithm available at EBI (http://www.ebi.ac.uk/Tools/clustalw2/;[27]).

4. Conclusions

The polymerase chain reaction is a suitable method enabling the detection of HPVs and differentiation between different subtypes. Many different HPV tests are available. All of the methods are able to give information about the presence of HPV in biological samples. The majority of carcinomas are caused by the most common and high risk HPV types, HPV-16 and HPV-18, which contain the E6-E7 oncogenes. The patients infected with one type of HPV were significantly more likely to harbor additional HPV types. Thus, evaluation of risk rate of heterogeneous coinfections has to be further done to determine the effects on head and neck cancer development.

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Conflicts of Interest

The authors declare they have no potential conflicts of interests concerning drugs, products,services or another research outputs in this study. The Editorial Board declares that the manuscript met the ICMJE „uniform reguirements“ for biomedical papers.

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