

## Human papilloma virus (HPV) and methods for its identification in head and neck cancers

Ana Maria Jimenez Jimenez<sup>a</sup>, Branislav Ruttkay-Nedecký<sup>a</sup>, Ondřej Zítka<sup>a,b</sup>, Vojtěch Adam<sup>a,b</sup>, René Kizek<sup>a,b</sup>

<sup>a</sup> Department of Chemistry and Biochemistry, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic - European Union

<sup>b</sup> Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic - European Union

### Human papilloma virus (HPV) and methods for its identification in head and neck cancers

Human papillomaviruses (HPV) are small circular, double-stranded DNA viruses infecting epithelial tissues. The viruses have been detected in humans and a variety of mammalian and avian species. The papillomavirus family that affects human skin and the moist membranes that line the body, such as the throat, mouth, feet, fingers, nails, anus and cervix. There are over 100 types, of which 40 can affect the genital area. Most known HPV types cause no symptoms to humans. Some, however, can cause verrucae (warts), while a small number can increase the risk of developing several cancers, such as that of the cervix, penis, vagina, anus and oropharynx. Researches are focused on the development of HPV detection assays specially designed for head and neck squamous cell carcinoma (HNSCC). Many different methodologies have been utilized to identify the HPV virus including polymerase chain reaction (PCR) testing, real time PCR, in situ hybridization analysis (ISH), immunohistochemical (IHC) staining for tumor suppressor protein p16, and southern blotting assays. The objective of this review is to explain HPV virology, the molecular mechanisms of carcinogenesis in head and neck cancer and the main methodology for HPV detection in these types of cancer

**Accepted:** 5. 12. 2014

**Keywords:** human papillomavirus, cancer, head, neck, PCR, In situ hybridization, immunohistochemical staining, southern blotting

### 1. Introduction

Human papillomavirus (HPV) selectively infects the epithelium of the skin and mucous membranes. Specific HPV types are associated with squamous cell carcinoma, adenocarcinoma, and dysplasias of the cervix, penis, anus, vagina and vulva<sup>1</sup>. The term head and neck cancer includes malignancy in an area that comprises the skin, oral cavity, salivary glands, lip, pharynx, larynx, nasal cavity, paranasal sinuses and soft tissues of the neck and ear<sup>2</sup>.

The first association of HPV with head and neck cancer was published in 1985<sup>3</sup>. HPV was also shown to play a role in the pathogenesis of a subset of head and neck squamous cell carcinomas (HNSCCs)<sup>4</sup>. Almost 650,000 patients worldwide are diagnosed with head or neck

cancer each year and 350,000 patients die of this disease as this cancer is the sixth most prevalent type of cancer worldwide.

The ratio of males to females is approximately 2:1<sup>2,3</sup>. From the point of view of the infection, HPVs have developed several molecular mechanisms to enable infected cells to suppress apoptosis<sup>5,6</sup>. Based on their potential for oncogenesis, HPV types can be classified both as high-risk or low-risk<sup>7</sup>. Precancerous lesions of the oral mucosa are epithelial changes that are able to undergo malignant transformation more likely than normal tissue at other mucosal sites<sup>8</sup>.

A total of 150 HPV genotypes have been identified<sup>9</sup>. The HPV 16 and 18 strains, which are known to cause nearly all cases of cervical cancer, also raise the risk of developing oro-

pharyngeal cancer<sup>10</sup>. The evident similarities between both cervical and head and neck tumors prompted the utilization of the same HPV diagnostic procedures. There is now compelling evidence that specially designed methodologies must be employed for prognosis<sup>11,12</sup>.

## 2. HPV virology

HPVs are quite small, non-enveloped double-stranded circular DNA viruses, that has diameter of 55 nanometers. The DNA of HPV has 8,000 nucleotide base pairs associated with histones. HPV genome is enclosed in an icosahedral capsid shell comprised of major and minor capsid proteins<sup>2,13</sup> and can be divided into 3 domains: an early region with 6 E genes E1, E2, E4, E5, E6 and E7; a late region with 2 L genes, L1 and L2; and a non-coding regulatory region (NCR) of approximately 1 kb. Organisation of HPV 16 genome is shown in Fig. 1<sup>1</sup>.

The L1 and L2 late proteins form capsomers of the virus that encapsidate the viral DNA. The early E proteins have different functions. The E1 protein binds to the origin of replication. The E2 protein binds to E1 protein and stimulates viral DNA replication. The E2 protein also acts as a transcriptional repressor of HPV E6 and E7 gene expression. The E4 protein is expressed in an later phase with a role in the assembly and release of the viral particle. E5 protein stimulates the transforming activity of the epidermal growth factor receptor resulting in the increased cell proliferation<sup>14,15</sup>.

The viral protein E6 and E7 are implied in tumorigenesis and are known to induce degradation of the tumor suppressors p53 and pRB, respectively<sup>16</sup>. They can suppress apoptosis and alter the function of factors involved in cell-cycle regulation, thereby facilitating prolongation of the proliferative stage of keratinocyte differentiation<sup>17</sup>. The E6 protein of HPV-16 is a small polypeptide of 150 amino acids. This protein can inactivate p53 by targeting the protein for ubiquitination and consequent degradation<sup>18</sup>. The HPV-16 E6 protein also activates the telomerase, an enzyme that maintains the telomeric DNA at the ends of linear chromosomes. Almost all human cancers and immortalized cell lines have highly active telomerase<sup>19-21</sup>.

The E7 protein of HPV-16 is a small, nuclear polypeptide of 100 amino acids. E7 binds to retinoblastoma protein (pRb)<sup>20</sup>. Besides pRb, E7 also interacts with various other proteins, most of which are important regulators of the cell growth. The E7 protein induces abnormal centrosome duplication, resulting in multipolar, abnormal mitoses, aneuploidy and genomic instability<sup>17,22</sup>.

The majority of cervical carcinomas caused by the two most common HPV types, HPV-16 and HPV-18, contain integrated viral sequences that express E6-E7 protein<sup>23</sup>. E6-E7 oncogene expression is considered necessary for carcinogenesis, and maintenance of the malignant phenotype of these cancers<sup>2,24</sup>.

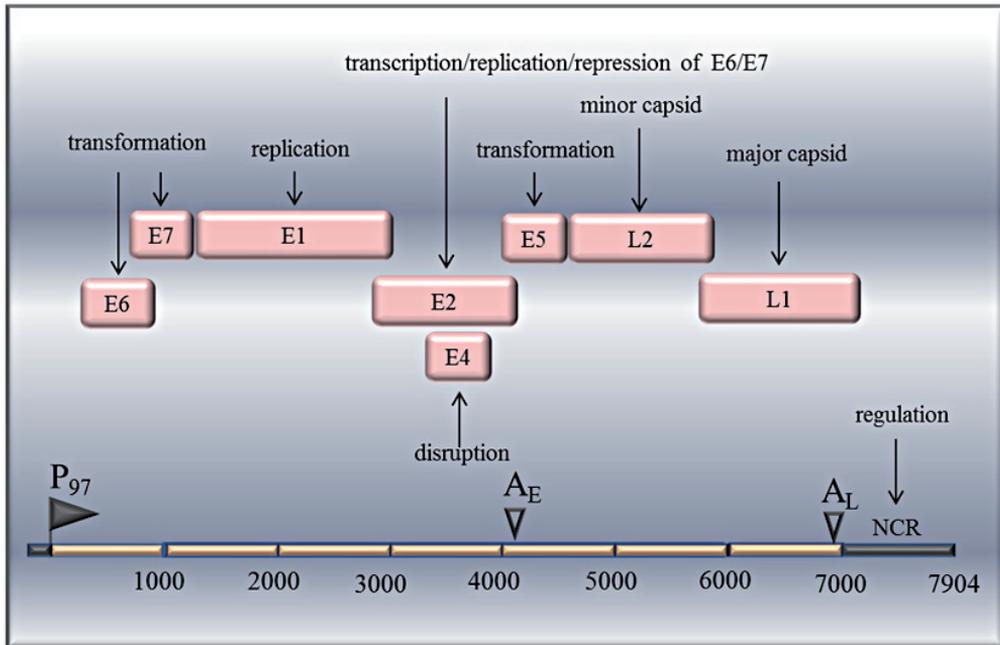
## 3. Methods for detection and identification of HPV

Some of the main methodologies for detection of HPV virus: southern blotting assay, polymerase chain reaction (PCR) testing, real time PCR, in situ hybridization (ISH) analysis, immunohistochemical (IHC) staining for p16 are shown in Table 1<sup>26</sup>.

### Southern Blotting Assay

Southern blotting is an assay that has long been one of the standard techniques for the detection of HPV DNAs, and it has the ability to differentiate between episomal and integrated DNA and can detect as little as 0.1 copies of viral DNA per cell<sup>27</sup>.

Southern blot has a theoretically higher specificity but is less sensitive than PCR. This method cannot be applied to formalin-fixed, paraffin-embedded tissue samples because they contain cross-linked, degraded nucleic acids. Nevertheless, the Southern blotting assay can be useful for comparing results of other methods for detection of viral integration, though this method has no practical or clinical utilization<sup>27,28</sup>.



**Figure 1.** Genetic Map of the human papillomavirus (HPV) type 16. HPV genome is a double-stranded circular DNA molecule. Early genes from the DNA sequence are designated E1 to E7 and late genes, L1, and L2, indicated in pink boxes. The non-coding region (NCR) is indicated by the black box. Adopted and modified according to Chen et al.<sup>25</sup>.

### Polymerase Chain Reaction (PCR) and real-time PCR

Polymerase chain reaction (PCR) is a sensitive marker for HPV DNA detection and RT-PCR may be a sensitive marker for HPV mRNA quantification<sup>29</sup>. PCR represents a highly-sensitive and cost-effective method for HPV detection. In theory, it can be used to detect as little as one copy of a DNA sequence and can be utilized in paraffin-embedded tissue or fresh tissue from biopsies<sup>27</sup>. The PCR techniques have a number of drawbacks in comparison to in situ hybridization (ISH); they have lower specificity, they do not allow distinction between HPV that is present in the neoplastic and non-neoplastic cells and they cannot distinguish between episomal and integrated HPV DNA<sup>30</sup>.

The presence of latent virus leads to false positive results due to the ability of PCR to detect

just a few copies of HPV DNA per cell. Attempts have been made to resolve this issue through use of real-time (RT)-PCR, which provides a quantitative analysis of viral load. RT-PCR amplification of viral E6/E7 mRNA is considered for the detection of clinically significant HPV infection within tumor specimens as it detects transcriptionally active HPV<sup>31,32</sup>. Nonetheless, PCR and RT-PCR cannot localize HPV in the area of neoplasia and other techniques like ISH can provide this information together with a higher clinical specificity<sup>33</sup>.

### In Situ Hybridization for HPV

*In situ* hybridization (ISH) is the only molecular method allowing reliable detection and identification of HPV in topographical relationship to their pathological lesions. Unlike in other molecular methods, in ISH the whole HPV detection procedure occurs within the nucleus of infected cells<sup>34</sup>. The result of the hybridization reaction is evaluated microscopically and the appearance of an appropriate precipitate within the nucleus of epithelial cells is indicative for the presence of HPV in the specimen. In addition, the physical state of the virus can be evaluated by the presence of punctuate signals for integrated virus and diffuse signals for

METHODS	ADVANTAGES	DISADVANTAGES
<b>Southern Blotting</b>	High specificity Differentiation between episomal and integrated DNA Detects 0.1 copies of DNA per cell	Not easily applied to FFPE samples Practical/clinical utilization
<b>PCR</b>	High sensitivity Cost effective Several commercially available primer sets	Low specificity No quantitative measure of viral load No distinction between episomal and integrated DNA
<b>Real time-PCR</b>	High sensitivity High specificity Ability to differentiate between episomal and integrated DNA	False positive and false negative products No direct evidence of viral integration
<b>ISH</b>	High specificity High sensitivity Ability to differentiate between episomal and integrated DNA	Technically difficult to be use in routine screening
<b>p16 Immunostaining</b>	High Sensitivity Easily applied to FFPE tissue	Surrogate marker Specificity not ideal

**Table 1.** Possible methods and techniques of HPV detection. Adopted and modified according Venuti and Paolini<sup>26</sup>

episomal virus. In this way, ISH may overcome some of the limitations of PCR by detecting only clinically relevant infection. Although the specificity of this method is high, the sensitivity is not ideal<sup>35</sup>. The sensitivity of ISH is still less than that seen in PCR analysis. However, ISH is more specific for HPV infection than p16 immunohistochemical staining<sup>30</sup>. In addition, current ISH-based assays are considered by many experts in the field to be too laborious and to have insufficient clinical sensitivity to be used in routine screening<sup>34</sup>.

### Immunohistochemical Staining for p16

The transcription of the viral oncoprotein E7 inactivate the function of the pRB gene, causing perturbation of other key components of the retinoblastoma pathway, and to induce upregulation of P16 protein expression, reaching levels that can be detected readily by immunohistochemistry. Accordingly, P16 immunohistochemistry is sometimes advocated as a surrogate marker of HPV infection<sup>36</sup>.

P16 immunostaining is an appropriate assay for elimination of HPV negative cases from any additional analysis. Since specificity is almost 100%, a finding positive for HPV 16 on in situ hybridization reduces the number of false-positive cases by P16 immunostaining alone. Whatever the method is used to establish the presence of non-HPV 16 virus types, upfront use of P16 immunostaining and HPV 16 in-situ hybridization accurately establishes the HPV status of most oropharyngeal cancers<sup>37</sup>.

Alternatively, P16 overexpression could suggest pRB pathway disturbances unrelated to HPV. Using E6 and E7 mRNA levels as conclusive evidence of HPV involvement, P16 immunostaining is 100% sensitive but only 79% specific as a surrogate marker of HPV infection<sup>36</sup>.

## 4. Conclusion

Many different HPV tests exist, and much more information about their analytical and clinical properties. All of the methods are able to give information about the presence of HPV in biological samples. At the present time, IHC

staining for p16 and PCR appear to be the most sensitive markers for HPV, while ISH confers the great specificity. The combination of a sensitive test, p16 IHC and a specific test, ISH could allow for the best potential to accurately establish the presence or absence of HPV.

This information is crucial for the prognostic assessment of these patients but it is ineffective for the individuation of people at risk of tumor after HPV infection. Thus, there is a need for improving these HPV diagnostic tools by detection not just of the virus, but also simultaneous detection of other biological markers like alterations in tumor suppressor gene pathways and the modification of the gene expression profiles.

*This work was financially supported by project SPINCANCER NT/14337.*

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 requirements“ for biomedical papers.

## References

1. Chen, R. W.; Aaltonen, L. M.; Vaheri, A., Human papillomavirus type 16 in head and neck carcinogenesis. *Reviews in Medical Virology* 2005, 15, 351-363.
2. Syrjanen, S., Human papillomavirus (HPV) in head and neck cancer. *Journal of Clinical Virology* 2005, 32, S59-S66.
3. Badulescu, F.; Crisan, A.; Badulescu, A.; Schenker, M., Recent data about the role of human papillomavirus (HPV) in oncogenesis of head and neck cancer. *Romanian Journal of Morphology and Embryology* 2010, 51, 437-440.
4. Fakhry, C.; Westra, W. H.; Cmelak, S. L. A.; Ridge, J. A.; Pinto, H.; Forastiere, A.; Gillison, M. L., Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *Journal of the National Cancer Institute* 2008, 100, 261-269.
5. Rose, B. R.; Thompson, C. H.; Tattersall, M. H.; O'Brien, C. J.; Cossart, Y. E., Squamous carcinoma of the head and neck: Molecular mechanisms and potential biomarkers. *Australian and New Zealand Journal of Surgery* 2000, 70, 601-606.
6. Yuan, C. H.; Filippova, M.; Duerksen-Hughes, P.,

- Modulation of Apoptotic Pathways by Human Papillomaviruses (HPV): Mechanisms and Implications for Therapy. *Viruses-Basel* 2012, 4, 3831-3850.
7. Smith, E. M.; Ritchie, J. M.; Pawlita, M.; Rubenstein, L. M.; Haugen, T. H.; Turek, L. P.; Hamsikova, E., Human papillomavirus seropositivity and risks of head and neck cancer. *International Journal of Cancer* 2007, 120, 825-832.
  8. Janicek, M. F.; Averette, H. E., Cervical cancer: Prevention, diagnosis, and therapeutics. *Ca-a Cancer Journal for Clinicians* 2001, 51, 92-114.
  9. Doorbar, J.; Gallimore, P. H., Identification of proteins encoded by the L1 and L2 open reading frames of human papillomavirus-1A *Journal of Virology* 1987, 61, 2793-2799.
  10. Syrjanen, S., Human papillomavirus infections and oral tumors. *Medical Microbiology and Immunology* 2003, 192, 123-128.
  11. Syrjanen, K.; Syrjanen, S., Detection of human papillomavirus in sinonasal papillomas: Systematic review and meta-analysis. *Laryngoscope* 2013, 123, 181-192.
  12. van Houten, V. M. M.; Leemans, C. R.; Kummer, J. A.; Dijkstra, J.; Kuik, D. J.; van den Brekel, M. W. M.; Snow, G. B.; Brakenhoff, R. H., Molecular diagnosis of surgical margins and local recurrence in head and neck cancer patients: A prospective study. *Clinical Cancer Research* 2004, 10, 3614-3620.
  13. zur Hausen, H., Human papillomavirus & cervical cancer. *Indian Journal of Medical Research* 2009, 130, 209-209.
  14. Foguel, D.; Silva, J. L.; de Prat-Gay, G., Characterization of a partially folded monomer of the DNA-binding domain of human papillomavirus E2 protein obtained at high pressure. *Journal of Biological Chemistry* 1998, 273, 9050-9057.
  15. Ustav, M.; Ustav, E.; Szymanski, P.; Stenlund, A., Identification of the origin of replication of bovine papillomavirus and characterization of the viral origin recognition factor-E1 *Embo Journal* 1991, 10, 4321-4329.
  16. Garnett, T. O.; Duerksen-Hughes, P. J., Modulation of apoptosis by human papillomavirus (HPV) oncoproteins. *Archives of Virology* 2006, 151, 2321-2335.
  17. Dayyani, F.; Etzel, C. J.; Liu, M.; Ho, C. H.; Lippman, S. M.; Tsao, A. S., Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC). *Head & Neck Oncology* 2010, 2.
  18. Strati, K.; Pitot, H. C.; Lambert, P. F., Identification of biomarkers that distinguish human papillomavirus (HPV)-positive versus HPV-negative head and neck cancers in a mouse model. *Proceedings of the National Academy of Sciences of the United States of America* 2006, 103, 14152-14157.
  19. Van Doorslaer, K.; Sidi, A.; Zanier, K.; Rybin, V.; Deryckere, F.; Rector, A.; Burk, R. D.; Lienau, E. K.; van Ranst, M.; Trave, G., Identification of Unusual E6 and E7 Proteins within Avian Papillomaviruses: Cellular Localization, Biophysical Characterization, and Phylogenetic Analysis. *Journal of Virology* 2009, 83, 8759-8770.
  20. Wiest, T.; Schwarz, E.; Enders, C.; Flechtenmacher, C.; Bosch, F. X., Involvement of intact HPV16 E6/E7 gene expression in head and neck cancers with unaltered p53 status and perturbed pRb cell cycle control. *Oncogene* 2002, 21, 1510-1517.
  21. Zimmermann, H.; Degenkolbe, R.; Bernard, H. U.; O'Connor, M. J., The human papillomavirus type 16 E6 oncoprotein can down-regulate p53 activity by targeting the transcriptional coactivator CBP/p300. *Journal of Virology* 1999, 73, 6209-6219.
  22. Dyson, N.; Howley, P. M.; Munger, K.; Harlow, E., The human papilloma virus-16 E7-oncoprotein is able to bind to the retinoblastoma gene-product *Science* 1989, 243, 934-937.
  23. Smith, E. M.; Pawlita, M.; Rubenstein, L. M.; Haugen, T. H.; Hamsikova, E.; Turek, L. P., Risk factors and survival by HPV-16 E6 and E7 antibody status in human papillomavirus positive head and neck cancer. *International Journal of Cancer* 2010, 127, 111-117.
  24. Syrjanen, S.; Rautava, J., HPV and oral health response *Journal of the American Dental Association* 2012, 143, 442-444.
  25. Chen, A. Y.; DeSantis, C.; Jemal, A., US Mortality Rates for Oral Cavity and Pharyngeal Cancer by Educational Attainment. *Archives of Otolaryngology-Head & Neck Surgery* 2011, 137, 1094-1099.
  26. Venuti, A.; Paolini, F., HPV detection methods in head and neck cancer. *Head and neck pathology* 2012, 6 Suppl 1, S63-74.
  27. Gagnon, D.; Fradet-Turcotte, A.; Archambault, J., A quantitative and high-throughput assay of human papillomavirus DNA replication. *Methods in molecular biology (Clifton, N.J.)* 2015, 1249, 305-316.
  28. Moreas, H.; Tsiambas, E.; Lazaris, A. C.; Nonni, A.; Karameris, A.; Metaxas, G. E.; Armatas, H. E.; Patsouris, E., Impact of HPV detection in colorectal adenocarcinoma: HPV protein and chromogenic in situ hybridization analysis based on tissue microarrays. *Journal of Buon* 2014, 19, 91-96.
  29. Haugg, A. M.; Rennspiess, D.; zur Hausen, A.; Speel, E. J. M.; Cathomas, G.; Becker, J. C.; Schrama, D., Fluorescence in situ hybridization and qPCR to detect Merkel cell polyomavirus physical status and load in Merkel cell carcinomas. *International Journal of Cancer* 2014, 135, 2804-2815.
  30. Rodel, F.; Wieland, U.; Fraunholz, I.; Kitz, J.; Rave-Frank, M.; Wolff, H. A.; Weiss, C.; Wirtz, R.; Balermipas, P.; Fokas, E.; Rodel, C., Human papillomavirus DNA load and p16(INK4a) expression predict for local control in patients with anal squamous cell carcinoma treated with chemoradiotherapy. *International journal of cancer. Journal international du cancer* 2015, 136, 278-288.
  31. Heidegger, I.; Pichler, R.; Muller, B.; Klocker, H.; Oswald, D.; Haid, B.; Zelger, B.; Horninger, W.; Oswald, J., Is real-time PCR the correct method to evaluate the incidence of human papillomavirus in prepuces of asymptomatic boys and men? *World Journal of Urology* 2014, 32, 1199-1204.
  32. Micalessi, M. I.; Boulet, G. A.; Bogers, J., A Real-

Time PCR Approach Based on SPF10 Primers and the INNO-LiPA HPV Genotyping Extra Assay for the Detection and Typing of Human Papillomavirus. *Methods in molecular biology* (Clifton, N.J.) 2015, 1249, 27-35.

33. Sahiner, F.; Kubar, A.; Gumral, R.; Ardic, M.; Yigit, N.; Sener, K.; Dede, M.; Yapar, M., Efficiency of MY09/11 consensus PCR in the detection of multiple HPV infections. *Diagnostic Microbiology and Infectious Disease* 2014, 80, 43-49.
34. Vega-Pena, A.; Illades-Aguilar, B.; Flores-Alfaro, E.; Lopez-Bayghen, E.; Reyes-Maldonado, E.; Alarcon-Romero, L. D., Correlation between KI-67 and telomerase expression with in situ hybridization for high-risk human papillomavirus. *Archives of Biological Sciences* 2013, 65, 81-90.
35. Singhi, A. D.; Westra, W. H., Comparison of Human Papillomavirus In Situ Hybridization and p16 Immunohistochemistry in the Detection of Human Papillomavirus-Associated Head and Neck Cancer Based on a Prospective Clinical Experience. *Cancer* 2010, 116, 2166-2173.
36. Salehinejad, J.; Sharifi, N.; Amirchaghmaghi, M.; Ghazi, N.; Shakeri, M. T.; Ghazi, A., Immunohistochemical expression of p16 protein in oral squamous cell carcinoma and lichen planus. *Annals of Diagnostic Pathology* 2014, 18, 210-213.
37. Shimizu, A.; Kato, M.; Takeuchi, Y.; Sano, T.; Kaira, K.; Uezato, H.; Ishikawa, O., Detection of human



This licence allows users to download and share the article for non-commercial purposes, so long as the article is reproduced in the whole without changes, and the original authorship is acknowledged.