Expression of Cell Cycle-Related Proteins p16, p27, p53 and Ki-67 in HPV-positive and -negative Samples of Papillomas of the Upper Respiratory Tract

TOMASZ ZATONSKI[1], URSZULA CIESIELSKA[2], KATARZYNA NOWINSKA[2], KATARZYNA RATAJCZAK-WIELGOMAS[2], CHRISTOPHER KOBIERZYCKI[2], BARTOSZ PULA[2], MARZENNA PODHORSKA-OKOLOW[2], TOMASZ KRECICKI[1] and PIOTR DZIEGIEL[2,3]

Departments of [1]Otolaryngology, Head and Neck Surgery and [2]Histology and Embryology, Wroclaw Medical University, Wroclaw, Poland;
[3]Department of Physiotherapy, University School of Physical Education, Wroclaw, Poland

Abstract. *Background: The role of human papillomavirus (HPV) infection as an etiological factor of respiratory tract papillomas has been described in numerous studies, however its role in malignant transformation has not been clearly defined. Depending on their oncogenic potential they have been classified as low- and high-risk HPVs. We analyzed the expression of four cell cycle-related proteins in order to understand the processes leading to malignant transformation. Materials and Methods: Fifty-six cases of pharyngeal and laryngeal papillomas were analyzed. Nested multiplex polymerase chain reactions to detect the presence of the HPV types, as well as immunohistochemical reactions were performed for the detection of cell cycle-related proteins p16, p27, p53 and Ki-67. Results: The presence of HPVs 6/11 and 16 was confirmed in 10/56 cases. The expression of all analyzed cell cycle-related proteins was increased in HPV-infected papillomas. Conclusion: HPV infection of the upper respiratory tract may influence the expression of cell cycle-related proteins, that could indicate its possible role in the process of malignant transformation.*

Over 120 different types of human papillomavirus (HPV) have been described and more than 50% manifest affinity to the epithelial tissues of genital and respiratory tract. Depending on their oncogenic potential, they have been classified as low-risk (LR) HPV types: 6, 11, 40, 42, 44, 54, 61, 72, 81 and 89; and high-risk (HR) types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 (1-5).

Although the association between HPV infection and presence of many benign and malignant lesions of the upper respiratory tract has been reported (4, 6-9) the pathways leading to malignant transformation of infected cells are still unclear. HPVs are present in cells as episomal or integrated forms. The tumorigenic transformation of epithelial cells may be induced by HR HPVs only when viral DNA is integrated into the host cell genome. In tumor cells, HPV DNA is detected mainly in the integrated form. The HR HPV proteins are potential factors which induce deregulation of the cell cycle (10-12). The malignant transformation of cells can result from deregulation of two main pathways of the cell cycle: p53 and retinoblastoma protein (pRb) mediated by $p16^{INK4A}$.

The p16 protein plays an important role in regulation of the cell cycle. It inhibits cyclin-dependent kinases (CDK) and also acts as an inhibitor of the cell cycle. The level of p16 expression changes due to the inactivation of pRb. In squamous cell carcinoma (SCC) of the head and neck region, decreased expression of p16 is often noted (13). The p27 and p53 proteins are CDK inhibitors, which inhibit the cyclin complexes of CDK2 and CDK4 and cause cell cycle arrest in the G_1 phase. Their increased or decreased expression is regarded as a prognostic factor in many types of malignant neoplasm *e.g.* head and neck (14), urinary bladder (15) and lung (16) cancer, as well brain tumors (17), and rhabdomyosarcoma (18). Literature shows that p53 expression in SCC differs from 35% up to 100% and its prognostic value by some reports was indicated by high expression of p53 correlated with shorter survival. In normal cells, the expression of p27 is observed in the nucleus. There are many studies indicating loss of p27 expression in SCCs

Correspondence to: Urszula Ciesielska, Ph.D., Department of Histology and Embryology, Wroclaw Medical University, Chalubinskiego 6a, 50-368 Wroclaw, Poland. Tel: +48 717841368, Fax: +48 717840082, e-mail: urszula.ciesielska@umed.wroc.pl

Key Words: Human papillomavirus, HPV, upper respiratory tract papillomas, malignant transformation.

Table I. *Polymerase chain reaction (PCR) targets and primers sequences.*

Target	Name sequences 5' → 3'	Size (bp)
Control/β-globin PCR	PC04 5' CAA CTT CAT CCA CGT TCA CC 3'	
GH20	5' GAA GAG CCA AGG ACA GGT AC 3'	268
HPV consensus/GP-E6/E7 gene	GP – E6 – 3F – 5' GGG WGK KAC TGA AAT CGG T 3'	602-666
	GP – E6 – 5B – 5' CTG AGC TGT CAR NTA ATT GCT CA 3'	
	GP – E6-6B – 5' TCC TCT GAG TYG YCT AAT TGC TC 3'	
HPV type-specific NMPCR	Cocktail primers	
HPV18	18 F – 5' CAC TTC ACT GCA AGA CAT AGA 3'	322
	18 R – 5' GTT GTG AAA TCG TCG TT TTC A 3'	
HPV16	16 F – 5' CAC AGT TAT GCA CAG AGC TGC 3'	457
	16 R – 5' CAT ATA TTC ATG CAA TGT AGG TGT A 3'	
HPV6/11	6/11 F – 5' TGC AAG AAT GCA CTG ACC AC 3'	334
	6/11 R – 5' TGC ATG TTG TCC AGC AGT GT 3'	

HPV, Human papilloma virus; NMPCR, nested multiplex polymerase reaction.

and some of them showing nuclear p27 positivity to be correlated with good prognosis (19). The Ki-67 is a non-histone nuclear protein, whose expression is observed in cells ranging from the G_1 phase of the cell cycle to mitosis, however, it is absent in interphase. Therefore, it is considered to be a reliable indicator of cell proliferation and often correlates with tumor growth (20-22). The Ki-67 proliferation index measured in immunostained tumor sections was shown to correlate with tumor aggressiveness and possess prognostic and predictive value in patients with breast and colon cancer, melanoma and lymphoma (23-24). However, its exact mechanism in cellular metabolism is still unknown.

In order to understand the processes leading to malignant transformation of the cells of the upper respiratory tract, we decided to focus on analysis of expression of the aforementioned proteins involved in cell-cycle regulation in the upper respiratory tract (pharyngeal and laryngeal) papillomas with regard to the presence of HPV infection. This study was conducted on one of the largest groups of patients with pharyngeal and laryngeal papillomas analyzed to date and facilitates the understanding of the mechanisms of malignant transformation leading to formation of head and neck squamous cell cancer (HNSCC).

Materials and Methods

Tissue samples (patients). Fifty-six formalin-fixed paraffin-embedded (FFPE) blocks containing tissues cases of pharyngeal (n=32) and laryngeal (n=24) papilloma samples were obtained from patients treated at the Department of Otolaryngology, Head and Neck Surgery, Wroclaw Medical University, Poland. The study population included 31 women (55.4%) and 25 men (44.6%). The average age of the study group was 43.7±18.9 (median 40; range 4-84) years.

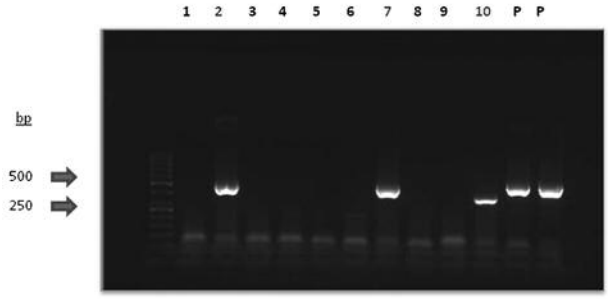

Figure 1. *Examples of nested multiplex polymerase chain reaction. Lane 10: human papilloma virus (HPV) 6/11, 334 bp; lane 2 and 7: HPV16 (not detected), 457 bp; lanes 1, 3-6, 8-9; P: positive control HPV16.*

Immunohistochemistry (IHC). For routine pathological examination with diagnosis verification, 7-μm thick sections (microtome RM 2145; Leica Biosystem, Nussloch, Germany) were stained with hematoxylin and eosin. Subsequently, 4-μm thick sections mounted on Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany) were used for IHC. After deparaffinization and hydration, endogenous epitopes were uncovered by 20 min heating of the sections in 97°C in Target Retrival Solution buffer (high pH for p16, p27, p53 and low pH for Ki-67) in Pre-Treatment Link Rinse Station (Dako, Glostrup, Denmark). The immunodetection was performed using specific mouse monoclonal antibodies directed against $p16^{INK4A}$ (clone G175-405, 1:50+linker; BD Pharmingen, CA, USA), $p27^{KIP1}$ (clone SX53G8, 1:25; Dako), p53 (clone DO-7, 1:50; Dako) and Ki-67 (clone MIB-1, 1:100; Dako). Antibodies were diluted in Antibody Diluent (Dako). The IHC reactions were carried with usage Autostainer Link 48 (Dako) automated staining platform and the EnVision FLEX™ visualization system (Dako). Negative controls were performed by omitting the primary antibody and tissue from cervical cancer served as positive control. The immunoreactivity of tested proteins was analyzed by two independent investigators using computer image analysis $Cell^D$

Table II. *The degree of expression of proteins p16, p27, p53 and Ki-67 in papilloma cells.*

Degree of expression	p16, n (%)	p27, n (%)	p53, n (%)	Ki-67, n (%)
0: Lack of expression	8 (14.3%)	1 (1.8%)	1 (1.8%)	1 (1.8%)
1: 1-5% of cells	10 (17.9%)	2 (3.6%)	23 (41.1%)	9 (16.1%)
2: 6-25% of cells	12 (21.4%)	3 (5.4%)	18 (32.1%)	19 (33.9%)
3: 26-50% of cells	10 (17.9%)	31 (55.3%)	12 (21.4%)	24 (42.8%)
4: ≥51% of cells	16 (28.5%)	19 (33.9%)	2 (3.6%)	3 (5.4%)

Software coupled with microscope BX41 and DP-12 camera (Olympus, Tokyo, Japan).

DNA extractions. DNA was isolated from FFPE blocks using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Quality control of extracted DNA was performed by polymerase chain reaction (PCR) with primers PC04 and GH20 specific for the gene of the human β-globin (Table I) according to the protocol of Husnjak *et al*. (20). Thermal cycling consisted of 30 cycles: initial denaturation at 94˚C for 4 min, denaturation at 94˚C for 30 s, annealing at 53˚C for 30 s, elongation at 72˚C for 30 s and the final elongation at 72˚C for 10 min.

Nested multiplex PCR (NMPCR). Identification of HPV6/11, -16 and -18 was performed using specific primers in the studied material. NMPCRs were carried out by performing the following two rounds of amplifying reaction (two single-round) using a 15 pmol pair of external primers complementary to the E6/E7 region genotyping HPV6/11 and HPV18 also pair of internal primers specific for HPV6/11, HPV16 and HPV18 (Table I). PCRs were performed in final volume of 50 μl reaction mixture and were conducted with Taq PCR Core Kit regents (Qiagen). Additionally, reaction mix consisted of 15 pmol pair of primers and a 100-200 ng/μl concentration of DNA extracted from paraffin sections as a DNA template, was used. The reaction procedure was carried out according to the protocol of Sotlar *et al*. (25). The first NMPCR conditions were: external PCR covered 40 cycles: initial denaturation for 4 min at 94˚C, denaturation for 1 min at 94˚C, annealing for 1 min at 40˚C, elongation for 2 min at 72˚C, final elongation for 10 min at 72˚C. The second NMPCR, with primers specific to the type of virus included 35 cycles with: initial denaturation for 4 min at 94˚C, denaturation for 30 s at 94˚C, annealing for 30 s at 56˚C, elongation for 45 s at 72˚C, with final elongation for 4 min at 72˚C. DNA extracted from cervical cancer samples with confirmed presence of HPV16, and 18 served as positive control. PCR amplification products were analyzed by electrophoresis on a 2% agarose gel with ethidium bromide staining using a horizontal electrophoresis unit in Wide Mini-Sub Cell (BioRad Laboratories, Hercules, CA, USA) and the gel documentation system GelDoc XR Quantity One software 1-D Analysis Software (BioRad Laboratories).

Statistical analysis. The obtained results were analyzed using Prism 5.0 statistical software (GraphPad, La Jolla, CA, USA). The Mann-Whitney U-test was utilized to compare the expression of studied markers between the tested groups. Correlations between the examined IHC markers were determined with the Spearman correlation test, whereas Pearson correlation test was used to analyze their expression with patient age. Differences were considered as significant when $p<0.05$.

Results

The presence of both HPV6/11 (LR) and oncogenic HPV16 (HR) in the studied papillomas of upper respiratory tract was confirmed. Using the NMPCR technique, HPV was found in 10/56 cases (17.85%). HPV6/11 was found in 6/56 cases (10.71%), and HPV16 in 4/56 (7.14%) cases. HPV18 was not detected in any of the studied cases (Figure 1).

The expression of the cell cycle-related proteins was localized in the nuclei of epithelial cells (Figure 2, Table II). Statistical analysis revealed that expression of all analyzed proteins was significantly stronger in HPV-infected cases (p16 at $p=0.0012$, p27 at $p=0.005$, p53 at $p=0.0182$ and Ki-67 antigen at $p=0.0021$) (Figure 3, Mann–Whitney test). Additionally, the level of expression of these proteins was compared with regard to LR (HPV6/11) or HR (HPV16) infected cases. The mean expressions of these proteins were higher in the group with LR HPV infections (Table III). Spearman correlation test revealed that the studied markers correlated positively with each other. The correlations between p53 protein and p16, p27 and Ki-67 were moderate (p16: r=0.37, $p=0.004$; p27: r=0.42, $p=0.001$; Ki-67: r=0.48, $p<0.001$). Moreover, Ki-67 correlated weakly with p16 (r=0.27, $p=0.0045$) and moderately with p27 (r=0.42, $p=0.002$). The correlations among the studied proteins are summarized in Table IV.

Furthermore, no statistically significant correlations between expression levels of the studied proteins and patient age were found.

Discussion

In our study, we extracted DNA from 56 FFPE papilloma samples to analyze by PCR the presence of HPV. The incidence of HPV was confirmed in 10/56 cases (17.85%). According to literature this is one of larger group of patients tested for the presence of HPV6/11, HPV16 and HPV18 in papillomas of upper respiratory tract. Majority of available publications

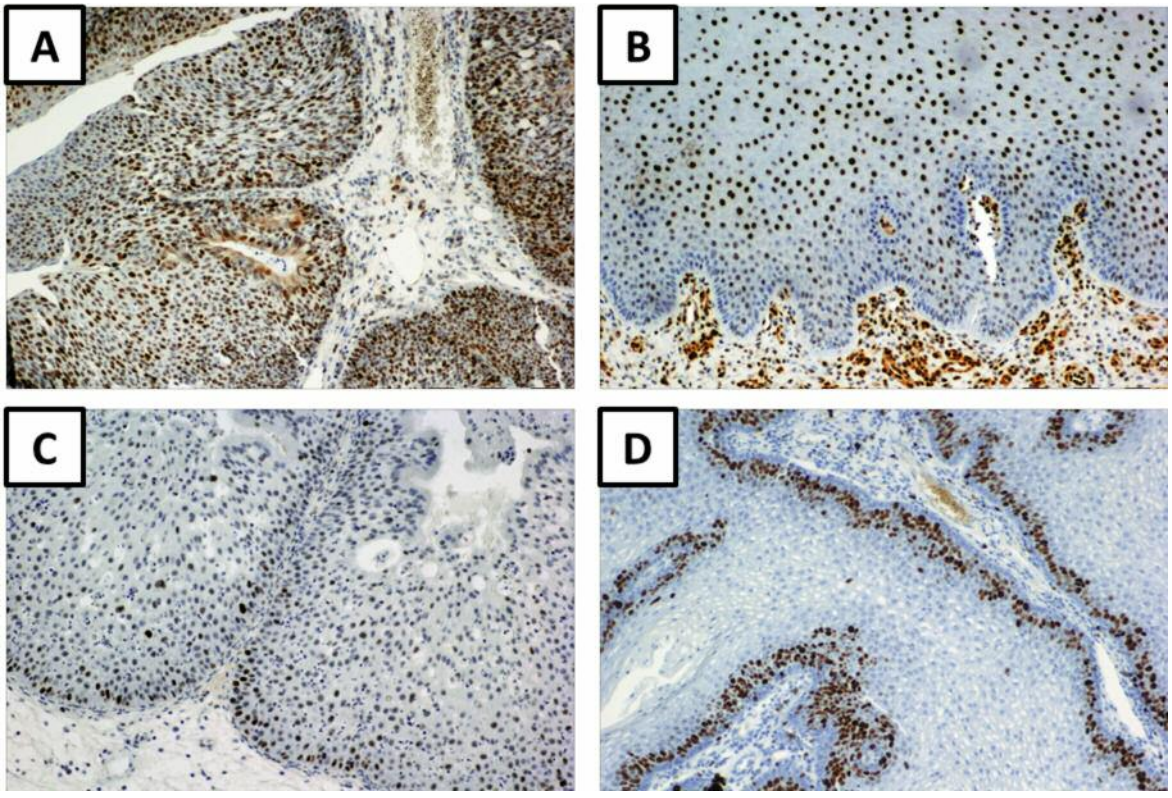

Figure 2. *Nuclear expression of p16 (A), p27 (B), p53 (C) and Ki-67 antigen (D) in human papilloma virus-positive samples. Magnification ×200.*

Table III. *Expression level of cell cycle-related proteins in regard to type low-risk (HPV6/11) and high-risk (HPV16) human papilloma virus infection.*

Marker	HPV6/11 (mean±SD)	HPV16 (mean±SD)	*p*-Value (Mann-Whitney *U*-test)
p16	3.83±0.41	3.25±0.50	**0.0031**
p53	2.83±0.41	1.75±0.50	**0.0153**
p27	3.83±0.40	3.25±1.50	0.0969
Ki-67	3.33±0.52	2.75±0.50	**0.0450**

Significant p-values are given bold. SD, standard deviation.

Table IV. *Spearman's rank correlation between expression levels of cell cycle-related proteins in pharyngeal and laryngeal papillomas.*

	Ki-67	p27	p53
p16	r=0.27, ***p*=0.045**	r=0.19, *p*=0.169	r=0.37, ***p*=0.004**
p53	r=0.48, ***p*<0.001**	r=0.42, ***p*=0.001**	
p27	r=0.42, ***p*=0.002**		

Significant p-values are given bold.

examine the presence of HPV in the specimens derived from HNSCC and less frequently from papillomas (7, 9, 26-34).

Kolmos *et al.* confirmed the presence of HPV6/11 in 139/152 (91.4%) of examined laryngeal papillomas (9). Shen *et al.* analyzed 62 benign and 76 malignant tumors of the upper respiratory tract, detecting HPV presence in 56% of cases (33). In the study of Fusconi *et al.*, HPV infection was confirmed in 24/118 (20.33%) cases of upper respiratory tract papilloma (28). Sun *et al.* detected HPV6/11 in 21/63 (33.3%) and HPV16/18 in 4/63 (6.3%) of laryngeal papillomas (29). The study by Gùltekin *et al.* concerning 46 papillomas (20 oral, 13 tonsillar and 13 laryngeal) detected HPV6/11 in 8/13 (61.5%) laryngeal papillomas, 3/20 (15%) HPV6 in oral and 4/13 (30.8%) HPV16 in tonsillar papilloma (34). Weiss *et al.* reported positive results for HPV6/11 in 35/44 (79.5%) patients, but none of the samples were positive for HPV16 (7). Major *et al.* described the presence of HPV in all of 10 upper respiratory tract papillomas, while Stephen *et al.* found HPV in all of their 25 studied laryngeal papillomas (31, 32).

In our study, in six out of the 10 positive cases were identified as having HPV6/11 (10.71%), and four as having

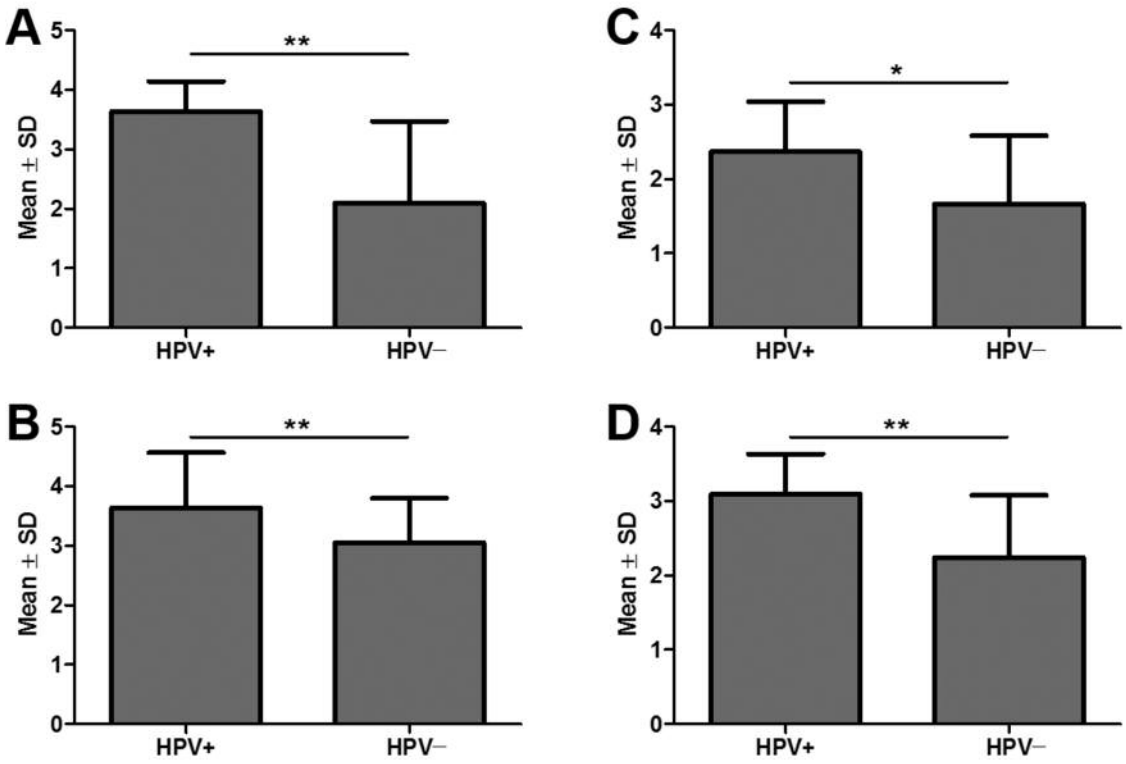

Figure 3. *Expression of p16 (A), p27 (B), p53 (C) and Ki-67 (D) in regard to presence of human papilloma virus (HPV) infection. Data are presented as mean±standard deviation (SD). *p<0.05, **p<0.01, Mann–Whitney U-test.*

HPV16 (7.14%). HPV18 was not confirmed in any case. Similarly, Fusconi *et al*. confirmed the presence of HPV6 in 10 cases, HPV11 in four, and HPV6/11 in 10 out of 118 patients. HPV18-positive cases were not observed (28). Dickens *et al*. investigated the same HPV types (6, 11, 16, 18) in laryngeal papillomas. Analogously with our results, they did not confirm the presence of HPV18 in any case (27). Lindeberg *et al*. also did not disclose the presence of HPV18 nor HPV16 in any of the examined samples (30).

Immunohistochemical expression of p16 protein was increased in all HPV-positive cases and there was a statistically significant correlation between the presence of HPV infection and overexpression of p16 protein. The obtained results suggest that overexpression of p16 protein could be a biomarker of HPV infection, often associated with HR types (35). p16 has been described to reflect the activity of HPV E7 oncoprotein, which leads to disruption of the pRb pathway. HPV E7 protein deactivates pRb, releasing E2F transcription factor, and as a consequence, these promote both progression in cell division and p16 overexpression (36). Mooren *et al*. pointed out that immunostaining of p16 in head and neck papilloma and laryngeal dysplasia is a reliable indicator of HR HPV- contrary to LR HPV-associated carcinomas (37). Moreover, Salazar *et al*. stated that only combined detection of p16 protein expression and the presence of HPV DNA better predicts survival in HNSCC, that may result in improved predictive value of p16 biomarker testing and stronger association with clinical outcome (38). On the other hand, Gültekin *et al*. demonstrated that p16 expression was negatively correlated with HPV prevalence in laryngeal papillomas (33). The authors suggest that the expression of p16 protein in the nuclei and cytoplasm of tumor cells may serve as a marker for the presence of HPV in tumor cells. The opposite opinion is presented by Venuti and Paolini, they found p16-positive SCCs without evidence of HPV infection (39).

Although p16 may not be a 100% specific marker of HPV infection, it can provide important prognostic information (34). Shah *et al*. describe IHC expression of p16 in the papillomas of the nose and sinuses. In their study, higher expression of p16 was confirmed in 14/16 of HPV-positive tumors (87.5%) and in 10/11 HPV-negative (90.9%) one without significant difference between analyzed groups (40). Mooren *et al*. also investigated the expression of p16 in 20 tonsillar and 27 laryngeal papillomas. In their study, p16 expression in HPV6-and HPV11-positive laryngeal papillomas varied from negative to strongly positive (37). Whereas, Gültekin *et al*. noticed overexpression of p16 in 26 out of 46 (56.5%) papillomas (34). Existing evidence indicates that this marker could be useful in detections of benign HPV-infected lesions of mucosa in the upper

respiratory tract and the expression of this protein should be taken into consideration as additional (besides PCR) marker of HPV infection.

The relationship between the presence of HPV infection and the severity of the expression of p27 protein was statistically significantly correlated. The p27 protein is a CDK inhibitor that inhibits CDK2– and CDK4–cyclin complexes and affects cell-cycle arrest at the G_1 phase. HPV oncoproteins disrupt cell-cycle regulation by acting on CDK inhibitors associated with cell cycle checkpoints: G_1 and G_2. E7 protein of HR HPV can bind to p27 cyclin inhibitors and disrupt the inhibition of the cell cycle (41, 42). Moreover, Zhao *et al*. indicated that cytoplasmic p27 expression promotes epithelial–mesenchymal transition and tumor metastasis (43). Low p27 expression was associated with many neoplasms including cervical, oral and gastric. In HNSCC, p27 expression was correlated with metastasis, poor prognosis and worse response to treatment (44). Literature data describing the relationship between p27 expression and respiratory papillomas (before malignant transformation) is rather rare. There are, however analyses of the relationship between p27- and HPV-positive HNSCC. Hafkamp *et al*. showed no difference in the expression of p27 in HPV-positive and HPV-negative tissue derived from tonsillar squamous cell carcinomas (45). However, the current investigation indicates that the subcellular locations of p27 expression could determine its ability to inhibit malignant transformation of the cells. Cytoplasmic location of this protein was associated with poor prognosis in oropharyngeal squamous cell carcinoma (46, 47). According to our results, p27 expression in papillomas of the larynx was higher in the HPV-positive group. In addition, we observed nuclear localization of p27 proteins. This may suggest that p27 plays an important role in the prevention of the malignant transformation of papilloma cells which were infected by HPV. Nuclear localization could suggest that in papillomas of the larynx, p27 protein was not mutated and increased expression may be a result of E7 effect on this protein.

The p53 protein is involved in many important cellular functions, such as cell cycle regulation, DNA-repair activation and induction of apoptosis. In normal cells, the level of expression of the protein is low but it increases due to DNA damage or viral infection (11, 47). The protein has very short half-life and for this reason may be difficult to detect in normal tissues. However, in response to mutation, the level of the modified p53 protein expression increases and is easily detectable by IHC (48). Mutation of the *p53* gene is one of the most common genetic abnormalities found in malignant neoplasms. Mutated p53 protein is inactive and unable to provide its proper functions, such as repair of genetic defects and apoptotic elimination of cells with damaged DNA (49).

In HNSCC cells, the p53 protein is inactive, which allows further cancer development. Rady and Schnadig described the relationship between a mutation in the gene encoding p53 protein and the presence of HPV11 in malignant lesions (50). In our study, the difference between the two analyzed groups (HPV-positive and HPV-negative) was statistically significant and the expression of p53 protein was higher in HPV-positive samples. This may indicate increased expression of p53 in response to increased cellular stress associated with an active viral infection. In all cases, the expression of p53 was observed in nuclei. Manjarrez *et al*. obtained similar results. In their study, p53 expression was significantly higher in papillomas in comparison to malignant lesions (51).

The mechanism of inactivation of the p53 protein is different between HPV-positive and -negative tumors. In HPV-positive HNSCC, genetic changes reflect the impact of oncogenic virus, where the wild-type of p53 is partially inactivated by the E6 oncoprotein (52-55). Whereas in HPV-negative tumors, specific p53 mutations caused by tobacco smoke carcinogens have been detected (52-55). Furthermore, passing a cell-cycle checkpoint by influencing p53 protein and the Rb protein allows the survival of cells with chromosomal abnormalities. In some cases, HPV infection persists for a long time, favoring accumulation of changes in the cell, which lead to malignant transformation (56, 57).

We also investigated the expression of Ki-67, which is detectable in both actively proliferating normal and neoplastic cells. It is also frequently correlated with tumor growth and facilitates in differentiating benign from malignant tumors (50). We also found a statistically significant positive correlation between the occurrence of HPV infection and increased expression of Ki-67, which may indicate the beginning of malignant transformation of infected cells. In the literature, increased proliferative index has been found to be a substantial indicator of malignant transformation, however, its single analysis seems to give insufficient evidence of this process (34).

Despite the increasing number of publications indicating the presence of HPV in HNSCC lesions, the mechanisms that lead to development of cancer from benign (pre-neoplastic) lesions still require explanation. The present study was conducted on one of the largest analyzed groups of patients with papillomas of the upper respiratory tract in correlation to specific cell-cycle proteins. The obtained results confirmed the usefulness of molecular indicators such as proteins p16, p27 and p53 in the diagnosis of precancerous lesions of the upper respiratory tract. The infection of HPV in the papillomas of the upper respiratory tract influences the expression of these specific cell-cycle proteins, which could indicate its role in malignant transformation of cells.

Acknowledgements

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