High-risk HPV DNA status and p16(INK4a) expression as prognostic markers in patients with squamous cell cancer of oral cavity and oropharynx

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Aim of the study: To determine and compare the influence of the HPV DNA status and p16(INK4a) expression on the outcome of postoperative radiotherapy for squamous cell cancer of the oral cavity or oropharynx.
Material and methods: 59 patients with high-risk squamous cell cancer of the oral cavity or oropharynx were enrolled. They underwent major surgery and postoperative radiotherapy. The HPV DNA status and p16(INK4a) expression were assessed with QPCR and immunohistochemistry and correlated with loco-regional control and overall survival.
Results: 15.3% of tissue samples were HPV positive. All positive patients were identified with HPV16 subtype infection, and no other subtypes of high-risk HPV were detected. 5-year LRC in HPV(+) patients was 100%, compared to 50% in the HPV(–) group. 17.9% of all samples had evident p16(INK4a) expression. Among HPV(+) cases, 55.6% showed p16(INK4a) expression.
5-year LRC in patients with p16(INK4a) expression was 89%, compared to 51% in the p16(INK4a) negative group but this tendency was not significant ($p = 0.055$).
Conclusion: These data show that the HPV status is a good predictor of loco-regional control and overall survival in patients treated with radical surgery and adjuvant radiotherapy. The study shows a strong correlation between high-risk HPV infection and p16(INK4a) expression, but detection of viral DNA with QPCR has stronger prognostic potential.

Key words: HPV, p16, PCR, oropharynx cancer, HNSCC, postoperative radiotherapy.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most common cancers and significant cause of morbidity in Europe and the USA. In the majority of cases it is associated with known risk factors such as smoking, alcohol abuse or betel nut chewing [1, 2]. In about 20-25% of HNSCC a high risk human papilloma virus (HPV) is identified as an etiologic agent, that corresponds with cases arising from the oral cavity or oropharynx.

Epidemiological data from the United States show that incidence for HPV-related oral squamous cell carcinomas has been increasing significantly since 1973 with an annual percentage change APC = 0.8 particularly among white men and at younger ages. By contrast, an incidence for HPV-unrelated oral squamous cell carcinomas remains constant [3].

In Europe similar trends are observed. The age-standardized sex-averaged incidence of tonsillar cancer increased from 1.3 to 3.6 (2.8-fold) per 100 000 between 1970 and 2002 in the Stockholm area. Dur-

ing the same period, a 2.9-fold increase in the proportion of HPV-positive tonsillar cancer was observed. In the 1970s, 23% of tonsillar cancers were recognized as HPV-positive in comparison to 68% in 2000 [4].

HPV-positive tumours of the upper aerodigestive tract are characterised by distinct histopathological features including lack or weak keratinization as well as basaloid morphology, which was described in varying proportions between 34% and 100% [5, 6].

Immunohistochemical profile is characterized by a decreased expression of pRb, cyclin D and up-regulation of p16(INK4A) due to Rb gene down-regulation by viral E7 protein. Among the above mentioned markers p16(INK4A) overexpression has the strongest correlation with active HPV infection and it is considered to be a surrogate marker of the viral infection [7]. Unfortunately, p16(INK4A) overexpression is also reported in some HPV-negative cases diminishing specificity of the method to about 85% [8].

Many recent studies show that from a clinical point of view HPV-dependent HNSCC defines distinct disease with characteristic features. The presence of HPV has been found to correlate with a small tumour size, presence of local metastases and more advanced TNM stage. Nevertheless, patients with HPV-positive HNSCC have a significantly lower risk of dying (HR = 0.85) and lower risk of local recurrence (HR = 0.62) than HPV-negative ones. Other studies that evaluated response to therapy of patients with HNSCC show that presence of HPV16 was associated with a better response to chemotherapy and chemoradiotherapy [9, 10].

Since the role of the HPV status in prognosis and treatment of HNSCC seems to be critical there is an urgent need for a highly sensitive and specific diagnostic method for the detection of clinically significant HPV infection. In this study we propose using a quantitative real-time PCR method (HR-HPV QPCR) for the detection of high risk HPV DNA in routine histopathological samples. We also present the prognostic value of the QPCR as compared to the prognostic value of p16(INK4a) expression assessed by immunohistochemistry. It is as well one of the first studies reporting prevalence of HR HPV infection in Poland in patients with squamous cell cancer of the oral cavity and oropharynx.

Material and methods

Patients

Between 2001 and 2004, 59 patients after major surgical intervention for squamous cell cancer of the oral cavity or oropharynx, with high-risk pathologic features and no gross residual disease were enrolled. After the surgery they received 63 Gy adjuvant radiotherapy in fractions of 1.8 Gy given in 5-days-a-week conventional manner (CONV, 30 patients) or 7-days-a-week (CAIR 29 patients) [11]. For every patient routine formalin fixed and paraffin embedded tissue samples were available for immunohistochemical assessment of p16(INK4a) and for high risk HPV DNA quantitative PCR (QPCR) assay.

QPCR

Detection of HPV DNA in paraffin embedded tumour histopathological samples was made using a RealTime High Risk HPV test (Abbott Molecular). It is a qualitative test that can detect 14 high-risk types and differentiate between HPV16, HPV18 and non-HPV16/18 high-risk types in the same reaction. The HPV target sequence is located in the conserved L1 region of the viral genome. The reaction mix contained three forward and two reverse primers designed to hybridize to an approximately 150 base HPV consensus region. A pair of primers targeting 136 bases in the human β-globin gene, serving as an internal control was also included. Detection of amplification products was achieved with the aid of 15 distinct fluorescently labelled probes.

The laboratory procedure consisted of three steps: DNA isolation, reaction assembly and real-time PCR. DNA was isolated from archival paraffin embedded tumour samples. For each sample, ten 10 μm thick sections were collected in an aseptic manner. They were deparaffinized in two changes of xylene, rehydrated in graded alcohols and digested in Proteinase K 100 μg/ml (Sigma-Aldrich), for 24 hours at 56°C. Next, samples were processed using mSample Preperation SystemDNA (Abbott Molecular) where DNA was immobilized on the surface of magnetic microparticles, purified and eluted ready for amplification. The DNA purity and concentration was evaluated with Nanodrop ND-1000 spectrophotometer.

At the completion of sample preparation, an amplification master mix was prepared with AmpliTaq Gold, $MgCl_2$ solution and oligonucleotide reagent containing primers, probes and dNTPs. The PCR reaction was then assembled in a 96-well optical reaction plate by combining 25 μl of master mix and 400 ng of extracted DNA diluted to the final volume of 25 μl with molecular biology grade purified water (Water Mol Biol grade, DNAse, RNAse and Protease-free, 5Prime).

Thermocycling and product detection were carried out in the m2000rt real-time PCR instrument (Abbott Molecular system based on ABI RT7500 instrument, Applied Biosystems Inc.).

Negative and positive controls were included in each run to verify that sample processing, amplification and detection were performed correctly. The negative control was formulated with DNA containing β-glo-

bin sequence and poly-dA:dT as carrier DNA. The positive control contained HPV16, HPV18, HPV58 and β-globin sequences tied to carrier DNA.

Samples were considered as positive when Ct value for any of the detected HPV type and β-globin was less or equal to the cut-off value of 35. Samples for which β-globin Ct value was greater than 35 were considered as non-informative.

Immunohistochemistry

Immunohistochemical detection of the p16INK4a antigen was assessed using CINtec Histology Kit (Mtm Laboratories). The immunostaining procedure was performed according to the kit manufacturer protocol. In detail:

Five-µm sections of formalin-fixed and paraffin-embedded samples mounted on slides were dewaxed in two changes of xylene and rehydrated by a graded series of ethanol. For heat-induced epitope retrieval, slides were immersed for 10 min in the Epitope Retrieval Solution (Mtm Laboratories, TRIS buffer/EDTA 10 mmol/l, pH 9.0) in a calibrated water bath at temperature of 95-99°C. Endogenous peroxidase activity was inactivated by 3% solution of H_2O_2 for 5 min. Presence of p16(INK4a) protein was detected by incubating sections for 30 min in monoclonal, mouse, anti-Human p16(INK4a) antibody, clone E6H4 solution in 50 mmol/l Tris. Finally, a mixture of polymer conjugated with horseradish peroxidase and affinity purified goat anti-Mouse Fab antibody fragments and DAB chromogen was used to visualize the reaction.

After counterstaining in haematoxylin, the specimens were covered with AquaMount (Dako Cytomation) mounting medium and evaluated by a pathologist according to the manufacturer recommendations.

The slides were considered to be positive for p16(INK4a) if the specimen showed continuous staining of at least 10% of tumor cells. Slides showing staining of isolated cells or small cell clusters were classified as negative.

Statistical analysis

Loco-regional control and overall survival curves were estimated using Kaplan-Meier method. Comparison of LRC and OS was performed with the Log-rank test. Correlation between the HPV status and p16(INK4a) expression was calculated according to the Spearman rank order correlation method. All tests with p value equal to or less than 0.05 were considered as statistically significant.

Results

For all patients enrolled we got informative results in respect of HPV DNA detection as well as p16(INK4a) protein expression. Out of 59 samples, 9 (15.3%) were HPV positive, 50 (84.7%) were negative. All 9 positive patients had HPV16 subtype infection. Presence of HPV DNA of types 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 was excluded in all tested samples.

In most cases – 5 (55.6%), the primary tumour was located in the tonsil, other locations were: base of tongue – 2 (22.2%) and fundus of the oral cavity – 1 (11.1%).

Evident p16(INK4a) expression was shown in 10 (17.9%) of all 59 histological slides compared to 49 (83.1%) p16(INK4a) negative ones. Among 9 HPV positive cases, only 5 (55.6%) were positive for p16(INK4a) expression assessed by immunohistochemistry. There were also 5 (10%) p16(INK4a) positive slides in the group of HPV-negative cases. These findings are summarized in Table I.

5-year LRC in HPV-positive patients was 100%, compared to 50% in the HPV-negative group. This difference was highly significant ($p = 0.011$ Log-rank test). Kaplan-Meier estimation of LRC curves in HPV-positive and HPV-negative groups is shown in Figure 4.

5-year LRC in patients with p16(INK4a) expression was 89%, compared to 51% in p16(INK4a) negative group but this tendency was not significant ($p = 0.055$ Log-rank test).

LRC curves in groups defined on the p16(INK4a) expression status are shown in Figure 5.

Similarly, in groups regarding the HPV status, 5-year overall survival in HPV-positive patients was 76.2% in contrast to 39.4% in HPV-negative ones. In a group of samples positive for p16(INK4a) and p16(INK4a) negative, 5-year survival was 66.7% and 40.3%, respectively.

Kaplan-Meier estimates of overall survival in those groups of patients are shown in Figures 6 and 7.

The HPV status and p16(INK4a) expression showed moderate positive correlation. Computed Spear-

Table I. Prevalence of p16(INK4a) positive and p16(INK4a) negative cases in HPV positive and HPV negative groups

	HPV16 POSITIVE (9 CASES)		HPV16 NEGATIVE (50 CASES)	
	p16(+)	p16 (–)	p16 (+)	p16 (–)
Number of cases	5 (55.6%)	4 (44.4%)	45 (90%)	5 (10%)

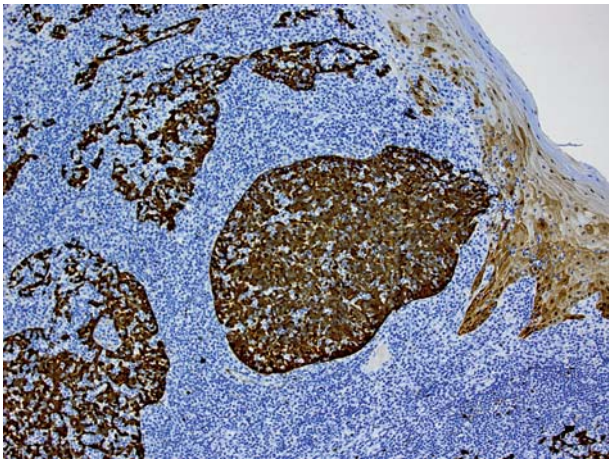

Fig. 1. Strong uniform expression of p16(INK4a) in SCC of the tonsil. Visible staining of squamous epithelium, IHC, primary magnification 10×

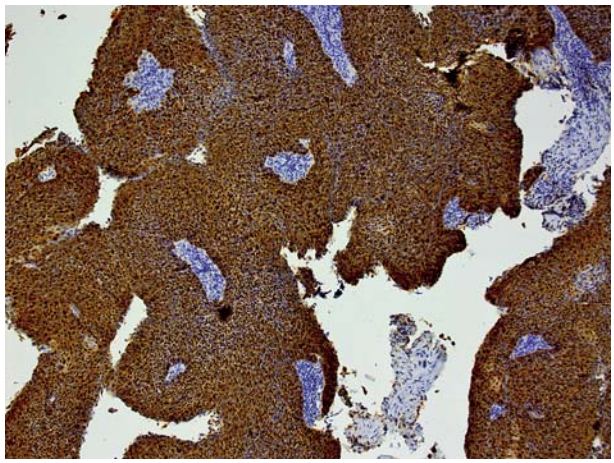

Fig. 2. Strong uniform expression of p16(INK4a) in SCC showing basaloid features, IHC, primary magnification 10×

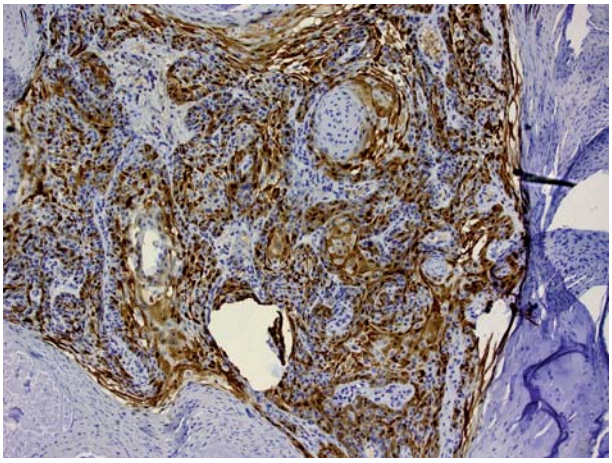

Fig. 3. Heterogeneous, moderate intensity expression of p16(INK4a) in SCC originating from the base of the tongue. IHC, primary magnification 20×

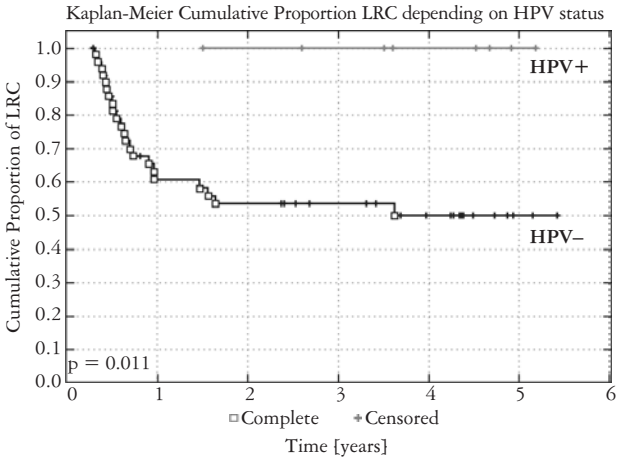

Fig. 4. Loco-regional control estimation in groups depending on HPV status

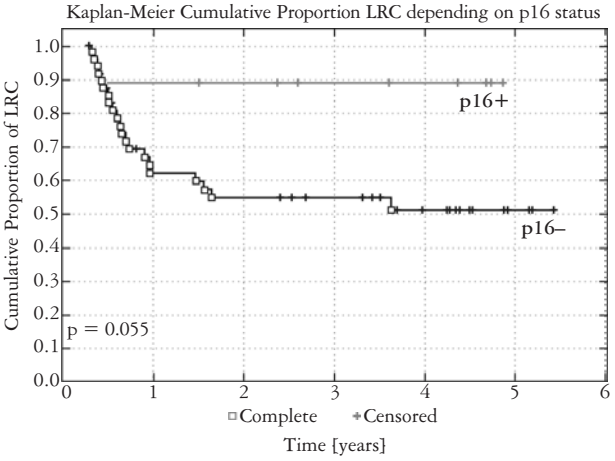

Fig. 5. Loco-regional control estimation in groups depending on p16(INK4a) expression

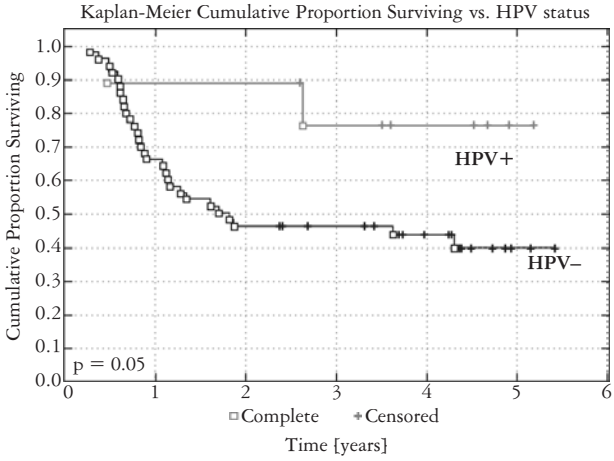

Fig. 6. Overall survival estimation in groups depending on the HPV status

man Rank Order Correlation Coefficient was 0.44 ($p < 0.05$).

Results summary

Our data show that the HPV status is a good predictor of loco-regional control and overall survival in patients treated with radical surgery and adjuvant radiotherapy. p16(INK4a) expression is correlated with the HPV status but it has lower power of predicting clinical outcome in this group of patients.

Discussion

Prevalence of HPV-dependent head and neck squamous cell cancer has been constantly increasing for several decades. Epidemiological data from the United States and Europe confirm that high-risk HPV especially of subtype 16 is detectable especially in oropharynx and the oral cavity [3]. Percentage of HPV-positive tonsillar cancer increased from 23% in the 1970s to 68% in 2002 according to Hamarstedt's study of the Swedish population [4]. Our data show a significantly lower (17.9% 3.8 fold) prevalence of HPV-positive tonsillar cancer in comparison to the mentioned data corresponding to the analogical period between 2002 and 2004. On the other hand, we are observing a dramatic increase in the incidence rate of HPV-positive cancer of the oral cavity and oropharynx between years 2004 and 2010 (data not shown). We have also noticed that the only virus type detected in our study was HPV16. This finding is in good accordance with other results. HPV16, the most common HPV type detected in biopsies from women with cervical SCC, is also the most wide-spread type detected in HNSCC biopsies. The second most common type, HPV18 detected in cervical cancers was never detected in our group. The extreme rarity of HPV18 in the oropharynx was already reported in the majority of published studies [12]. Other oncogenic HPV types (e.g. HPV31, 33, 35 and 58), commonly detected in invasive cervical cancer, are rarely detected in HNSCC samples [13].

A lot of evidence supports the thesis that HPV-dependent HNSCC is a distinct entity with a mechanism of carcinogenesis other than that in a group of tobacco and alcohol driven tumours. This fact is reflected in distinct clinical features like an increased radio and chemosensitivity and favourable outcome despite adverse histopathological features [14, 15]. El-Mofty *et al.* suggested that a low differentiated basaloid subtype of HNSCC could be surrogate of HPV infection [5]. This was not confirmed in other studies which indeed showed a strong positive correlation between HPV positivity and basaloid appearance but it was proven that a group of basaloid tumours can be further divided into two clinically

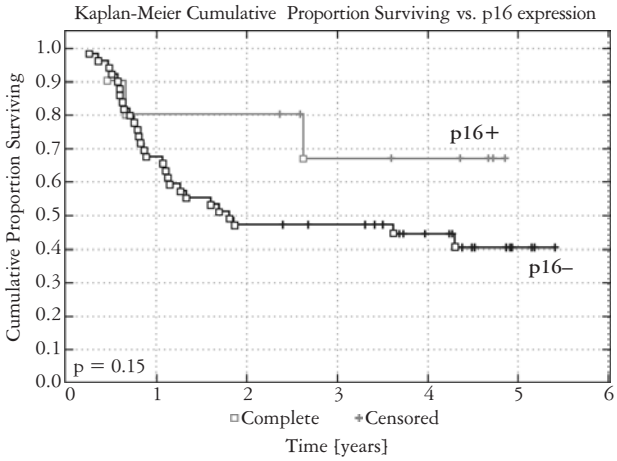

Fig. 7. Overall survival estimation in groups depending on p16(INK4a) expression

distinct subgroups defined based on the HPV status [16].

Multivariate analysis showed that HPV is the most significant prognostic factor in the studied groups of patients with oropharyngeal tumours (HR = 0.27, 95% CI: 0.12-0.61) along with other well-known histopathological features like regional lymph node involvement, extracapsular spread and tumour size [17]. Our study supports these findings. We showed that 5-year loco-regional control as well as 5-year overall survival were common in a group of HPV-positive patients treated with radical surgery and postoperative radiotherapy. In multivariate analysis including other molecular features such as EGFR, PTEN, nm23, p53 and Ki-67 expression, the HPV status remained the strongest prognostic marker of LRC in HNSCC (RR = 4.8 p = 0.05) [18, 19].

Because of some technical limitation of widely used PCR methods such as very high sensitivity, a HPV DNA-positive assay may not always reflect biologically meaningful viral involvement [20]. For this reason many alternative techniques were proposed including RT-PCR and FISH. Both of them have their limitations. First one is characterized by excellent sensitivity and specificity but requires fresh or frozen tissue samples, second has perfect specificity but compromised sensitivity of about 85% [21]. Therefore, surrogate markers of the HPV infection were proposed, among them p16(INK4a) as the most promising one.

The p16(INK4a) protein encoded by tumour suppressor gene CDKN2A(INK4A), located on human chromosome 9p21, inhibits binding of cyclin D1 to the cyclin-dependent kinases (CDK4 or CDK6), thus preventing the phosphorylation of the Rb protein [22, 23]. The underphosphorylated Rb inhibits transcriptional activation of the S phase genes, which are necessary for cell proliferation. The observation that cell lines with a defect in pRb overexpress

p16(INK4a) led to further studies demonstrating that pRb acts as a negative regulator of p16(INK4a) expression at the transcriptional level. The causal role of human papilloma viruses in squamous cell carcinogenesis depends on the activity of the viral oncoproteins E6 and E7, leading to inactivation of the cellular tumour suppressor p53 and the retinoblastoma gene product pRb. Because of the negative feedback mechanisms, the pRb inactivation causes an increase of p16(INK4a) [24]. This fact originated the thesis that p16(INK4a) expression could be a surrogate marker of HPV infection. The concept was proven by many researchers in cases of cervical cancer as well as in HNSCC [21, 25, 26]. Estimated analytical sensitivity of the method is 85-100% and specificity in the range of 74-81%. In our study analytical sensitivity and specificity with respect to the HPV DNA status was 69% and 91%, respectively, which is close to ranges showed in the mentioned studies. Fischer and Lassen suggested that p16 expression could affect prognosis and predict response to radio- and chemotherapy in patients with HNSCC [27, 28]. This was not evident in our data. We showed a tendency towards longer locoregional control in patients demonstrating expression of p16(INK4a). In contrast, quantitative PCR was a far better standalone predictor of LRC and overall survival in this group of patients.

In conclusion, we can state that High Risk HPV QPCR is a reliable standalone method of predicting outcome and response to radiotherapy in patients with squamous cell cancer of the oral cavity and oropharynx.

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