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Comparison of PI3K Pathway in HPV-Associated Oropharyngeal Cancer With and Without Tobacco Exposure

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Objectives: The aim of the study was to evaluate whether HPV associated OPSCC with tobacco exposure follows a different carcinogenic pathway compared to HPV associated OPSCC without tobacco exposure and to investigate its prognostic significance. The question was addressed with focus on components of the PI3K pathway.

Methods: 184 patients with newly diagnosed OPSCC treated with curative intent were consecutively enrolled. The expression level of p16, p53, PI3K, mTOR, and PTEN was assessed by immunohistochemistry and analyzed in relation to the risk factors HPV status and tobacco exposure.

Results: 94 of 184 (51%) patients were p16 positive, p53 overexpression was detected in 48 of 184 (26%) cases. PI3K overexpression with 70 of 184 (38%) cases was significantly higher in p16 positive tumors. mTOR overexpression was present in 90 of 184 (49%) cases and significantly higher in p16 negative tumors. PTEN loss was found in 42 of 184 (23%) cases without association to p16 expression. p16 positive OPSCC showed lower rates of p53 expression and mTOR expression as well as higher rates of PI3K expression irrespective of tobacco exposure. Survival analysis showed a distinct intermediate survival rate of p16 positive smokers. The markers PI3K, mTOR, and PTEN did not have a significant impact on survival.

Conclusion: HPV associated OPSCC with tobacco exposure follows the same expression level of the PI3K pathway as HPV associated OPSCC without tobacco exposure. The impaired survival rate of the intermediate risk group cannot be explained by different expression patterns of PI3K, mTOR, and PTEN.

Key Words: Oropharyngeal cancer, HPV, PI3K, tobacco.

Level of Evidence: 2b

INTRODUCTION

During the last decades infection with high risk type human papilloma virus (HR-HPV) has emerged as an important cause for the development of oropharyngeal squamous cell carcinoma (OPSCC).[1,2] HPV-associated tumors are known to have improved treatment response and better outcomes.[3–8] Possible explanations include both host-intrinsic factors such as superior performance status, lower rate of comorbidities, and lower incidence of second primary tumors, as well as tumor-intrinsic factors such as distinct genetic pathogenesis and enhanced radiation sensitivity.[9]

Although the National Comprehensive Cancer Network (NCCN) recommends routine HPV testing in OPSCC, suggestions for modified treatment depending on the HPV status are currently lacking, but several prospective trials underway.[10]

As HPV-related carcinogenesis differs substantially from traditional smoking- and alcohol-induced pathways, it is crucial to understand the carcinogenic mechanisms of the tumors in order to potentially establish individualized therapeutic modalities. While HPV-negative, tobacco- and alcohol-related OPSCC are often characterized by genetic alterations of the TP53 tumor suppressor gene,[1] HPV-associated OPSCC follow a different carcinogenic pathway. HPV-positive tumors usually harbor the p53 wild-type gene,[11,12] and are characterized by inactivation rather than mutation of p53 and pRb (retinoblastoma protein) by viral oncoproteins E6 and E7.[13] According to a study by Ang et al.[5] and other publications,[14,15] HPV-associated OPSCC with exposure to tobacco show a risk for tumor progression and disease-related death which is intermediate between the low-risk group of HPV-associated OPSCC in non-smokers and the high-risk group of HPV-negative OPSCC. The question arises whether this intermediate risk cohort tumor type is characterized by a carcinogenic pathway different from the one causative for HPV-associated OPSCC in non-smokers.

One of the most frequently altered carcinogenic pathways in cancer[16,17] is the PI3K (Phosphoinositid 3

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Kinase) pathway which regulates physiologic cellular processes including cell proliferation, differentiation, motility, metabolism, and apoptosis.[18,19] Its central components PI3K, a key signaling activator, and mTOR (mammalian target of rapamycin), a key downstream regulator, drive tumor metastasis by promoting cell motility.[17] In contrast, PTEN (phosphate and tensin homolog), a tumor suppressor gene, acts as a negative regulator of this pathway.[10,16] In head and neck squamous cell cancer (HNSCC) this pathway is mutated in 10% to 30%.[1,18,20–22] Due to its frequent activation, investigations about agents targeting this pathway are of highest interest.[23] Of note, HNSCC with PI3K pathway mutation seem to harbor increased genomic instability which eventually effects the susceptibility of a targeted therapy.[22]

Several mutational and immunohistochemical studies were able to demonstrate that HPV-associated tumors showed more frequent mutations and alterations of the PI3K pathway compared to HPV-negative tumors.[21,24–26]

Therefore, the aim of our study was: (1) to analyze the immunohistochemical expression patterns of p53 and central components of the PI3K pathway (PI3K, mTOR, PTEN) in the low-risk group of HPV-associated OPSCC in non-smokers compared to the intermediate-risk cohort of HPV-positive smokers and the high-risk group of HPV-negative OPSCC; and (2) to assess the impact on survival of these different markers.

MATERIALS AND METHODS

Patients

The total number of 184 consecutive patients treated in curative intent for a newly diagnosed OPSCC at the University Hospital of Zurich were evaluated. Tumor tissue from the primary tumor was collected from all patients at the time of diagnosis. The treatment modalities included primary radio(chemo)therapy, primary surgery, or a combined approach consisting of surgery followed by adjuvant radio(chemo)therapy. Smoking was defined as current smoking or a history of more than 10 pack-years not longer than 10 years ago. Alcohol consumption was defined as the intake of $\geq$3 units of alcohol per day.

Tumors were classified according to the 7th edition (2010) of the Union Internationale Contre le Cancer (UICC) TNM classification of malignant tumors.[27]

Tissue Microarray and Immunohistochemical Staining

Formalin-fixed and paraffin-embedded tissue of a pretreatment biopsy or a resection specimen was available for all patients. A tissue microarray (TMA) block was constructed in order to ensure uniform staining of antibodies. For each patient, two regions from the core of the tumor were selected for TMA construction. The preparation of the TMA slides as well as antibody staining for p16 as a surrogate marker for HPV-positive tumors and p53 were performed as previously described.[28]

For the expression staining analysis of PI3K, mTOR, and PTEN, following antibodies were used: mouse monoclonal anti-human PTEN (Dako A/S) diluted 1:100, rabbit monoclonal PI3K Kinase p110alpha (Cell Signaling Technology) diluted 1:200, rabbit monoclonal anti phospho-mTOR (Cell Signaling Technology) diluted 1:50. The staining intensity was scored from 0 to 2 (0 = no staining, 1 = intermediate staining, 2 = strong staining) for each of the three markers. The sum of the staining score of the two specimens was used to define the total staining intensity. A PTEN loss was defined as a total staining intensity of 0. Overexpression of mTOR and PI3K was defined as a sum of the two scores $\geq$2.

TABLE I.
Correlation of Antibodies and p16 Status

	p16-positive (n = 94)	p16-negative (n = 90)	*P*-value
PI3K overexpression	44 (63%)	26 (29%)	.02*
mTOR overexpression	36 (40%)	54 (60%)	.005*
PTEN loss	22 (23%)	20 (22%)	.86
P53 positive	8 (8%)	40 (44%)	<.001

Statistical Analysis

A descriptive analysis was performed by cross tables and Fisher's exact test to calculate significant differences and correlations between immunohistochemical and clinical parameters. Categorial variables were compared using a Chi-square test.

Survival analyses for overall survival (OS), and disease-specific survival (DSS) were performed by Kaplan Meier curves. To compare different risk groups, a log rank test was used. Univariate and multivariate analysis using cox proportional hazard models to identify the impact of different risk factors on survival were outlined. A *P*-value of $\leq$ .05 was considered statistically significant. All statistical analyses were calculated with SPSS (Version 22).

RESULTS

Expression Status in Entire Cohort

A total of 184 consecutive patients with OPSCC were included with a male to female ratio of 3.6:1 (78% vs. 22%). The median age was 60 years and ranged from 42 to 91 years. 94 of 184 (51%) of the patients had a p16-positive tumor. P53 overexpression was detected in 48 of 184 (26%) cases. PI3K overexpression was detected in 70 of 184 (38%), mTOR overexpression in 90 of 184 (49%), and PTEN loss in 42 of 184 (23%) cases.

Expression Status in p16-Related Groups

P53 overexpression was significantly more often present in p16-negative compared to p16-positive tumors. PI3K overexpression was significantly higher in p16 positive tumors whereas mTOR overexpression was more frequent in p16 negative tumors. PTEN loss was not significantly different in the two groups (Table I).

Expression Status in p16 and Smoking-Related Subgroups

Expression of p53, PI3K, mTOR, and loss of PTEN were analyzed in three subgroups according to p16 expression and smoking status. Subgroups were defined as A = p16-positive non-smokers, B = p16-positive smokers, and C = p16 negative. The characteristics of the subgroups and the results are given in Table II.

TABLE II.
Subgroup Analysis in Different Risk Groups According to p16 Expression and Tobacco Exposure

		p16-pos-nonsmoking (A) n = 44	p16-pos-smoking (B) n = 50	p16-neg (C) n = 90	*P*-value			
					A vs. B vs. C	A vs. B	B vs. C	A vs. C
PI3K overexpression		22 (50%)	22 (44%)	26 (29%)	.05*	.62	.08	.02*
No PI3K overexpression		22 (50%)	27 (54%)	61 (68%)				
mTOR overexpression		17 (39%)	19 (38%)	54 (60%)	.01*	.93	.02*	.02*
No mTOR overexpression.		26 (59%)	28 (56%)	33 (37%)				
PTEN loss		11 (25%)	11 (22%)	20 (22%)	.82	.59	.99	.56
No PTEN loss		33 (75%)	39 (78%)	70 (78%)				
Mean age (range 42-91)		62.4	61.5	60.3	.47	.45	.46	.46
Gender	**M**	36 (82%)	41 (82%)	67 (74%)	.47	.98	.31	.34
	F	8 (18%)	9 (18%)	23 (26%)				
T-category	**T1/T2**	32 (73%)	32 (64%)	44 (49%)	.02*	.37	.08	.01*
	T3/T4	12 (27%)	18 (36%)	46 (51%)				
N-category	**N0/N1/N2a**	15 (34%)	18 (36%)	36 (40%)	.77	.85	.64	.51
	N2b/N2c/N3	29 (66%)	32 (64%)	54 (60%)				
Site	**Tonsil**	34 (77%)	36 (72%)	56 (62%)	.18	.56	.22	.17
	Base of tongue	10 (13%)	14 (28%)	27 (30%)				
	Other	0 (0%)	0 (0%)	4 (4%)				
Alcohol	**>3U**	6 (14%)	21 (42%)	46 (51%)	<.01*	<.01*	.3	<.01*
	<3U	38 (86%)	29 (58%)	44 (49%)				
Second cancer	**Yes**	6 (4%)	8 (16%)	21 (23%)	.33	.75	.31	.19
	No	38 (86%)	42 (84%)	69 (77%)				
p53	**Positive**	4 (9%)	4 (8%)	40 (44%)	<.01*	.85	<.01*	<.01*
	Negative	40 (91%)	46 (92%)	50 (56%)				
Therapy	**Surgery**	7 (16%)	3 (6%)	12 (13%)	.01*	.23	.02*	.01*
	Radiotherapy	20 (45%)	29 (58%)	63 (70%)				
	Surgery+RT	17 (39%)	18 (36%)	15 (17%)				

U = unit; RT = radiotherapy.

P53 overexpression was significantly more often present in the p16-negative subgroup C compared to both p16-positive subgroups A and B. The presence of the risk factor smoking did not have an impact on p53 overexpression in the p16-positive subgroup.

PI3K was comparably overexpressed in both p16-positive subgroups A and B irrespective of the risk factor smoking. In contrast, the expression rate was significantly lower in the p16-negative subgroup C. An overexpression of mTOR was significantly more often detected in the p16-negative subgroup C compared to the p16-positive subgroups A and B. No significant difference was seen between the two p16-positive subgroups A and B. PTEN loss was a rare event and occurred at a comparably low frequency in all three subgroups.

Survival Analysis in Entire Cohort

The median observation time was 61 months (range 6 to 144 months). A total of 68 of 184 (37%) patients died, 45 of 68 (66%) died of disease and 23 of 68 (35%) of other causes. The 5-year overall survival (OS) and disease specific survival (DSS) rates in the entire cohort were 69% and 82%, respectively.

In the Kaplan-Meier analysis, p16 overexpression had a favorable impact on OS (78% vs. 59% $P = .001$) and DSS (84% vs. 66% $P = .001$). In contrast, p53 was a poor prognostic marker for OS (10% vs. 77% $P = <.001$) and DSS (26% vs. 83% $P = .001$). mTOR overexpression was a negative predictor for OS (58% vs. 77%, $P = .02$) but not for DSS (68% vs. 83%, $P = .07$). PI3K overexpression did not have prognostic impact on OS (70% vs. 67%, $P = .7$) or DSS (81% vs. 74%, $P = .16$). PTEN loss did not influence OS (49% vs. 73%, $P = .17$) and DSS (77% vs. 81%, $P = .48$). Other factors with prognostic impact were T- and N-category as well as treatment modality whereas gender, age, smoking, and alcohol intake did not affect the survival rates (data not shown).

In univariate analysis based on cox regression model, p16 overexpression showed a favorable impact on OS (OS HR 0.42 95% CI 0.25–0.69, $P = .001$) and DSS (DSS HR 0.37 95% CI 0.19–0.70, $P = .001$). In contrast, p53 overexpression was a negative predictor for OS (OS HR 2.33 95% CI 1.43–3.77, $P = .001$) and DSS (DSS HR 2.63 95% CI 1.46–4.75, $P = .002$). mTOR overexpression revealed a negative impact on OS (OS HR 1.81 95% CI 1.10–2.95, $P = .01$), but not on DSS (DSS HR 1.72 95% CI 0.95–3.13, $P = .07$). PI3K overexpression and PTEN loss did not play a prognostic role for OS and DSS (PI3K OS HR 0.91 95% CI 0.55–1.49, $P = .7$; DSS HR 0.64 95% CI 0.34–1.19, $P = .15$) (PTEN loss OS HR 0.65 95% CI 0.35–1.21, $P = .15$; DSS HR 0.78 95% CI 0.37–1.59,

TABLE III.
Univariate and Multivariate Analysis of Overall Survival for Patients with OPSCC. (Therapy modality was divided in 3 groups as in Table II. T- and N- category are defined as in Table II.)

	Univariate analysis		Multivariate analysis	
Parameters	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
p16 positivity	0.42 (0.25–0.69)	.001*	0.59 (0.32–1.10)	.09
p53 positivity	2.33 (1.43–3.77)	.001*	1.83 (1.04–3.22)	.03*
PI3K overexpression	0.91 (0.55–1.49)	.7	0.87 (0.50–1.48)	.61
mTOR overexpression	1.81 (1.10–2.95)	.01*	1.13 (0.62–2.04)	.67
PTEN loss	0.65 (0.35–1.21)	.15	0.68 (0.34–1.37)	.28
Smoking	1.59 (0.92–2.76)	.96	1.07 (0.56–2.05)	.83
Alcohol	1.27 (0.78–2.05)	.32	0.98 (0.56–1.71)	.94
T-category	1.31 (1.05–1.63)	.01*	1.25 (0.96–1.63)	.09
N-category	1.16 (0.97–1.38)	.09	1.18 (0.99–1.41)	.06
Therapy modality	0.67 (0.45–1.00)	.05*	-	-
Gender (male)	1.30 (0.73–2.30)	.36	-	-
Age (>60y)	1.02 (1.00–1.05)	.02*	-	-

$P = .47$). T- and N-category were prognostic parameters for DSS, whereas therapy modality could reach significance only for OS ($P = .05$) and not for DSS ($P = .06$). Further parameters such as smoking, alcohol intake, gender, and age did not influence survival (Tables III and IV).

In multivariate cox regression model, only p53 overexpression kept its negative prognostic significance whereas the other factors such as p16 overexpression ($P = .06$) and mTOR overexpression ($P = .12$) lost its prognostic impact. After adding T-, N-category, smoking, alcohol, PTEN loss, and PI3K in the multivariate cox regression analysis, these parameters also did not have prognostic influence (Tables III and IV).

Survival Analysis in Risk-Related Subgroups

The 5-year OS for p16-positive non-smokers (Group A), p16-positive smokers (Group B), and p-16 negative patients (Group C) were 83%, 72%, and 58%, respectively ($P = .001$) (Fig.1). There was no significant difference between Groups A and B ($P = .19$) but between Group B and C ($P = .03$) and Group A and C ($P = .001$). Similar results were observed for 5-year DSS (Group A 88%, Group B 81%, and Group C 66%, $P = .006$) (Fig 2) with a significant difference between Groups B and C ($P = .02$) and Groups A and C ($P = .008$) but not Groups A and B ($P = .66$).

In Kaplan Meier analysis, there were no important prognosticators of the tested markers p53, PI3K, mTOR, or PTEN loss in subgroups A to C. Same results could be demonstrated in univariate analysis.

Further parameters such as gender, T-status and therapy modality did also not show a prognostic impact on OS and DSS in the subgroups A, B, and C.

DISCUSSION

HR-HPV related OPSCC go along with a particular tumor biology and risk profile when compared to HPV-

TABLE IV.
Univariate and Multivariate Analysis of Disease-Specific Survival for Patients with OPSCC. (Therapy modality was divided in 3 groups as in Table II. T- and N- category are defined as in Table II.)

	Univariate analysis		Multivariate analysis	
Parameters	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
p16 positivity	0.42 (0.25–0.69)	.001*	0.53 (0.25–1.14)	.10
p53 positivity	2.33 (1.43–3.77)	.001*	1.88 (0.94–3.75)	.07
PI3K overexpression	0.91 (0.55–1.49)	.7	0.67 (0.37–1.33)	.26
mTOR overexpression	1.81 (1.10–2.95)	.01*	0.85 (0.43–1.70)	.66
PTEN loss	0.65 (0.35–1.21)	.15	0.80 (0.37–1.76)	.59
Smoking	1.59 (0.92–2.76)	.96	0.92 (0.42–2.00)	.84
Alcohol	1.27 (0.78–2.05)	.32	1.49 (0.77–2.87)	.23
T-category	1.31 (1.05–1.63)	.01*	1.51 (1.09–2.10)	.01*
N-category	1.16 (0.97–1.38)	.09	1.42 (1.13–1.78)	.002*
Therapy modality	0.67 (0.45–1.00)	.05*	-	-
Gender (male)	1.30 (0.73–2.30)	.36	-	-
Age (>60y)	1.02 (1.00–1.05)	.02*	-	-

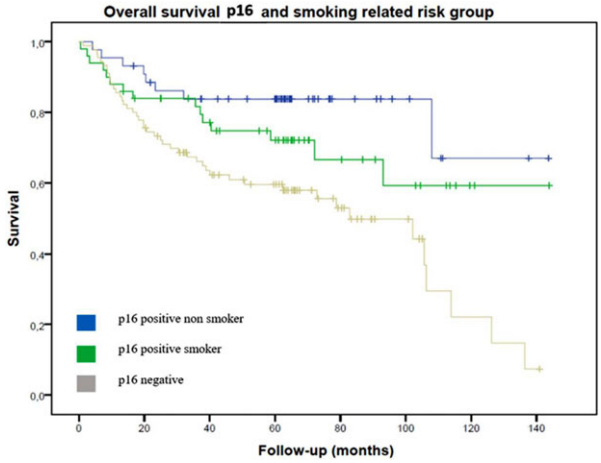

Fig. 1. Overall survival of p16 positivity and smoking-related risk groups

negative, smoking-, and alcohol-induced OPSCC.[29–31] HPV-positivity is an independent and strong risk factor associated with improved survival of OPSCC patients.[32]

The oncogenic proteins E6 and E7 are consistently expressed in HPV-associated cancer targeting the retinoblastoma (RB1) and TP53 tumor suppressor networks,[33–37] while smoking- and alcohol-induced, HPV-negative tumors harbor genetic alterations of the TP53 gene.[38] Beside the low-risk group of HPV-associated OPSCC in non-smokers and the high-risk group of HPV-negative OPSCC there exists a group of HPV-associated OPSCC with exposure to tobacco and alcohol. According to recent studies this group shows an intermediate risk profile[5,15,39] in relation to survival outcomes. The exact carcinogenic pathway of this intermediate-risk tumor type has not been investigated so far and the question arises if the intermediate risk OPSCC rather follows the pathway of HPV-associated or that of HPV-negative OPSCC.

The PI3K pathway is a major regulator in tumorigenesis of head and neck squamous cell cancer (HNSCC) with mutations in 10% to 30%.[1,18,20–22] This pathway is

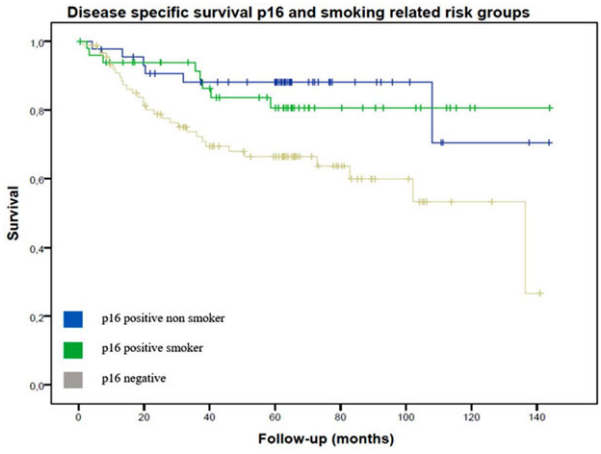

Fig. 2. Disease specific survival of p16 positivity and smoking-related risk groups

frequently altered in OPSCC, in particular in HPV-associated tumors.[21,40,41] The exact molecular interaction between an activated PI3K pathway and the HPV-associated carcinogenesis is poorly understood.

Beside mutational analyses of genetic alterations of the PI3K pathway in OPSCC a high correlation of immunohistochemical expression of signaling molecules of the PI3K pathway with genetic alterations has been demonstrated in recent studies.[42,43] Because of its cost and time efficacy and proven strong correlation to DNA mutation analysis also in other cancer types,[44–46] this method was used in our study.

Therefore, the aim of our study was to assess the immunohistochemical expression pattern of p53 as surrogate marker for a tobacco-induced cancer pathway and p16 as surrogate marker for a HPV-induced cancer pathway as well as the central components of the PI3K signalling pathway PI3K, mTOR, and PTEN to compare the pattern between the low-, intermediate-, and high-risk groups of OPSCC. In addition, the prognostic impact of these markers on survival has been addressed.

Overall, the p16-positive group revealed significantly higher PI3K overexpression and a significantly lower mTOR overexpression compared to the p16-negative group. In studies by Won et al. and Chun et al. no correlation of PI3K and mTOR expression with HPV status, but a more frequent PTEN expression in HPV-associated tumors was found.[42,43] However, compared to our study, the number of patients included in both studies was smaller, possibly explaining the contradictory results. As PTEN acts as a negative regulator in the PI3K pathway, loss of PTEN rather than overexpression seems to have a clinical significance. Therefore, in our study PTEN loss was assessed with no difference between the groups. Mutational studies have demonstrated more prevalent alterations and mutations of downstream signalling genes of the PI3K pathway in HPV-associated OPSCC[41,47,48] supporting our results. The expression pattern of PI3K, mTOR, and PTEN was quite heterogenous. Our data underline the fact that downstream molecules are not simultaneously upregulated. In the report by Won et al., expression status of each marker (PI3K, mTOR, AKT, PTEN) did not show a homogenous pattern either.[43] The same results have been already described in lung and gastric cancer.[45,49] It should be taken into account that our study assessed expression and not activity of the markers, which does not necessarily go along. Measurement of other downstream effectors of the PI3K pathway such as pS6 or 4EBP1 would potentially allow to analyze pathway activity.

The division of our cohort into three different risk groups according to p16 overexpression and the risk factor smoking[5,28] showed as expected significant survival differences in favor of p16-positive non-smokers.[50,51]

To the best of our knowledge this is the first study investigating the above mentioned expression patterns in the three risk groups based on the HPV positivity and tobacco exposure.

P53 and mTOR overexpression were significantly more often present in the p16-negative subgroup,

whereas PI3K overexpression was more predominant in both p16-positive groups irrespective of tobacco exposure. In conclusion, p16-positive OPSCC in smokers show the same immunohistochemical expression patterns and seem to follow the same carcinogenic pathway as p16-positive OPSCC in non-smokers. The impaired survival rates of the intermediate risk group compared to the p16-positive non-smokers can therefore not be explained by different expression patterns of PI3K pathway molecules but rather by smoking-associated second primary tumors and comorbidities.

Deriving therapeutic consequence based on our result is preliminary, as the cohort is rather small. Our results suggest that patients with HPV-associated OPSCC could benefit from the same targeted therapies irrespective of smoking status. Clinical trials have to prove, if differences in mutational status and marker expression translate into different outcomes. It has been shown in a study that a PI3K inhibitor (Alpelisib) shows the same response rate independent of the mutation status.[52]

In univariate survival analysis neither PTEN loss nor PI3K overexpression were prognostic, whereas mTOR expression was shown to be a negative prognosticator. However, this significance was lost in multivariate analysis. A recent study by García-Carracedo et al. found significant better disease specific survival in patients with p-s6 expression, a surrogate marker of mTOR1 activity, in laryngeal cancer.[20] Nevertheless, none of their tested proteins of the PI3K pathway (PDK1, PTEN, p-AKT, p-s6) had a prognostic significance on survival for OPSCC. Neither in breast cancer, gastric cancer, or renal cell cancer, expression status of the PI3K pathway revealed a prognostic impact.[46,49,53]

The limitation of our study is that HPV-positivity of the tumor was only based on p16 overexpression instead of HPV-DNA or HPV-RNA detection by either polymerase chain reaction (PCR) or in situ hybridization (ISH). Numerous studies have demonstrated a high correlation between HPV and p16 expression status in HNSCC[5,15,54–56] and stated that p16 is a reliable biomarker of HPV-associated HNSCC. Moreover p16 overexpression has recently been introduced into TNM staging.[57] In addition, p53 activity was assessed by immunohistochemistry and not by mutational status which might yield further information on p53 activity.

CONCLUSION

Based on our results it seems that HPV-associated OPSCC in smokers show the same expression pattern of key molecules of the PI3K pathway as HPV-associated OPSCC in non-smokers. The impaired survival rates of the intermediate risk group compared to HPV-positive non-smokers can probably not be explained by different expression patterns of key molecules of the PI3K pathway but might be associated with other factors like second primary tumors and comorbidities in smokers.

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