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Prognostic Impact of p16 Alterations and Pretreatment Anemia in Head and Neck Cancer Patients Undergoing Definitive Radiochemotherapy

Martina E. Becker-Schiebe[1, 3, *], Matthias Sperling[2], Fabian Wetzel[1], Uwe Pinkert[1], Wolfgang Hoffmann[1]

[1]Department of Radiotherapy and Radio-Oncology, Klinikum Braunschweig, Hannover Medical School, Braunschweig, Germany
[2]Department of Pathology, Klinikum Braunschweig, Hannover Medical School, Braunschweig, Germany
[3]Radiation Oncology, Hannover Medical School, Hannover, Germany

Abstract

Aim: Up to now, the incidence of HPV-associated oropharyngeal cancer is rising, indicating the increased impact of the viral etiology, but prognostic significance of HPV/p16 in other pharyngeal sites remains unclear. In addition, hypoxia might be important for carcinogenesis of head and neck cancer (HNSCC) and treatment response as well. This evaluation was carried out to explore, whether there is any correlation between pretreatment factors like pre-RT anemia and p16 expression and to determine the prognostic value of these factors in advanced HNSCC pts. *Methods:* A total of 79 locally advanced HNSCC patients (pts.) who underwent definitive RCT- or RT-antibody-therapy were retrospectively analysed. p16 (INK4A) expression was detected by immunohistochemical analysis. Factors predisposing for treatment response were examined and survival curves were compared. *Results:* The follow-up period ranged from 16 to 48 months, with a median of 25.3 months. Pretreatment anemia was apparent in one third of pts..P16 overexpression was detected in 32 cases. A significant correlation was found between p16 expression and pretreatment hemoglobin level. Only 3 pts. were characterized by both pre-RT anemia and p16 overexpression. Two-year locoregional control, progression-free survival (PFS) and overall survival (OS) were 75%, 62% and 70%, respectively. The prognostic value of p16 in the entire HNSCC patient cohort was confirmed; Kaplan-Meier analysis proved significant differences in progression-free survival (PFS) depending on p16 overexpression and PFS was significantly improved in the non-anemic patient group (p=0.023). *In conclusion,* this study demonstrated that p16 expression and pretreatment anemia are related to HNSCC subgroups. The prognostic value of this variables was confirmed for a patient cohort consisting of advanced naso-/oro-and hypopharyngeal carcinomas undergoing definitive RCT.

Keywords

Anemia, p16, Head and Neck Cancer, Radiochemotherapy

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) has been shown to present as clinically and histologically heterogeneous disease. Currently, the potential value of tumor suppressor genes, microsatellite instability and cell proliferation as biologic markers in the treatment of HNSCC is being investigated [1-4]. Hereby p53 gene mutations, p16 overexpression and chromosome loss of 9p21 and 10q22-26

* Corresponding author
E-mail address: martina_schiebe@web.de (M. E. Becker-Schiebe), m.schiebe@klinikum-braunschweig.de (M. E. Becker-Schiebe)

are the most frequent alterations in these tumours [1-3]. Furthermore, p16 expression has been shown to be a surrogate biomarker for HPV infection, which might play an important role in carcinogenesis of oropharyngeal cancers as well [4-6].

Besides risk factors like smoking or alcohol, there is some evidence, that hypoxia enhances the genetic instability of cells and stimulates carcinogenesis. This may induce the expression of a variety of genes such as tumour suppressor gene p53 and p16.

Recent studies discussed that the oxygenation status influences local control and overall survival rates in patients (pts.) treated with radiotherapy [7, 8].

A significant correlation between hemoglobin level and tumour oxygenation in HNSCC pts. was reported as well. The loss of apoptotic potential in hypoxic cells might be important for carcinogenesis and their resistance to radiotherapy [7].

Multimodal treatment including hyperfractionated radio/-chemotherapy (RCT) is the standard treatment for patient with locally advanced HNSCC. Although promising rates of tumour control were achieved with this regimen, mean overall survival rates have not been improved significantly over the last decade [8-11].

The identification of subgroups might be helpful to determine important prognostic factors and to individualize therapeutic procedures.

This retrospective evaluation was carried out to explore, whether there is any correlation between pretreatment factors like pre-RT anemia and p16 expression and to determine the prognostic value of these parameters in advanced HNSCC pts.

2. Material and Methods

Data from 79 patients with histologically proven advanced head and neck squamous cell carcinoma were analysed. HNSCC pts. treated in our department between 2012 and 2014 were recruited randomly for this retrospective evaluation. All pts. had a newly diagnosed, locally advanced HNSCC and had undergone definitive RCT- or RT-antibody-therapy. 38 (48%) pts. were diagnosed at stage III and 41 (52%) were diagnosed at stage IV.

In most cases IMRT/RapidArc® planning (76 pts) were performed using the Eclipse™ Treatment Planning System (Varian Medical Systems). 3 pts. Were irradiated using 3D conformal RT (3D-CRT). The accumulated radiation dose to the primary tumour and metastatic lymph nodes was 70.2 Gy in fractions of 1.8Gy daily, 5 times per week. The second and third order target volumes encompassed the regions of high and low risk for lymphatic spread and received a total dose of 59.4 and 50.4 Gy, respectively.

Systemic treatment consisted of concurrent, continuous infusion 5-FU of 600 mg/m (2) on days 1-5 and 6 cycles of weekly Cisplatin (30 mg/m^2). Due to inadequate renal function 5/72 pts. received 4 cycles of weekly Cisplatin only. Platin-based treatment was interrupted in 1 patient due to reduced general condition after one course of chemotherapy. In 9% (7 pts.) of cases Cetuximab was administered with an initial dose of 400 mg/m2, followed by seven weekly doses at 250 mg/m concurrent to RT [11].

Laboratory parameters such as a complete blood count, liver function tests, kidney function tests, and coagulation parameters were checked prior treatment and then weekly. Pts. were classified as being anemic, when hemoglobin levels prior to combined treatment were below 13 g/dl (men) or 12 g/dl (women).

Pts. were assessed at regular intervals during and after the completion of RT. Follow-up assessments began 4 weeks after the completion of therapy and included history, physical examination, and CT imaging of the head and neck. Histological remission of the gross tumour was examined between weeks 10 to 12.

Furthermore, treatment-related side effects were recorded according to the Common Toxicity Criteria Version 3.0 (CTCAEv3.0) [12]. Clinical information was collected from medical records maintaining patient anonymity.

p16 (INK4A) expression was detected by immunohistochemical analysis with CINtec^{R}p16 Histology ready to use VENTANA Antibody-Kit, that contains 5.0 µg of the mouse monoclonal primary antibody E6H4 for 50 tests. For visualization of the primary antibody the VENTANA ultraView Uninversal Alkaline Phosphatase Red Detection Kit was used. Stains were performed on a Benchmark XT VENTANA stainer. P16 (INK4A) overexpression was considered positive if a diffuse continuous nuclear and cytoplasmic staining of more than two thirds of the detectible tumor cells was visible. Focal staining represented by non-continuous staining of isolated cells or small cell clusters was considered as a negative staining result as outlined in the CINtecR p16 Histology VENTANA product information.

The data were evaluated by descriptive statistical methods using the SPSS software package (Windows) and the Real Statistics Data Analysis Tool ™/Excel/Microsoft Word. The different variables were evaluated by contingency tables. Associations between the different variables were calculated using the chi-square test and Fisher's exact test (two-tailed,

categorical variables). P < 0.05 was considered to be statistically significant. The prognostic effects of parameters were assessed by plotting Kaplan-Meier curves. Log-rank-test was used to compare the survival probabilities. Approval of the study (no. 2377-2014) was given by the ethics committee of the Hannover Medical School.

3. Results

The follow-up period ranged from 16 to 48 months, with a median of 25.3 months. All pts. underwent definitive RT combined with systemic treatment and concluded radiation treatment up to a total dose of 70.2 Gy. 92% of pts. received ≥ 5 cycles of planned Cisplatin, Cetuximab was administered according treatment schedule for 7 pts.. The majority of enrolled pts. suffered from oropharyngeal or hypopharyngeal cancer in 43% and 41% of cases, respectively. 38 pts. had stage III and 41 pts. had stage IV disease without any evidence for distant metastasis. The complete demographic and clinical characteristics are presented in table 1. History of smoking was reported in 89% of pts.. P16 overexpression was detected in 32 samples without any association to history of smoking. 5 of 9 non-smokers were p16 positive, 3 non-smokers were p16 negative (one patient with unknown p16 status). The associations between p16 overexpression, pre RT anemia and smoking are presented in table 2. Pretreatment anemia was apparent in one third of cases, 18 of 24 pts. with reduced pre-RT hemoglobin levels were treated due to hypopharyngeal cancer. Positive p16 status was more common in nasopharyngeal, oropharyngeal and oral cavity tumours, the highest frequency of p16 expression was found in nasopharyngeal cancer (60%), but correlation analysis did not reveal statistical significance (p>0.05) (table 3). A significant correlation was found between p16 expression and pretreatment hemoglobin level. Only 3 pts. were characterized by both pre-RT anemia and p16 overexpression, whereas in 49% of cases p16 overexpression was combined with non-hypoxic pre-RT blood values (p< 0.05, fig.1, table 2).

No difference in tumour stage was seen neither between the subgroups of patients with anemic or non-anemic blood values nor depending on p16 overexpression.

Within the observation period 35% (28/79) of pts. developed progressive disease (local and distant metastasis). 6 pts. developed distant metastasis and were treated with first-line chemotherapy, 5 of these 6 pts. demonstrated further progressive disease with pulmonal and hepatic metastasis.

After a median follow-up of 25.3 months, 69% of pts. were alive. Two-year locoregional control, progression-free survival (PFS) and overall survival (OS) were 75%, 62% and 70%, respectively. The subgroup of p16 positive oropharyngeal and oral cancer pts. developed only 2 recurrences during follow-up, whereas 64% (7/11) of p16 negative oral/oropharyngeal pts. suffered from recurrent disease.

p16 expression was significantly correlated with local control. 75% of p16 positive pts. revealed complete remission at 6-month follow-up after the end of RCT (table 4) compared to 52% of pts. with p16 negative tumours (p=0.03). Kaplan-Meier analysis proved significant differences in PFS depending on p16 overexpression for all patients. In addition, PFS was improved in the non-anemic patient group (p<0.05, figure 2 and 3).

P16 positive pts. tended to have a better OS, but statistical analysis was not able to confirm significant differences in OS depending on p16 expression and hemoglobin-levels prior treatment.

Table 1. Patients baseline of the study cohort (n=79).

Characteristics	number of patients n (%)
Age	61.7 y. (44-78y.)
<50 y.	9 (11%)
>50 y.	70 (89%)
Gender	
Female	15 (19%)
Male	64 (81%)
TNM stage	
III	38 (48%)
IV	41 (52%)
Tumor location	
Nasopharynx	5 (6%)
Oral cavity	2 (3%)
Oropharynx	34 (43%)
Hypopharynx	33 (41%)
Larynx	5 (6%)
Differentiation	
well	5 (6%)
moderate	63 (80%)
poor	11 (14%)
History of smoking	
Yes	70 (89)
No	9 (11)
Pretreatment anemia	
yes	24 (30%)
no	55 (70%)
P16 expression	
Positive	32 (41%)
Negative	27 (34%)
Unknown	20 (25%)

Table 2. Distribution of p16 overexpression, pre RT anemia and smoking.

Characteristics	p16 overexpression		pretreatment anemia		Non-smoker	
p16 expression						
positive (n=32)	-		9%		16%	
negative (n=27)	-		56%	(p=0.01)	11%	(p=0.68)
unknown (n=20)	-		30%		5%	
pretreatment anemia						
yes (n=24)	13%	(p=0.03)	-		13%	(p=0.91)
no (n=55)	53%		-		11%	

Table 3. Relationship between pretreatment anemia, p16 status and tumour location, number of patients n (%).

Tumor entitiy	patients, n (%)					
	total	p16 expression			anemia prior RT	
		positive	negative	unknown	yes	no
Hypopharyngeal cancer	33	12 (36)	12 (36)	9 (27)	18 (54)	15 (46)
Laryngeal cancer	5	0 (0)	3 (60)	2 (40)	0 (0)	5 (100)
Oropharyngeal cancer and oral cavity tumours	36	17 (47)	11 (31)	8 (22)	5 (14)	31 (86)
Nasopharyngeal cancer	5	3 (60)	1 (20)	1 (20)	1 (20)	4 (80)

Table 4. Local control for patients with positive and negative p16 expression, histopathological evaluation at 6 months follow-up, % of patients, p=0.03.

Local control	total	p16 positive	p16 negative
Complete remission	66%	75%	52%
Partial remission/no change	28%	21%	40%
Progressive disease	6%	4%	8%

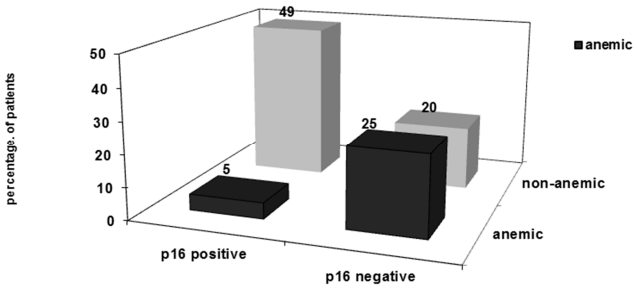

Figure 1. Relationship between p16 expression and pretreatment anemia (percentage of patients (%), n=59, p=0.02).

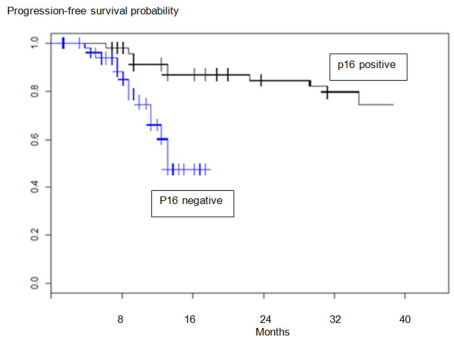

Figure 2. Progression-free survival curves for 79 HNSCC patients in correlation to p16 overexpression (Kaplan-Meier analysis, p=0.026).

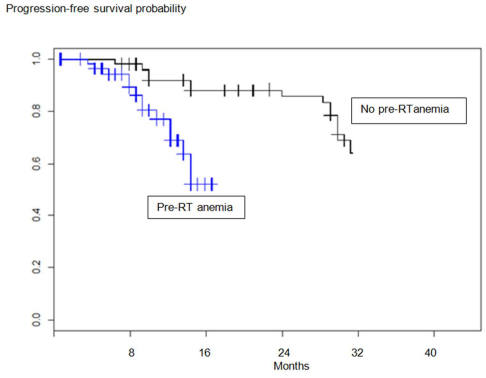

Figure 3. Progression-free survival curves for 79 HNSCC patients in correlation to pretreatment anemia (Kaplan-Meier analysis, p=0.023).

4. Discussion

Definitive combined treatments (RCT /RT+ Cetuximab) have been proven as highly active regimens in patients suffering from advanced HNSCC [11, 13-15]. However five-year survival rates less than 50-55% are reported [4, 8] and the identification of predictive markers is warranted to optimize treatment. Traditionally, prognosis stratification of HNSCC has been determined by clinicopathologic variables such as tumour stage, nodal involvement and histopathological grade [9]. However, these factors have been shown to predict clinical behaviour inconsistently [1]. HNSCC pathogenesis is driven by an accumulation of genetic events supporting the hypothesis of multistep progression [5]. Recent studies analysed several genetic markers, which might allow the differentiation of squamous cell carcinomas of the head and neck in molecular-biological subsets with probably prognostic and clinical implications [1, 2, 9]. Studies indicated, that HPV positive oropharyngeal carcinomas have favourable survival rates [9, 16]. The prognostic impact of HPV positivity was proven in randomized trials as well [2, 8, 16].

P16 expression assessed through immunohistochemistry has now been accepted as a reliable biomarker for the HPV E7 oncoprotein, thus acting as a surrogate marker for high risk HPV infection [2]. As shown by Lassen et al. [17] the prognostic significance of p16 expression is kept even in the absence of HPV DNA detection. Up to now, the incidence of HPV-associated oropharyngeal cancer is rising, indicating the increased impact of the viral etiology, but prognostic significance of HPV/p16 in hypopharyngeal and nasopharyngeal cancers remains unclear [6, 16]. In our study group p16 overexpression was detected in 41% of all pts. and 47% of oropharyngeal cancers, but p16 positivity was seen in hypopharyngeal and nasopharyngeal cancers in 36% and 60% of cases as well.

Baseline characteristics of our study cohort were typical for HNSCC stage III/IV with a majority of male pts. with moderate differentiated oropharyngeal/ hypopharyngeal tumours. HPV/p16 positive tumours are less likely to have a history of significant tobacco use [18-20]. It is noteworthy, that we did not found any correlation between p16 expression and tobacco use due to the small number of non-smokers in

our patient cohort (table 2).

In several analysis of radiation trials variables were found to be independent predictors of survival including hemoglobin levels prior RT [21-24]. Pretreatment anemia has been shown to adversely impact tumour control of pts. treated with RT +/- CT. The relative risk of death was significantly associated with decreasing serum hemoglobin levels, which supported the hypothesis that hypoxic squamous epithelium might restrain a significant number of malignant cells, which are biologically more aggressive [25].

We demonstrated that patients suffering from pretreatment anemia had worse progression-free survival rates (p=0.023, figure 3).

Our results (fig. 1, table 2) suggested that p16 expression and pre-RT anemia were predictors for different HNSCC subtypes, as we could identify pre-RT anemia combined with p16 overexpression in 3 pts. only.

In our study two-year locoregional control, progressivion-free survival (PFS) and overall survival (OS) were 75%, 62% and 70%, respectively, which is in line with published data [8,11,15],

Currently, the RTOG study 0129 analyzed the efficacy and toxicity of Cisplatin plus accelerated vs. standard fractionation in locally advanced head and neck carcinoma and reported 2-year and 5-year PFS of 66% and 55% [8].

Three-year locoregional control, disease-free survival and overall survival rates of 72%, 54, and 61% were reported for accelerated RCT [26].

In these trials the p16 status was confirmed as predictive marker in oropharyngeal cancers only. The prognostic advantage might be conferred by p16 upregulation and an association with lower exposure to tobacco and less hypoxic tumour volumes [2, 7, 8, 16, 23]. Comparable to this, Rosenthal et al. [27] reported that p16 overexpression was strongly prognostic for patients with oropharyngeal cancers, but the influence of p16 was not seen in nasopharyngeal and hypopharyngeal cancers [28]. Accordingly, we demonstrated an advantage for the subgroup of p16 positive oropharyngeal and oral cancer pts., who developed only 2 recurrences during follow-up, whereas 64% (7/11) of p16 negative oral/oropharyngeal pts. developed recurrent disease. However, it is noteworthy that we were able to confirm the prognostic value of p16 in the entire HNSCC patient cohort; a significant impact of p16 overexpression to PFS and local control was demonstrated by Kaplan-Meier analysis (figure 2, table 4).

5. Conclusion

Advanced head and neck squamous cell carcinomas are challenging to treat effectively. Several randomized trials have confirmed the value of definitive radiochemotherapy and the need to adapt treatment strategies depending on stratifying variables.

Even though this study was retrospective, we consider our conclusions to be of significant informative value. Based on our data drawn from an uniformly treated pool of patients, we were able to identify and confirm prognostic factors in advanced head and neck cancer treated with definitive combined radiation.

This study demonstrated that p16 expression and pretreatment anemia are related to different HNSCC subgroups. The prognostic value of these variables was confirmed for a patient cohort consisting of naso-/oro-and hypopharyngeal carcinomas as well and should be evaluated in future deintensification trials [29].

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