RESEARCH ARTICLE

HPV-Associated p16 INK4A Expression and Response to Therapy and Survival in Selected Head and Neck Cancers

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Abstract

Background: Development of squamous cell cancer of head and neck (SCCHN) is associated with human papillomavirus (HPV) infection, which in turn is closely related with expression of p16 INK4A. Loss of p16 INK4A expression by deletion, mutation, or hypermethylation is common in SCCHN. We here evaluated p16 INK4A as a prognostic marker of treatment response and survival in our SCCHN patients with laryngeal, hypopharyngeal or nasopharyngeal cancers. Materials and Methods: 131 patients diagnosed with SCCHN between January 2,2006 and July 17, 2010 were examined for p16 INK4A. The median age was 60 years (15-82 years). Fifty one patients were stage I-II and 80 were stage III-IV. Immunohistochemical expression of p16 INK4A was analyzed in pretreatment paraffin-embedded tumor blocks. The influence of p16 INK4A status on disease-free survival, and overall survival after treatment was evaluated. Results: P16 INK4A positivity was found in 58 patients (44%). Tumor-positivity for p16INK4A was correlated with improved disease free survival (70.1 months *vs* 59 months) and improved overall survival (2, 3 and 5-year values; 77% *vs* 72%, 70% *vs* 63% and, 63% *vs* 55%; respectively). On multivariate analysis, stage was determined as independent prognostic factor for disease-free survival. Conclusions: Stage was the major prognostic factor on treatment response and survival in our patients. P16 INK4A status predicts better outcome in laryngeal, hypopharyngeal or nasopharyngeal cancer cases treated with surgery plus adjuvant radiochemotherapy as well as with definitive radiation therapy and/or chemotherapy.

Keywords: p16 INK4a - hpv - head and neck cancer - radiotherapy - outcome

Asian Pac J Cancer Prev, **16 (1)**, 253-258

Introduction

The correlation between human papillomavirus (HPV) and head and neck cancer was first suggested in the late 1980s. The first report demonstrated that, seven tongue base cancers, three of them being HPV-positive (Villiers et al., 1985). In following years, many studies published which shows a various number of HPV-positive cases in squamous cell cancer of the head and neck cancers (SCCHN) more common in oral cavity and oropharyngeal cancers (Elongo et al., 2011; Huang et al., 2012; Ahmed et al., 2012; Rushatamukayanunt et al., 2014).

Recent studies showed that in SCCHN with HPV-positive tumors have distinct clinicopathologic behavior (Gillison et al., 2000; Dahlstrand et al., 2008; Huang et al., 2014). HPV-positive patients tend to be younger age, lower exposure to tobocco and alcohol and also they have more advanced stages (Gillison et al., 2000; Huang et al., 2012) . These biologic differences impact the prognosis of HPV-positive SCCHN patients and they have a better prognosis compared with patients with HPV-negative tumors (Licitra et al., 2006; Kumar et al., 2008; Fakhry et al., 2008; Lassen et al., 2009; Huang et al., 2012).

Approximately 90% of HPV-positive SCCHN are infected with high-risk HPV 16 (Gillison et al., 2000, Elongo et al., 2011). HPV-positive SCCHN are characterised by high expression of the p16 INK4A tumor suppressor protein and also named cyclin-dependent kinase inhibitor 2A (CDKN2A) (Licitra et al., 2006; Kumar et al., 2008; Fakhry et al., 2008; Lassen et al., 2009; Rushatamukayanunt et al., 2014). Viral oncoproteins E6 and E7 cause to inactivation of p53 and retinoblastoma (Rb) tumor supressor gene functions and this situation up-graded CDKN2A gene activation and increased expression of p16 INK4A (Mantovani et al., 2001; Munger et al., 2001; Rushatamukayanunt et al., 2014).

Base on these findings p16 INK4A positivity considered as a biomarker for tumors origin clinically and oncogenetically correlate with HPV infections. Several studies determined this issue and they found p16 INK4A usefull surrogate biomarker for HPV- associated disease in SCCHN (Smeets et al., 2007; Klussmann et al., 2009; Ahmed et al., 2012).

With the present study we aimed to evaluate our

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institutional series of SCCHN treated with radiotherapy, the impact of HPV-associated tumor cell p16 ^{INK4A} expression on response to therapies and treatment outcomes including disease-free survival (DFS) and overall survival (OS).

Materials and Methods

Patients and Tissues

We retrospectively analyzed the data of patients who have been treated between January 2006 and July 2010 at the Radiation Oncology Department of Gazi University Medical School. Tissue samples of clinico-pathologically comfirmed, one hundred thirty-one patients with SCCHN were obtained. Oral cavity and oropharynx cancers were excluded from this study. All the informations e.g., history, tumor location, tumor size, histology, grade, stage, nodal involvement, p16^{INK4A} status and treatment were collected from patient's medical and pathological records.

The anatomical location of primary tumors were as allows: larynx (n=103), nasopharynx (n=19), and hypopharynx (n=9). The patients treated with curatively intents were included in this study. Treatment modality of these patients were based on interdisciplinary advice. The patients were treated with primary or postoperatively; conventionally fractionated radiotherapy (RT) or radio-chemotherapy (RCT). The radiation dose of primary tumor and pathological lymph nodes were 68-70 Gy in 34 to 35 fractions. Clinically uninvolved regional lymph node doses were up to 50 Gy.

For this study, all events were retrospectively examined for details about locoregional control and distant metastasis during the follow-up. The survival state was last updated on November 13, 2013. Overall survival was measured to date of death. Disease-free survival was measured to date of first treatment failure or death.

Immunohistochemical p16 analysis

Immunohistochemical staining for p16 protein was performed on 4-µm-thick formalin-fixed paraffin-embedded tissue sections with CINtec® p16 Histology kit (Ventana Medical Systems), a monoclonal anti-p16^{INK4A} antibody clone E6H4. The immunostaining procedure was carried out using an authomatised system (Ventana Medical Systems). Positive tissue controls were included in each assay. The paraffin sections of cervical biopsy with high-grade squamous intraepithelial lesion were used as positive tissue control. After immunostaining, tissue sections were evalauted by 2 pathologists. Either nuclear staining only or nuclear and cytoplasmic staining were regarded as positive.

Statistical methods

Statistical analyses were computed using SPSS version 18. Comparition of p16 ^{INK4A}- positive or p16^{INK4A}- negative tumors with clinico-pathological variables were done by using the Chi-square test. Actuarial survival curves were obtained by using Kaplan-Meier plots and long-rank test. Cox regression analysis was performed to evaluate the

Table 1. Clinico-Pathologic Characteristics of HNSCC Patients

	All patients (n=131)		P16INK4A-positive (n=58)		P16INK4A-negative (n=73)		
	n	%	n	%	n	%	p[a]
Age (years)							
Median	60		60		59		ns
Range	(15-82)		(15-70)		(17-82)		
Age ≤55	53	40	25	43	28	38	ns
>55	78	60	33	57	45	62	
Gender							0.01
Female	15	11	11	18	4	5	
Male	116	89	47	82	69	95	
Primary site							ns
Larynx	103	78	46	79	57	78	
Hypopharynx	9	6	3	5	6	8	
Nasopharynx	19	16	9	16	10	14	
T –stage[b]							ns
T1-2	67	51	30	52	37	50	
T3-4	64	49	28	48	36	50	
N-stage[b]					ns		
N0	78	60	32	55	46	63	
N1-3	53	40	26	45	27	37	
TNM-stage[b]							ns
I-II	51	39	21	36	30	41	
III-IV	80	61	37	64	43	59	
Tumour differentiation							ns
Well or moderate	110	84	47	81	63	86	
Poor	21	16	11	19	10	14	
Cigarette							ns
Yes	96	73	45	77	53	72	
No	35	27	13	23	29	28	
Alcohol							ns
Yes	14	10	5	9	9	12	
No	117	90	53	91	64	88	

[a]Chi-square test for comparition between P16INK4A- positive and P16INK4A- negative tumours; b International Union of Cancer Research (UICC) 1982. Classification

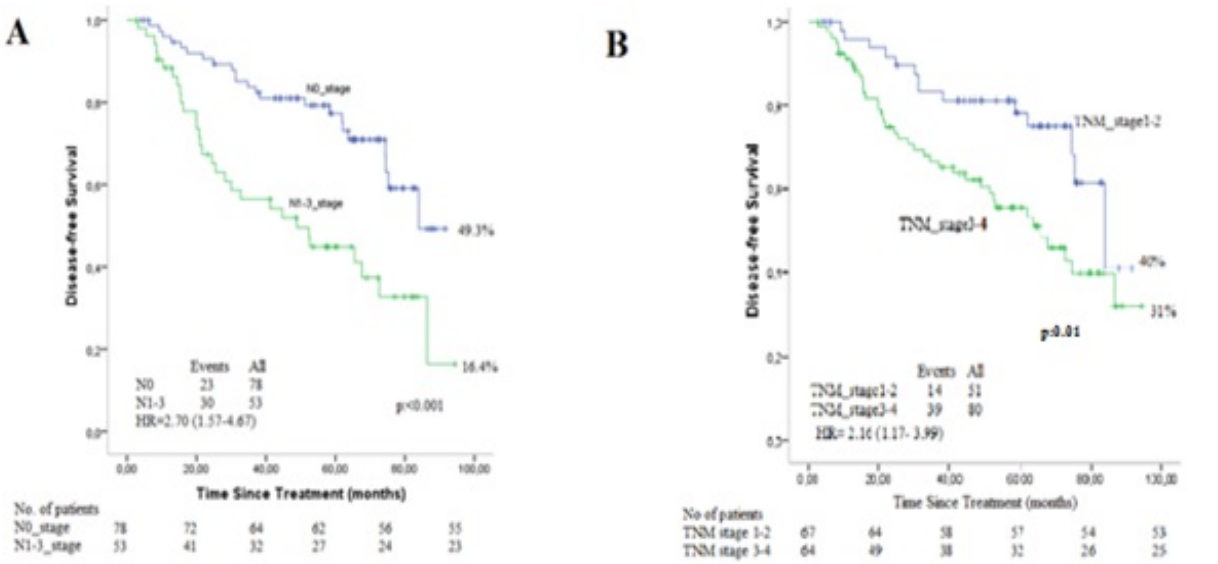

Figure 1. (A) Actuarial Estimated Disease-Free Survival Rates in Patients with N0 stage vs NI-III Stage Patients and (B) TNM Stage I-II *vs* TNM Stage III-IV

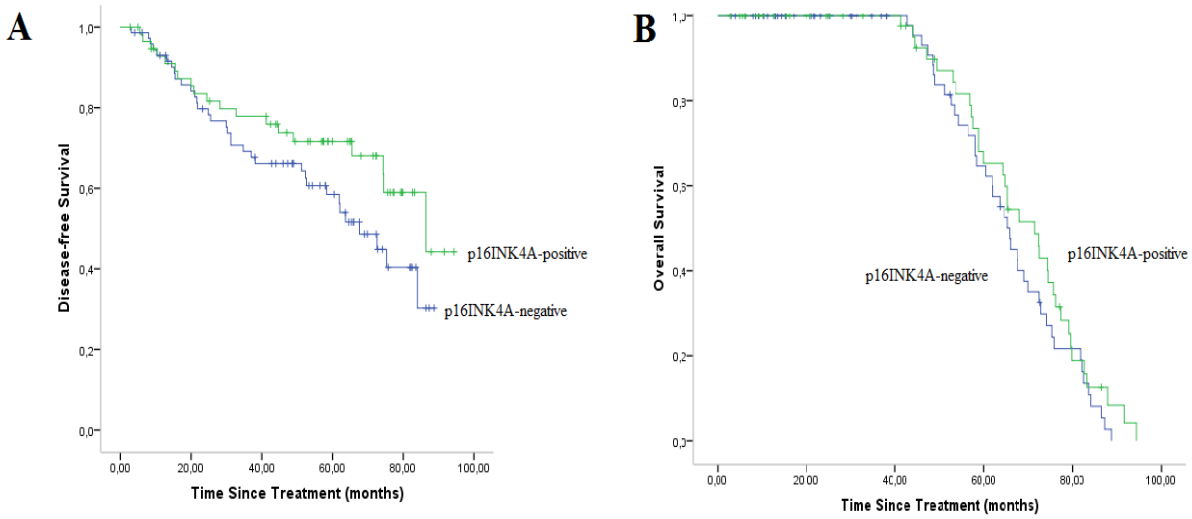

Figure 2. The Kaplan-Meier Curves for DFS A) and OS B) with regard to p16^{INK4A}

multivariate comparitions, with an estimated hazard ratio (HR) and 95% confidence interval (CI). All statistics were two-tailed, and the choosen significance level was 5%.

Results

In total, 131 patients were included in this study, 15 was female (11%) and 116 was male (89%). Median age of patents was 60 years (range; 15-82 years). One hundred three patients (78%) were larynx, 19 patients (16%) were nasopharynx and 9 patients (6%) were hypopharynx cancer. Fifty one (39%) patients were had TNM-stage I-II and 80 patients (61%) were had TNM-stage III-IV tumor.

The characteristics of patient and tumor are shown in Table1. Of the 131 patients, 58 were p16 ^{INK4A}- positive. There were significantly more women in p16 ^{INK4A}- positive group (p:0.01). The median age of p16 ^{INK4A}- positive group was 60 years and p16 ^{INK4A}- negative group was 59. The distribution of tumor location was similar between these groups. Furthermore p16 ^{INK4A}- positive tumors had been more advanced nodal (N)-stage (45% *vs* 37%) than p16 ^{INK4A}- negative tumors. However there was no difference in tumor (T)-stage. p16 ^{INK4A}- positive tumors were associated with a slightly higher TNM- stage compared with p16 ^{INK4A}- negatives (64% *vs* 59%). Otherwise there were no differences in tumor differentiation, cigarette status, and alcohol use between this groups.

Median follow-up time was 52.6 months (range; 2.7- 94.3 months). At the time of follow-up; 74 patients (56.4%) survived; 53 patients (40.4%) died because of the disease; and the 4 patients (0.03%) died because of another cause (cause of myocardial enfarction). In total, mean OS of patients was 67.1 months. Mean DFS of patients was 64.9 months.

The N0 stage subgroup (78 of 131 patients; 60%) was compared to NI-III stage (53 of 131 patients; 40%). Mean DFS was 72.6 months in N0 stage, 51.1 months in NI-III stage. The resuls are statistically significant (p<0.001). Two, 3, and 5 year values were; 90%, 83%, 77% and 67%, 56%, 44%; respectively.

Similarly, patients with TNM stage I-II subgroup (67 of 131 patints; 51%) was compared with TNM stage III-

IV subgroup (64 of 131 patients; 49%). Mean DFS was 73.08 months in TNM stage I-II, 58.68 months in TNM stage III-IV. Two, 3, and 5 year values were; 91%, 83%, 78% and 74%, 66%, 55%; respectively. The resuls are statistically significant (p:0.01). The Kaplan-Meier curves for DFS and OS, depending for N-stage and TNM-stage was shown in Figure 1.

There were no statistically significant difference in survival according to patient age, gender, primer tumor site, T-stage, tumor differentiation, cigarette and alcohol status between the patients. Also the female gender, the younger age, the earlier T-stage (TI-II stage), the lower use of cigarette and alcohol were association with higher DFS and OS.

When we compared survival outcomes according to variable of the $p16^{INK4A}$ status of patients; we obtained that $p16^{INK4A}$ positivity can be predict of better DFS and OS. Mean DFS was 70.1 months in $p16^{INK4A}$- positive group and 59 months in $p16^{INK4A}$-negative group (p:0.1). Two, 3, and 5 year DFS rates were 83%, 77%, 71% in $p16^{INK4A}$-positive group and 78%, 68%, 58% in $p16^{INK4A}$- negative group. Also improved overall survival was found in $p16^{INK4A}$- positive group. The median OS was 68.7 months in $p16^{INK4A}$- positive group and 64.1 months in $p16^{INK4A}$-negative group (p:0.2). Two, 3 and 5-year values were; 77%, 70%, 63% in $p16^{INK4A}$- positive group and 72%, 63%, 55% in $p16^{INK4A}$- negative group. But these results did not reach to statistical significance. The Kaplan-Meier curves for DFS and OS, depending for $p16^{INK4A}$ is shown in Figure 2.

In the final Cox proportional hazard analysis with risk of disease-free survival as the end point, low nodal classification (N0-stage, hazard ratio [HR], 2.70; 95% CI, 1.57 to 4.67) and earlier TNM stage (HR, 2.16; 95% CI, 1.17 to 3.99) were found independent factors associated with good prognosis.

Discussion

The potential role of HPV in carcinogenesis of head and neck cancers is studied in several trials and relatively high prevelance was found especially in oropharyngeal, flour of the mouth, oral cavity cancers and carcinoma of the tonsil (Brandsma et al., 1986; Brachman et al., 1992; Ogura et al., 1993; Noble-Topham et al., 1993; Brandwein et al., 1994; Gillison et al., 2000; Elongo et al., 2011; Huang et al., 2012; Ahmed et al., 2012; Rushatamukayanunt et al., 2014)

The correlation between $p16^{INK4A}$ and HPV in oral cavity and oropharengeal cancers has been so close that $p16^{INK4A}$ considered to be not only a surrogate marker for HPV infection, but also a prognostic factor in SCCHN (Licitra et al., 2006; Smeets et al.,2007; Kumar et al., 2008; Fakhry et al., 2008; Lassen et al., 2009; Klussmann et al., 2009; Ahmed et al., 2012). Retrospective studies have signed improved outcomes in HPV associated SCCHN especially in oropharyngeal cancer (Lassen et al., 2009; Mantovani et al., 2001; Huang et al., 2012)

We analyzed correlation between the tumor $p16^{INK4A}$ expression and treatment response of patients with SCCHN except for oral caviy and oropharynx cancer. In our knowledge this is the first study examine that $p16^{INK4A}$ can be prognostic marker of treatment responce and survival in this subgroup of SCCHN patients like oral cavity and oropharynx cancers.

The etiologic role of HPV infection in larengeal carcinoma is unclear. Altough a correlation was determined with verrucous larengeal carcinoma, a rare well-differentiated variant of squamous carcinoma with a low malignant potential, and HPV-6, HPV-11, or HPV-16 or related DNA (Brandsma et al., 1986; Noble-Topham et al., 1993; Fliss et al., 1994), the etiologic role of HPV is unclear because of the lack of more spesific confirmatory methods for HPV detection and also viral DNA loads are rarely detected in laryngeal dysplasias and cell cultures derived from laryngeal cancers (Fouret et al., 1995; Poljak et al., 1997; Atula et al., 1999). In a one study, altough a 2.4 fold increase in the risk of laryngeal cancer have found in HPV 16 positive patients, 14.4 fold increase have determined in the risk of orofarengeal cancer (Mork et al., 2001).

Clayman et all. examined the association between HPV status and treatment outcomes in their larengeal and hypopharyngeal carsinomas. Fifty -nine patient were with larynx cancer in this study. They were demostrated that, %46 of all cohort were HPV-positive and HPV-positivity to be a negative prognostic indicator for survival (Clayman et al., 1994).

Wilson et al investigated the association between hyopharyngeal carsinoma and $p16^{INK4A}$ status. Twenty seven patients with hypopharyngeal carsinoma treated with radiochemotherapy (22 of 27 patients) or radiotherapy alone (5 of 27 patients) included in this study. They were found $p16^{INK4A}$- positivity in 9 patients. There was no significant difference in OS, DFS and loco-regional control (LRC) between $p16^{INK4A}$- positive or $p16^{INK4A}$-negative groups. They were also demostrated that $p16^{INK4A}$ was not a useful prognostic biomarker in hypopharyngeal cancers (Wilson et al., 2012).

Also there is lack of information regarding the prognostic significance of $p16^{INK4A}$ and HPV in nasopharynx carsinomas other than oral cavity and oropharynx cancers too.

In our trial, we examined the impact of $p16^{INK4A}$ expression on OS and DFS in 131 SCCHN patients out of oral cavity and oropharyngeal cancer. In our study the patients were especially in larynx cancer; 103 patients with larynx cancer. Improvement in DFS and OS were found in $p16^{INK4A}$- positive group but the results did not show a statistical significance. Because the exception of oral cavity and oropharynx cancer and higher proportion of laryngeal patient numbers and Questionable relationship with HPV and the other site of SCCHN may be the reason of dissimilarity with current data on survival in our study.

In Gillison et all studies; they were compared HPV-positive with HPV- negative SCCHN, HPV- positive SCCHN patients tend to be more advanced stage (Gillison et al., 2000). In this study, we observed that $p16^{INK4A}$-positive patients were associated with more advanced nodal stage (45% *vs* 37%), and more advanced TNM stage (64% *vs* 59%). The nodal stage and TNM stage associations, although shown in previous studies (Fakhry

C et al., 2008; Lassen et al., 2009) (4,8). In our study we were also found that p16 ^{INK4A}- positive patients were associated with more female gender (18% *vs* 5%; p:0.01).

'The extent of primary lesion and neck disease are the major determinants of prognosis. The likehood of local control is determined primarily by T-stage; there are conflicting data pertaining to a possible inverse relationship between N-stage and local control. The likehood of locoregional control is impacted primarily by the overall AJCC stage, which accounts for both T- and N-stages, AJCC stage and N-stage are the major determinants of cause-spesific survival' (Halperin et al., 2008) (28).

In our study TNM-stage and N-stage were the most important factors associated with improved DFS. Mean DFS was 72.6 months in N0 stage, 51.1 months in NI-III stage (p<0.001). Similarly mean DFS was 73.08 months in TNM stage I-II, 58.68 months in TNM stage III-IV (p:0.01). The results were suggested from literature.

The primary limitations of this study are retrospective approach and examining different primary tumor locations. In future, our study and other retrospective trials examinig the role on survival of p16 ^{INK4A} in SCCHN out of oral cavity and oropharynx cancers, may lead larger prospective randomized trials and the impact of p16 ^{INK4A} on prognosis, risk stratification and staging should can be modified with these datas.

In conclusion, in contrast to oral cavity and oropharyngeal cancers, p16 ^{INK4A}- positivity in patients with our study indicated improved OS and DFS but without statistical significance.

Acknowledgements

The authors wish to thank Prof. Dr. M. Koc (Necmettin Erbakan University Meram Medical School, Head of Radiation Oncology Department) for continuous support.

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