Cent. Eur. J. Med. • 7(3) • 2012 • 349-353
DOI: 10.2478/s11536-011-0148-4

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Central European Journal of **Medicine**

Survival of patients with head and neck squamous cell carcinoma in association with human papillomavirus and p53 polymorphism

Rapid Communication

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Received 2 September 2011; Accepted 29 December 2011

Abstract: Survival of patients with head and neck squamous cell carcinoma (HNSCC) is dependent on many factors – stage of the disease, treatment regimen, operation technique etc. Many authors discuss on association of survival with various biomarkers as HPV infection, p53 mutation and polymorphism or p16 expression. The objective of our study was to analyze the survival of HNSCC patients in association with HPV infection and p53 polymorphism. Methods. 39 patients with primary diagnosed HNSCC were investigated. HPV DNA was detected using PCR with general primers MY09/11; p53 polymorphism was analyzed using single nucleotide polymorphism assay by PCR. Results. Of the 39 patients, 12 (30.8%) had detectable HPV. After p53 polymorphism analysis heterozygous *Pro*/*Arg* type was found in 34 cases (87.2%). Survival was higher in laryngeal cancer patients and in patients when tumour was classified as T_{1-2}. Somewhat higher survival was in the HPV positive patients, however difference was not statistically significant ($P = 0.7$). Only significant factor influencing survival in our study group was site of primary tumour ($P < 0.05$). Conclusion. HNSCC patients' survival in our study depend on primary tumour site; HPV infection and p53 SNP was not associated with better survival.

Keywords: *Head and neck cancer • Survival • HPV infection • p53 polymorphism*

1. Introduction

Worldwide incidence rates of head and neck squamous cell carcinoma (HNSCC) are still high with some differences in geographical distribution [1,2]. High incidence was reported in northern France, Hong Kong, the Indian sub-continent, Central and Eastern Europe, Spain, Italy, Brazil, and among US blacks [3]. Recent increases of oral and pharyngeal cancers have been reported in Eastern Europe and in Japan [4]. Main risk factors of HNSCC are tobacco and alcohol [5,6]. However, high risk type human papillomavirus infection is closely related with some cancers and HNSCC as well. Despite treatment advances over the past decades, the overall 5-year survival rate in the Europe remains low - from 25 to 45% for sites with fair (tongue, oral cavity, oropharynx, and nasopharynx) and poor prognosis (hypopharynx) [7]. Patient's survival depends on many factors – stage of the disease, treatment regimen, operation technique *etc*. The association between HNSCC survival, HPV infection and some molecular biomarkers – p53, $p16^{INK4a}$ has been suggested, but the results are contradictory [8]. The objective of our study was to analyze the survival of HNSCC patients in accordance to HPV positivity and p53 single nucleotide polymorphism (SNP).

2. Materials and methods

39 patients with a primary diagnosis of head and neck cancer, from the previously conducted study, [9] were included in the analysis. Patients were recruited in the Institute of Oncology, Vilnius University (period from March to November of 2006). All participants were comprehensively informed about the tests, possible risks and advantages of the study, agreed to take part in this study and had to sign the informed agreement form. The protocol of the study, invitation and patients agreement

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form were approved by Lithuanian bioethics committee of Ministry of Health (2006 02 23, No 7).

HPV DNA was detected using PCR; p53 SNP for proline (Prol) or arginine (Arg) allele at codon 72 of exon 4 was analyzed using SNP assay by PCR.

2.1 HPV detection

DNA from fresh tumor cells was extracted using column method (*SorpoClean™ Genomic DNA Extracion Module*, SORPO Diagnostics, Lithuania) according to the manufacturer protocol. The concentration of extracted DNA was measured before PCR.

Polymerase chain reaction was carried out using the *Ready to use Master Mix* for consensus HPV detection (SORPO Diagnostics, Lithuania). The PCR *Master Mix* was designed to detect many of high, low and intermediate risk HPV (HPV 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 70, 71, 72, 73, 82, IS39, CP8304, CP6108, MM4, MM7, MM8 *etc*.). In the composition of *Master Mix* the internal control for β globine gene was included. PCR was performed starting from the initial denaturation step at 95ºC for 7 min followed by 42 cycles of denaturation step at 94ºC for 30 sec, primer annealing step at 50ºC for 30 sec and a chain elongation step at 72ºC for 30 sec. A final extension for 2 min at 72ºC was used. For HPV typing the *Ready to use Master Mix* for HPV 16 and 18 (SORPO Diagnostics, Lithuania) was used. The PCR protocol consisted from the initial denaturation step at 95ºC for 7 min followed by 35 cycles of denaturation step at 94ºC for 30 sec, primer annealing step at 56ºC for 45 sec and a chain elongation step at 72ºC for 45 sec. A final extension for 2 min at 72ºC was used. Positive (CaSki and HeLa cells) and negative (PCR mix without DNA) controls were used during all cycles of PCR.

2.2 P53 polymorphism detection

The p53 gene SNP at codon 72 resulting in the presence of Prol and/or Arg were detected with PCR followed by MvnI enzymatic digestion (Roche, Germany) of the PCR product followed by restriction fragment analysis (RFLP). For p53 amplification these primers paires were used: sense oligonucleotide 5'-TTGCCGTCCCAAGCAATGGATGA-3' antisense oligonucleotide 5'TCTGGGAAGGGACAGAAGATGAC-3'. Target sequences were amplified in 50 µl reaction volume containing 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$, 0.002% gelatin, 0,4 mM dNTP mix, 0.06 units Taq DNA Polymerase/µl (SIGMA, USA). The amplification was performed for 35 cycles with an annealing temperature of 60ºC. The PCR product was digested with MvnI (Roche, Germany) for 2 hour at 37ºC. The amplified fragment identifies two alleles: Prol – 199 bp and Arg – 113 bp +86 bp.

All amplified PCR products were analyzed by electrophoresis in 2% agarosis gel stained by ethidium bromide. After electrophoresis the stained products were analyzed in transiluminator (HEROLAB, German) using UV light. All results were photographed and documented.

2.3 Statistical analysis

The vital status of the study group was assessed as of September 1, 2009, by passive follow-up, using data from the population registry. It was found that 20 (33.9%) of the patients had died. Descriptive statistics were used to summarize study data. Survival was estimated by the Kaplan–Meier method. The statistical difference between the survival curves was determined using the log-rank test. P-value lower than 0.05 was considered statistically significant.

3. Results

39 patients with HNSCC were included in the survival analysis: there were 37 cases in men and 2 women. The mean age of patients was 60.8±10.0 years (median 60; range 44-80 years). Women in the study group were younger than men - mean age 48.0±5.7 and 61.5±9.7 respectively.

Clinical and molecular characteristics of tumours are presented in Table 1.

Table 1. Clinical and molecular characteristics of head and neck tumours (*n = 39*).

Variable	No. of patients	% of total
Primary site		
Oropharynx	6	15.4
Lingua	9	23.1
Hypopharynx	6	15.4
Larynx	14	35.9
Other[a]	4	10.3
Tumor classification		
T_{1-2}	8	20.5
T_3	20	51.3
T_4	11	28.2
Lymph node classification		
N_0	21	53.8
N_{1-3}	18	46.2
M status		
M_0	39	100.0
M_1	0	-
HPV status		
positive	12	30.8
negative	27	69.2
P53		
Prol or Arg	5	12.8
Prol/Arg	34	87.2

The most common sites of tumours were larynx (35.9%) and lingua (23.1), oroharynx and hypopharynx composed 15.4%. A high proportion of analysed tumours were diagnosed at advanced stages - almost 80% of tumours were evaluated as T_3 and T_4. Of the 39 patients, 12 (30.8%) had detectable HPV. After p53 SNP analysis heterozygous Prol/Arg was found in 34 cases (87.2%): in the remaining 5 cases (12.8%) homozygous Prol or Arg was found.

The overall survival of HNCC patients was 76.9% (95% CI 60.3-87.3) and 43.6% (95% CI 27.9-58.3), respectively, 1 year and 3 years after diagnosis.

3 year survival rates by demographic and clinical characteristics of HNSCC patients are shown in Table 2. Survival was higher in laryngeal cancer patients and in patients when tumour was classified as T_{1-2}. Only significant factor influencing survival in our study group was site of primary tumour (P < 0.05). Somewhat higher survival was in the HPV positive patients, however difference was not statistically significant (P = 0.7) (Figure 1). There were no significant differences in survival according p53 polymorphism, but higher survival was found in the group P/A - heterozygous (Figure 1).

Table 2. Survival by demographic and clinical characteristics of HNSCC patients (*n = 39*).

Variable	3 year survival (95% CI)	Log rank test
Gender		
Male	43.2 (27.2 – 58.3)	P = 0.7
Female	50.5 (0.6 – 91.0)	
Age, years (median, 60 years)		
≤ 60	44.4 (21.6 – 65.1)	P = 0.8
> 60	42.9 (21.9 – 62.3)	
Primary site		
Oropharynx	16.7 (0.8 – 51.7)	P = 0.02
Lingua	22.2 (3.4 – 83.3)	
Hypopharynx	33.3 (4.6 – 67.6)	
Larynx	64.3 (34.3 – 83.3)	
Other[a]	75.0 (12.8 – 96.1)	
Tumor classification		
T_{1-2}	75.0 (31.5 – 93.1)	P = 0.09
T_3	40.0 (19.3 – 60.1)	
T_4	27.3 (6.5 – 53.9)	
Lymph node classification		
N_0	47.6 (25.7 – 66.7)	P = 0.2
N_{1-3}	38.9 (17.5 – 60.0)	
HPV status		
positive	50.5 (20.9 – 73.6)	P = 0.7
negative	40.7 (22.5 – 58.2)	
P53		
Prol or Arg	40.0 (5.2 – 75.3)	P = 0.8
Prol/Arg	44.1 (27.3 – 59.7)	

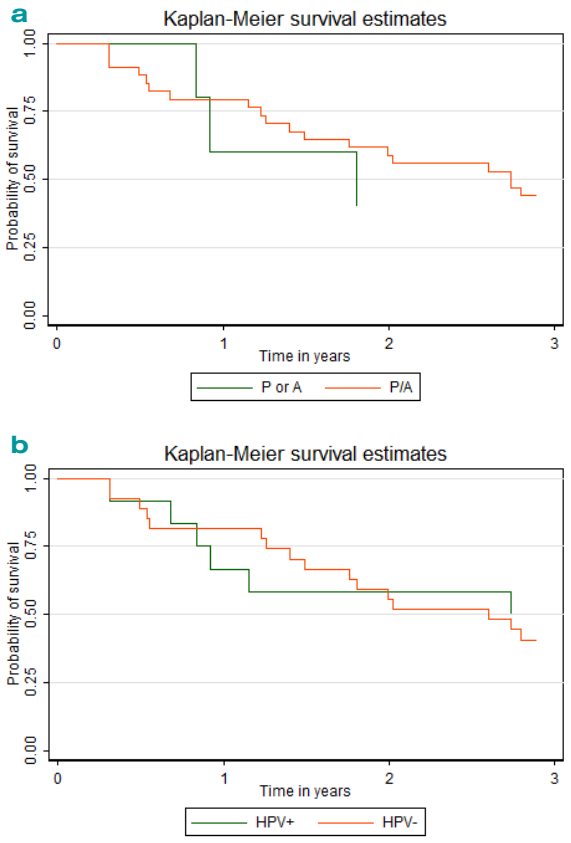

Figure 1. Survival of HNSCC patients according HPV positivity and p53 SNP.

a Patients survival according HPV positivity.

b Patients survival according p53 SNP (P – homozygous for proline, A – homozygous for arginine, P/A – heterozygous).

3. Results

Many authors show the association between HNSCC, HPV infection and other molecular biomarkers – p53, $p16^{INK4a}$ and others. HPV oncoproteins E6 and E7 increase degradation of p53 and interfere with pRb function leading to upregulation of $p16^{INK4a}$ by loss of negative feedback control [8]. Few studies show better survival prognosis in HPV positive HNSCC patients [10-13], some of them analyze p53 importance in the head and neck carcinogenesis. However, the results are controversial: some studies showing p53 expression linked to decreased HNC survival [13-15] and others find no correlation [16-18]. Authors conclude that TP53 wild type has been shown to be highly correlated with HPV infection in HNC whereas TP53 mutations are rare in infected tumors.

Regarding patients survival it was stated that infection with high risk HPV is associated with better prognosis of HNSCC patients [19,20]. However, regarding p53

analysis, some authors show that over expression of p53 is related to worse [21-23] or to better prognosis as well [24,25]. Gillison ML *et al*. [19] says that p53 mutation and HPV infection are independent factors in HNSCC carcinogenesis and prognosis. Smith EM *et al*. [15] evaluated differences in prognosis of HNSCC patients associated with the joint assessment of HPV status and p53 protein over expression. Their results show that evaluating multiple biomarkers give greater variation in clinical outcomes in comparison with assessing the individual biomarkers separately. Authors stated that patients with p53 negative/HPV-HR positive tumors had the highest survival and lowest recurrence rates whereas those with p53 positive/HPV negative tumors had significantly worse outcomes.

On the other hand, not only TP53 mutations, but also single nucleotide polymorphism could impact the HNSCC carcinogenesis. In our small size study 3 year survival of HNSCC patients was significantly associated only with site of primary tumour ($P< 0.05$): it was higher in laryngeal cancer patients and also in patients when tumour was classified as T_{1-2}. HPV infection and p53 SNP didn't show any significant impact to the survival of our patients probably due to small sample size.

In summary, the findings of the authors should assist clinicians in the applying of treatment strategies based on the molecular markers of the tumor. However, these tests should be of low-cost, quick and easily performed. We, as all authors, agree that these findings need further assessment in study with large numbers of HNSCC cases.

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