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ORIGINAL ARTICLE

HPV infection in oropharyngeal squamous cell carcinomas: correlation with tumor size

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Summary

Purpose: *Human papillomavirus (HPV) is implicated in carcinogenesis of a variety of epithelia, including oropharyngeal and laryngeal. High risk (HR) HPV persistent infection in head and neck squamous cell carcinomas (HNSCC) is a significant event, but its influence regarding the prognosis and survival in these patients remains under consideration. Our aim was to analyze a series of oropharyngeal (OP) SCCs at the HPV DNA level, correlating them to the survival status of the corresponding patients.*

Methods: *Using HPV DNA polymerase chain reaction (PCR) microarray technology, 28 formalin-fixed, paraffin-embedded primary OPSCCs were cored and analyzed.*

Results: *Positive DNA amplicons for HPV infection were detected in 3 SCC cases (sub types: HPV 31/35/70). Interestingly, HPV persistent infection was associated with larger tumors (p=0.029) which also affected survival status (p=0.007) in the corresponding patients. Overall survival was also significantly dependent on the stage of the malignancies (p=0.022). Furthermore, tumor size was significantly and negatively correlated with age (p=0.015), meaning that younger patients will probably develop larger tumors.*

Conclusions: *HPV-depended OPSCCs - although not so common as the laryngeal ones, but still not so rare in the rural population in Greece - are characterized by a combination of specific features. Our results showed that survival was adversely effected by the stage of the disease and tumor size and indirectly by the presence of HPV - especially in young adults - while the combined surgery/radiotherapy/chemotherapy therapy seems to prolong survival. Additionally, HPV co-existence seems to be associated with larger tumors and poor survival.*

Key words: *DNA, head and neck, HPV, squamous cell carcinoma*

Introduction

Extensive clinico-molecular analyses have shown that HPV-dependent HNSCCs, demonstrate significant differences regarding sex, genetic, epidemiological, and prognostic features compared to alcohol and tobacco depended ones [1-3]. HR HPV genotype persistent infection leads to neoplastic transformation of the infected host cells due to aberrant expression of viral oncogenes in the corresponding nuclei as directly detected by applying not HPV DNA testing but E6/E7 viral oncogenes mRNA analysis [4,5]. Although HR HPV - mainly HPV 16/18/31/45 types - are molecularly

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recognized in laryngeal SCCs frequently, genotype analyses in oropharyngeal (OP) and tonsilar carcinomas detect a variety of viral subtypes [6]. It is also well known that those SCCs are characterized by a geographic heterogeneity regarding predominantly the prevalence of HPV16 in the corresponding infected sub-populations [7]. Additionally, a crucial parameter in these patients is the impact of HPV infection on disease outcome. According to molecular studies focused on survival analysis and prognostic estimation, HPV-positive patients have been shown to have better responses to radiotherapy and better overall and disease-free survival than HPV-negative patients [8,9]. In conjunction to those significant observations, the role of HR HPV in handling OPSCCs by applying specific targeted therapeutic agents, such as anti-EGFR monoclonal antibodies, remains under investigation [10]. Similarly, the result of HPV infection and prominently modification of the host-cell DNA critically influences the therapeutic regimens including surgery/radiotherapy/chemotherapy or combinations of them in the corresponding patients [11,12]. In the current molecular study, we explored the presence of HPV genotypes in a series of OPSCCs specimens by implementing HPV DNA PCR microarray technology correlating also the results with clinicopathological parameters and survival of the examined patients.

Methods

Patients

Concerning the study group, 28 patients were included (23 males with a mean age of 61.5 years, 5 females with a mean age of 68.2 years). Among them 16 were treated with a combination of radiotherapy and chemotherapy, 5 with a combination of surgery, radiotherapy and chemotherapy, 6 with radiotherapy, whereas one patient was not treated because of advanced stage and rapid malignant progression.

Tissue specimens

For the purposes of this study, 28 formalin-fixed, paraffin-embedded archival tissue specimens of histologically confirmed primary OPSCCs were used. The Medical School, Dept of Pathology ethics committee consented to the use of these tissues for research purposes, according to World Medical Association Declaration of Helsinki guidelines. The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the cor¬responding samples were reviewed for confirmation of histopathological diagnoses. All lesions were classified according to the histological typing, grading and staging criteria of World Health Organization (WHO). Clinicopathological data of the examined cases are demonstrated in Table 1.

HPV DNA analysis (HPV testing)

Thirty-μm tissue sections -focused on carcinoma areas- were removed by microtome slicing and then centrifuged for 5 min at 14,000 rounds per min. The tissue sections were re-suspended in a mix of 180 μl phosphate buffered saline (PBS) (10× concentration (pH 7.4) and 20 μl proteinase K (Roche Diagnostics, Rotkreuz, Switzerland). Samples were vortexed and incubated for one hr at 56°C and one hour at 90°C. DNA was purified using MagNa Pure LC 96 instrument with MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Basel, CH). Aliquots of extracted DNA were stored frozen and used accordingly for HPV CLART genotype arrays testing (Genomica, Madrid, Spain). PCR amplification was performed using CLART HPV2 Amplification kit (Genomica). Five μl of purified DNA were used as template per reaction. Prior to visualization, the PCR products were denatured at 95°C for 10 min. Hybridization was performed using 10 μl of the denatured PCR products on the CLART microarray, and subsequent visualization was done according to manufacturer's specifications. The genotyping results - analyzed and reported automatically on the Clinical Array Reade(Genomica) - are demonstrated in Table 1.

Statistics

The sample consisted of 28 patients (23 males, 5 females) aged between 48 and 82 years (mean±SD 65.5±9.0).The primary outcome was survival time, expressed either in months that ranged from 7 to 38 months (mean±SD 18.9±8.7) or in terms of the binary variable of survival over 12 months, with 7/28 (25%) having deceased within the first year. Independent variables were gender, grade, stage, presence of HPV, kind of therapy and tumor size expressed both as the maximum diameter or the volume of the tumor through the formula: volume = (Height X Width X Length) /2. Depending on the nature of the dependent and independent variables, associations were sought with the independent samples t-test, Pearson's correlation coefficient, the ANOVA procedure with *post-hoc* Bonferroni comparisons, as well as receiver operating characteristics (ROC) analysis and Fisher exact test with odds ratios (OR) with their 95% confidence intervals (CI). The level of significance was set at $p<0.05$. Overall HPV DNA testing results and statistical correlations of the examined cases are demonstrated in Table 2.

Results

According to HPV DNA testing results, positive DNA amplicons for HPV infection were detected in 3 SCC cases (subtypes: HPV 31/35/70). Interestingly, in 2 of them a co-existence of 2 genotypes was confirmed. Survival in months did not depend on the grade or gender (independent samples t-test, p = nonsignificant/NS). Age was also not a significant predictor of survival (r=-0.055, p=NS). ANOVA analysis showed that survival in months was significantly dependent on the stage (p=0.022).

Table 1. HPV DNA testing results and clinicopathological data of the examined cases

a/a	Sex	*Age*	*Anatomic location*	*Grade*	*Stage*	*HPV type*	*Tumour size (diam)*	*Therapy*	*Survival (months)*
1	M	53	Base of tongue	2	T3N1M0	NEG	4X2,2X1,8	S+R+C	31
2	M	59	Base of tongue	2	T4N1M0	NEG	4X2,6X2,I	R+C	28
3	M	48	Tonsil l/ soft palate	2	T4N1M1	**PO-31,70**	5X3X2	R+C	7
4	M	64	Base of tongue	3	T2N0M0	**PO-70**	2X1,7	S+R+C	38
5	M	69	Tonsil/ glosso-epiglottic fold l	3	T4N1M1	**PO-35,70**	4X2X2,5	R+C	9
6	M	72	Tonsil l/ soft palate	3	T2N0M0	NEG	2X1,3	S+R+C	29
7	M	58	glosso-epiglottic fold/base of tongue l	2	T4N1M0	NEG	3,2X1.6X2,8	R+C	11
8	M	78	Base of tongue	2	T4N1M0	NEG	3X1,8X2,1	R+C	13
9	M	63	Base of tongue/ glosso-epiglottic fold l	3	T4N0M0	NEG	2,8X2X1,2	R+C	18
10	F	82	Tonsil l	3	T4N0M0	NEG	2,5X2X2,3	S+R+C	34
11	M	58	retromadibular tiangle l	3	T4N0M1	NEG	1,7X1,2X2	R+C	8
12	M	70	Base of tongue	3	T3N1M0	NEG	1,6X1,8	R+C	15
13	M	57	Base of tongue	3	T4N0M0	NEG	3,1X2,1	R+C	27
14	M	77	Tonsil/base of tongue	3	T4N0M0	NEG	1,4X1,6X2,3	R+C	17
15	M	80	Base of tonque	3	T4N1M0	NEG	2,2X1,3X2,1	R+C	21
16	M	64	Tonsil l/ glosso-epiglottic fold	3	T4N2M0	NEG	2,6X1,8	R	13
17	F	59	Base of tongue	2	T4N1MO	NEG	2,6X1,8	R+C	16
18	M	63	glosso-epiglottic fold l	3	T3N1M0	NEG	1,7X2,1	R+C	22
19	M	76	retromadibular tiangle l	3	T4N1M1	NEG	2,9X2,4	R	12
20	F	56	Tonsil r	3	T4N1M0	NEG	3,1X2,1	R+C	18
21	M	68	Tonsil l/ soft palate	3	T3N1M0	NEG	1,8X2,1	R	15
22	M	66	Base of tongue	3	T4N0M0	NEG	2,3X1,8	R	23
23	M	54	Tonsil l	3	T4N0M0	NEG	2,8X2X1,2	R+C	28
24	F	77	glosso-epiglottic fold l	3	T3N1M0	NEG	1,5X1,9	R	14
25	F	67	retromadibular tiangle l	3	T4NIM1	NEG	3,1X2,1X2	R	9
26	M	61	glosso-epiglottic fold l	3	T4N0M0	NEG	2,8X2X1,2	R+C	17
27	M	60	Tonsil r	3	T3N1M0	NEG	1.6X2,2	S+R+C	29
28	M	75	glosso-epiglottic fold/base of tongue l	3	T4N0MI	NEG	3,2X1.6X2,8	-	8

M: male, F: female, S: surgical operation, R: radiotherapy, C: chemotherapy. PO: positive for HPV infection cases (corresponding HPV types).

Table 2. HPV DNA testing results and statistical correlations (neg=25, pos=3)

Variables	p value
Sex	NS
Age	NS
Grade	NS
Tumour size	0.029
Therapy	NS
Survival	0.007

NS: non significant

The mean survival time of the 20 stage 4 patients was significantly lower than that of the 2 stage 2 patients (16.9±7.8 *vs* 33.5±6.4 months, Bonferroni p=0.023), while the survival time of the 6 patients with stage 3 laid between the above two extremes (21.0±7.6 months). Likewise, survival time was significantly dependent on the kind of therapy (p<0.001). Since the significant difference in survival times was affected by therapy (surgery+radiation+chemotherapy), it was decided to group all other treatments (radiation+chemotherapy without surgery ,only radiation or no treatment at all) due to age or staging. All patients who underwent surgery received also radiation+chemotherapy into one group called “all other treatments”. The

independent samples t-test proved that the 5 patients under surgery + radiation + chemotherapy had doubled the survival time compared to the 23 patients under all other therapies (32.2±3.8 *vs* 16.0±6.4 months, p<0.001).

Furthermore, tumor size, expressed as tumor volume and, as Figure 1 shows, especially the maximum tumor diameter had a significant discrimination ability on the 12 month survival (Area under the curve, AUC=0.789, which is significantly greater than 0.5 of the dotted reference line depicting no discrimination). Subsequent analysis revealed that the cutoff value of the maximum tumor diameter that yielded the best tradeoff between sensitivity and specificity regarding the 12-month survival curve was 2.9 cm. Only one out of the 17 patients (5.9%) with a tumor size less than 2.9 cm deceased during the first year, while among the 11 patients with a tumor size larger than 2.9 cm 6 (54.5%) deceased during the first year (Fisher exact test, p=0.007). The OR was 19.2 (95% CI 1.8-199.9), meaning that the odds of patients dying to patients surviving in the first year was 19 times higher amongst patients with large tumor size greater than 2.9 cm than amongst patients with small tumor size.

Interestingly, tumor size was significantly and negatively correlated with age (r=-0.455, p=0.015), meaning that younger patients would probably have larger tumors. There were only 3 HPV positive patients who did not show to have a direct effect on survival, although 2 of them (66.7%) had a survival time less than 12 months, while from the 25 HPV negative patients only 5 (20%) had a survival time less than 12 months. However, the presence of HPV seemed to be associated with larger tumors. The HPV positive patients had a mean maximum tumor diameter of 3.7±1.5 cm, in contrast to HPV negative patients with a mean maximum tumor diameter of 2.7±0.6 cm (independent t-test samples, t(26)=2.3, p=0.029). Equally, HPV positive patients had a mean tumor volume of 9.5±5.8 cm^3, in contrast to HPV negative patients with a mean tumor volume of 5.4±2.7 cm^3 (independent t-test samples, t(26)=2.1, p=0.042). Thus the effect of HPV presence on survival was expressed rather indirectly through its effect on the size of malignancy, which in its turn, as shown above, had a significant effect on survival.

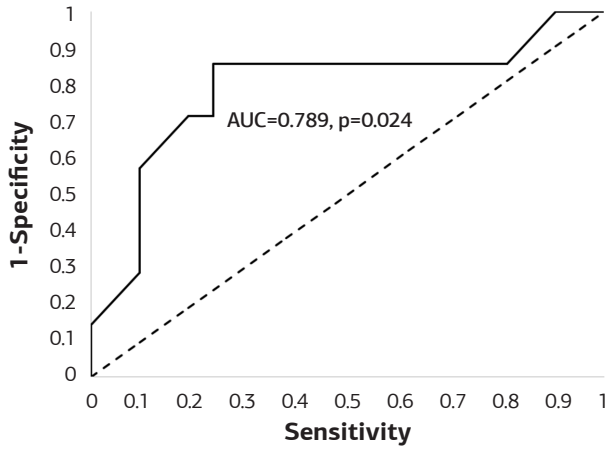

Figure 1. ROC curve showing the significant discrimination ability of the maximum tumor diameter on the 12-month survival.

Discussion

HPV-mediated carcinogenesis in HNSCCs seems to be related with the anatomic location of the infected cells. Genetic analyses have shown that oropharynx predominantly, oral cavity and to a lesser degree larynx are targets for HR-HPV persistent infection [13,14]. The mechanism of host cell malignant transformation includes initially a simple (episomal) viral infection followed by HPV-DNA integration into the host cell genome leading to aberrant oncogene E6/E7 expression. Molecular approaches are based on two main distinct techniques: HPV DNA and mRNA testing, respectively. HPV DNA analysis confirms or not the presence of specific HPV sub-types as a result of true viral intra-cellular infection. In fact, this method detects the DNA volume of isolated or diffuse clusters of episomal viral particles before the corresponding viral genomes penetrate the nuclear micro-area and modify the DNA of the target host-cells [15]. In contrast to HPV DNA detection, HR-HPV oncogenic activity - due to E6/E7 overexpression - is measured by implementing mRNA analysis [16]. Identification of HR-HPV DNA and also genome transcripts at the mRNA level combined to p16 aberrant overexpression are used as surrogate markers for evaluating the impact of the viral infection and progression in the corresponding patients [17].

In the current study we implemented an HPV DNA PCR microarray-based technology assay in a series of OPSCC tissue specimens focused on a Greek sub-population in order to confirm or not the presence of HPV genotypes in the corresponding patients. According to our findings, 3 patients were positive regarding the HPV infection at the DNA level, demonstrating 3 specific subtypes (HPV 31/35/70). This represents a relatively low incidence level of HPV involvement in the current group of OPSCC patients. No analyzed specimen was found to be infected by HPV 16 at the DNA level. Recently published studies focused on HPV DNA analysis in HNSCCs have identified "exotic'' - very rare - subtypes, such as HPV43 and HPV62

in subgroups of laryngeal SCCs, reflecting a broad spectrum of sexually transmitted and persistent HPV infections in different anatomical regions of the head and neck [18]. Interestingly, co-infection was observed in 2 of our examined specimens. Tonsil and base of the tongue were the main anatomical regions in which viral infection was detected. Concerning topography in OPSCC, another previous study demonstrated these two locations as predominant in the development of HR-HPV-depended carcinomas [19]. We also observed that survival was adversely effected by the stage of disease and tumour size and indirectly by the presence of HPV, while the combined surgery/radiotherapy/chemotherapy regimen seems to prolong survival. Although there are controversial conclusions in similar molecular studies, HPV-positive patients demonstrated better responses to radiotherapy and better overall and disease-free survival than HPV-negative patients [20,21]. In contrast, the 2 cases demonstrated HPV co-infection (HPV DNA 31/70 and HPV DNA 35/70, respectively) were characterised by advanced stage and also very poor life span. It seems that HPV multi-infection is correlated with an aggressive phenotype and biological behaviour of the malignancy. Furthermore, the presence of HPV was associated with larger tumours, as tumour size was estimated by tumour volume. Interestingly, younger patients developed larger tumours than the older ones. This is an indirect evidence of a rapid neoplastic proliferation due to HR-HPV DNA transcription and malignant genomic transformation in the affected host cells.

Novel genomic analyses in HPV-related malignant or not neoplasms have shown that oral and oropharyngeal papillomas are not associated with HR HPV infection [22]. It seems that there are different genetic mechanisms in the progression of these HPV-related pathological entities. Furthermore, co-existence of HPV and other viruses, such as human polyomaviruses (HpyVs), is unclear regarding their involvement in the carcinogenetic process. A study analyzing the co-infection of HPV and Merkel cell polyomavirus (MCPyV) concluded that although detection of these two viruses was relatively frequent in tonsillar carcinomas, MCPyV persistent infection was not correlated significantly with carcinoma development presented also in normal tonsillar tissue [23]. Concerning the heterogeneous response to treatment, differences in recurrence and survival that HPV-dependent OPSCCs demonstrate compared to non-HPV ones, there are novel genomic data explaining partially the genetic complexity that characterizes them. A study group based on genome-wide loss of heterozygosity (LOH) and DNA copy number aberration (CNA) in HPV-negative malignancies identified specific regions in 4q, 8p, 9p and 11q that seem to play an important role in oral cancer biology and survival regarding the disease [24]. Additionally to these significant molecular reports, a recently published extensive genome-wide association analysis showed that OPSCC is characterized by a spectrum of specific alterations including specific gene loci such as 6p21.32, 10q26.13, and 11p15.4. Especially, OSCCs are related also with 2p23.3 and 9q34.12, and with known cancer-related loci-9p21.3 and 5p15.33. Interestingly, in HPV-positive SCC cases, the presence of HLA allele imputation showed a protective association with the class II haplotype HLA-DRB1*1301-HLA-DQA1*0103-HLA-DQB1*0603 [25]. Such novel molecular information are very important and promising for creating unique genetic signatures in HPV-positive and - negative OPSCCs that could affect prognosis and response to specific treatment regimens in the corresponding patients.

In conclusion, analyzing a sub-group of patients with OPSSC in a rural Greek population we observed that survival was adversely effected by the stage of disease, tumour size and indirectly by the presence of HPV while the combined surgery/radiotherapy/chemotherapy treatment seemed to prolong survival. HR-HPV persistent infection is correlated to a rapid progression of the malignancy mainly in young patients, especially in cases of co-infection reflecting an increased rate of cancer cell proliferation.

Conflict of interests

The authors declare no conflict of interests.

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