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Human papillomavirus tumor-infiltrating T-regulatory lymphocytes and P53 codon 72 polymorphisms correlate with clinical staging and prognosis of oropharyngeal cancer

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SUMMARY

The association between human papillomavirus (HPV) DNA positivity, p53 codon 72 polymorphisms, and the type of leukocyte infiltration in head and neck squamous cell carcinomas (HNSCC) and their combined impact upon patient survival is poorly investigated. For this reason, leukocyte infiltration profile and p53 codon 72 polymorphisms were assessed in freshly removed HNSCC specimens (N=71 patients). HPV detection was performed by nested-PCR followed by DNA sequencing. Viral loads were determined by quantitative RT-PCR. The choice to investigate fresh instead of archive paraffin-embedded specimens was privileged to avoid possible artifacts due to sample processing. HPV DNA was detected in 14% of cases. Oropharyngeal carcinomas were the most frequently associated with the presence of HPV16 DNA (41%) and were associated with p53 Pro/Pro or Pro/Arg polymorphisms. In HPV16-positive oropharyngeal carcinomas increased infiltrations of CD3+ and FoxP3+ T-cells correlated with higher HPV16 copy numbers. The presence of HPV may trigger a stronger immune response and may be considered a reliable marker for clinical staging and a more favorable prognosis of oropharyngeal carcinoma.

KEY WORDS: HNSCC, HPV, Tumor-infiltrating lymphocytes, p53, Prognosis.

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with more than 600,000 patients diagnosed with cancer of the oral cavity, oropharynx and larynx each year (Li *et al.*, 2003). Carcinogenesis in HNSCC is a multifactorial process, involving mutations in oncogenes or tumor suppressor genes, as well as environmental factors such as tobacco chewing or smoking, poor oral hygiene, alcohol abuse, or infection by certain human papillomavirus (HPV) genotypes (Serefoglou *et al.*, 2008). More than 180 papillomaviruses have been identified to date, with approximately 120 genotypes isolated from humans (Bernard *et al.*, 2010). The oncogenic types are classified as either high- or low-risk according to their ability to induce cancer growth. Most HPV-associated HNSCCs tend to occur in the oropharynx, with the tonsil area being the most commonly affected area and where HPV is present in 45-70% of cases (Mellin *et al.*, 2002). HPV16 is the predominant genotype in HNSCC tumors, although its prevalence changes for different anatomical sites. These different subsets of HNSCC present clinical and molecular distinctions, as well as distinct patient

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prognoses. For example, the presence of HPV in oropharyngeal tumors is associated with more favorable prognoses compared to HPV-negative tumors (Chung *et al.*, 2009, Fakhry *et al.*, 2006, Gillison. 2009, Rautava *et al.*, 2012).
Molecular studies have shown how expression of the viral oncoproteins E6 and E7 disrupts crucial cellular events involved in the control of the cell cycle and apoptosis. Specifically, the E6 oncoprotein inactivates p53, whereas E7 inactivates Rb. The p53 protein, encoded by the TP53 gene, plays a pivotal role in cell regulation pathways, influencing the transcription and activity of several replication and transcription factors. p53 activation leads to the induction of DNA repair mechanisms, the induction of cell cycle arrest, and the prevention of cancer growth via enhanced apoptosis (Orsted *et al.*, 2007). The p53 polymorphism at codon 72 of exon 4, causing an Arg72Pro substitution in the putative SH3 binding domain of p53, influences the binding capacity and functional properties of p53 (Marin *et al.*, 2000). The Arg (R) allele presents a greater ability of p53 to locate to mitochondria and induce cell death, whereas the Pro (P) allele exhibits a lower apoptotic potential and promotes cell cycle arrest in the G1 phase (Dumont *et al.*, 2003, Pim *et al.*, 2004, Storey *et al.*, 1998). Despite the greater susceptibility of the Arg72 form to degradation mediated by the HPV-E6 oncoprotein, the relationship between p53 codon 72 polymorphism and HPV-related HNSCC is still unclear. Some studies have failed to demonstrate a major role of the p53 polymorphism in head and neck carcinogenesis (either taken alone or in association with HPV status) (Hamel *et al.*, 2000, McWilliams *et al.,* 2000, Shen *et al.*, 2002), whereas others have shown that the RP genotype is associated with a reduced risk of developing oropharyngeal SCC (Hoffmann *et al.*, 2009, Perrone *et al.*, 2007). Differences in study sample size, sampling methods, and geographical distribution may account for the discrepancies reported in the recent literature.
The infiltration of leukocytes into the neoplastic microenvironment is a common feature of many epithelial malignancies. For this reason, HNSCC have been extensively investigated and the strong inflammatory component of the tumors is well established. In addition to the presence of macrophages, a strong degree of T lymphocyte infiltration, presenting all the components of an adaptive immune reaction (i.e. CD3, CD4, and CD8), can be detected in some tumors (Fridman *et al.*, 2011). In patients with head and neck cancers, the antitumor functions of T lymphocytes are often compromised (Rabinowich *et al.*, 1998, Reichert *et al.*, 1998), but the presence of functional T-cells, as assessed by the expression of the CD3-ζ chain or a good proliferative response of lymphocytes to CD3 antibodies, has been associated with better patient survival and prognosis (Badoual *et al.*, 2006). Moreover, the level of regulatory CD4+CD25+ T-cells has been found to be elevated in both peripheral blood and tumors of patients with HNSCC (Strauss *et al.*, 2007).
The present study attempted to verify whether the presence of HPV associated with the p53 codon 72 polymorphism and the type of leukocyte infiltration could:

1) provide a reliable array of markers capable of predicting the development of head and neck tumors;
2) represent a prognostic tool for following tumor progression. For this purpose fresh instead of archive paraffin-embedded specimens were employed to avoid possible artifacts due to sample processing.

MATERIALS AND METHODS

Patients

Investigations involved 71 patients with primary SCCs of the head and neck district, diagnosed and treated in the Department of Otolaryngology, San Giovanni Battista Hospital, Turin, between 2005 and 2010. Written informed consent was obtained from the patients, and ethical approval for this study was granted by the San Giovanni Battista Hospital Research Ethics Committee, Turin. The present study was conducted according to the Declaration of Helsinki principles.
All the patients underwent surgery as first-line treatment. All the tumors included in the study were histopathologically diagnosed as squamous cell carcinoma. Tumors were staged according to the AJCC/UICC TNM Staging System 2009 (Sobin *et al.*, 2009). Information on patient tobacco and alcohol consumption was obtained from patient medical files. Patients who currently smoke were classified as light (<10 cigs/day), medium (10-20

cigs/day), heavy (>20 cigs/day) or non-smokers. Patients were classified as moderate, heavy, or non-drinkers, according to the American Dietary Guidelines on alcohol consumption (Services *et al.*, 2005).

Tumor samples

Tumor samples were collected at the time of surgery. A fragment was excised and immediately snap-frozen in liquid nitrogen and stored at -70°C. The tissue specimens comprising the remaining tumor tissue were fixed in buffered formaldehyde 4% and paraffin-embedded for histopathological analysis. All specimens were stained with hematoxylin and eosin to determine histology and analyzed by two independent pathologists.

DNA extraction, HPV detection and viral load determination

DNA extraction from fresh-frozen samples was performed by using the Nucleo Spin Tissue extraction kit (Machery-Nagel, Germany), and in accordance with the manufacturer's protocol. All specimens were examined by β-globin PCR to estimate the quantity of DNA and to control its quality, as previously described (Rittà *et al.*, 2009). HPV DNA analysis was performed by nested PCR assays, using MY09/MY11 as the outer and GP5+/GP6+ as the inner primers, the latter amplifying a 140-bp fragment within the HPV L1 open reading frame (Fuessel Haws *et al.*, 2004, Rittà *et al.*, 2009). All PCR reactions were carried out using REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich).

Positive PCR products were purified using a purification kit (NucleoSpin Extract II, Macherey Nagel) and verified by direct sequencing with the GP5+ primer on a DNA sequencer (PRIMM, Milan, Italy). HPV genotypes were determined on the basis of >95% homology with sequences deposited in GenBank using the BLAST network service at NCBI (http://blast.ncbi.nlm.nih.gov/).

Tumor DNA p53 codon 72 polymorphisms were genotyped using PCR followed by Restriction Fragment Length Polymorphism (RFLP) analysis. Amplifications were performed with the primers 5'-ATCTACAGTCCCCCTTGCCG-3' and 5'-GCAACTGACCGTGCAAGTCA-3'. The PCR reaction was performed in a 25 µL final volume, containing 25 pmol of each primer, ~100 ng of extracted DNA, and 12.5 µL of Ready Mix PCR Reaction Mix (Sigma). The PCR thermal profile comprised an initial denaturation for 4 minutes at 94°C, followed by 30 cycles of: 94°C for 40 sec, 56°C for 30 sec, and 72°C for 40 sec; a final elongation step occurred at 72°C for 10 min. The reaction products were digested with *Bsh1236I (BstUI)* (Fermentas, Vilnius, LT) for 3 hours at 37°C. The digested DNA was subjected to electrophoresis in 2% agarose gels, followed by ethidium bromide staining. The *Pro* allele resulted in an undigested 296-bp band, whereas the *Arg* allele was indicated by the presence of two bands of 169 and 127 bp (Ji *et al.*, 2008, Li *et al.*, 2010).

To determine HPV16 load, a type specific Q-PCR protocol was performed with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) according to the manufacturer's instructions, using the Mx3000P thermocycler (Stratagene). A specific primer set was designed to amplify the *E7* region of HPV16 (5'-GAGATACACCTACATTGC-3' and 5'-CACACAATTCCTAGTGTG-3'). A Standard curve was generated in the same PCR run with HPV16 plasmid dilutions ranging from 1 to 10^6 copies per sample in an HPV-negative human DNA solution. To correct for PCR efficiency and DNA integrity and to determine the number of input cell equivalents, the gene GAPDH was quantified using the following primers: 5'-AACGTGTCAGTGGTGGACCTG-3' and 5'-AGTGGGTGTCGCTGTTGAAGT-3'. The samples were run in duplicate and the final result expressed as the number of HPV16 copies per cell.

Immunohistochemistry (IHC)

IHC was performed on formalin-fixed and paraffin-embedded biopsy samples (2 µm-thick), using the completely automated Leica BOND-MAX™ instrument. The following antibodies were applied: anti-CD3 (clone PS1, working dilution 1:50; Novocastra, Newcastle Upon Tyne, UK); anti-CD4 (clone 1F6, working dilution 1:40; Novocastra); anti-CD25 (clone 4C9, working dilution 1:50; Novocastra); anti-CD8 (clone C8/144B, working dilution 1:50; DakoCytomation, Denmark); anti-CD20 (clone L26, working dilution 1:200; DakoCytomation); anti-CD68 PGM1 (clone PGM1, working dilution 1:50; DakoCytomation); anti-FoxP3 (clone 236A/E7, working dilution 1:100; eBioscience, Inc. San Diego, CA, USA). Immunostaining was evaluated by three independent pathologists (MR, JM

and VL). To confirm the reproducibility, 25% of the slides were selected at random and scored twice. Positive cells were stained dark brown with cytoplasmic positivity for CD3, CD4, CD8, CD25, CD20, CD68 PGM1 and nuclear for FoxP3. Numbers of labeled tumor infiltrating cells are reported as percentages of positively stained tumor infiltrating cells.

Statistical and overall survival analysis

All statistical tests were performed using GraphPad Prism version 5.00 for Windows. Chi-square and Fisher's exact test were used to test for differences between the groups. The Kaplan Meyer method was used to estimate survival curves, which were compared using a 2-sided log-rank test. Overall patient survival times were defined as the interval between the date of surgery and the last date when the patient was known to be alive or the date of death for any reason.

RESULTS

Clinical-pathological features

The 71 lesions included 22 cases of SCC located in the oropharynx (31%), 22 SCC in the oral cavity (31%) and 27 cases in the larynx (38%). The

TABLE 1 - *HPV status and clinical and histopathological parameters of the HNSCC patients.*

			HPV status		
		*patients no. (%)**	*HPV negative no. (%)**	*HPV positive no. (%)**	*p value*
Gender	female	12 (16.9)	9 (12.7)	3 (4.2)	
	male	59 (83.1)	52 (73.2)	7 (9.9)	0.3565
Smoking	non smokers	10 (14.1)	7 (9.9)	3 (4.2)	
	<10 cigs/day	9 (12.7)	7 (9.9)	2 (2.8)	
	10-20 cigs/day	25 (35.2)	20 (28.2)	5 (7)	
	>20 cigs/day	27 (38)	27 (38)	0	0.0518
Alcohol consumption	no	16 (22.5)	15 (21.1)	1 (1.4)	
	moderate	46 (64.8)	38 (53.5)	8 (11.3)	
	heavy	9 (12.7)	8 (11.3)	1 (1.4)	0.5239
Tumor site	oral cavity	22 (31)	21 (29.6)	1 (1.4)	
	oropharynx	22 (31)	13 (18.3)	9 (12.7)	
	larynx	27 (38)	27 (38)	0	<0.0001
Grading of differentiation	g1	12 (16.9)	12 (16.9)	0	
	g2	42 (59.2)	35 (49.3)	7 (9.9)	
	g3	17 (23.9)	14 (19.7)	3 (4.2)	0.3047
T classification	T1	15 (21.1)	13 (18.3)	2 (2.8)	
	T2	29 (40.8)	24 (33.8)	5 (7)	
	T3	13 (18.3)	11 (15.5)	2 (2.8)	
	T4	14 (19.7)	13 (18.3)	1 (1.4)	0.8443
N classification	N0	32 (45.1)	30 (42.3)	2 (2.8)	
	N1	10 (14.1)	7 (9.9)	3 (4.2)	
	N2	29 (40.8)	24 (33.8)	5 (7)	0.1384
M classification	M0	71 (100)	61 (85.9)	10 (14.1)	
	M1	0	0	0	NA
Age at diagnosis (mean)			61 yrs	61.9 yrs	0.8116

*Number of patients (percentage of patients).

average age of the patients was 68 years for females (range 55-80) and 60 years for males (range 38-85); total number of females was 12, compared to 59 males. The characteristics of the study group and the histopathologic parameters are summarised in Table 1.

HPV DNA detection and viral load analysis

The global prevalence of HPV in HNSCC and its distribution across the various anatomical carcinoma sites remain unclear. The frequency of HPV-positive tumors in HNSCC varies depending on the population studied, the location of the tumors, and the HPV detection method used (Kreimer *et al.*, 2005, Wang *et al.*, 2012). The results of PCR-based HPV detection methods may be influenced by the anatomical source of the clinical specimen and by the DNA-extraction method used, since the nucleic acids in formalin-fixed, paraffin-embedded tissues are only amplified in a reliable manner when PCR products are generated that are smaller than 150-bp due to fixative-induced cross links (Brink *et al.*, 2007). In the present study, the prevalence of HPV infection was therefore investigated only using fresh-frozen specimens to avoid the pitfalls caused by the use of formalin and paraffin reagents. As a consequence, the number of samples processed is lower, but still reliable for a correct statistical analysis.

All 71 frozen-tissue samples turned out to be β-globin positive, and were consequently investigated using high sensitivity nested-PCR for HPV detection; MY09-MY11 were used as the outer and GP5+-GP6+ as the inner primers. HPV DNA was detected in 10 of the 71 cases (14%). Sequencing revealed all 10 HPV DNA-positive samples to be infected by HPV16, with only one case of co-infection by HPV16 and HPV33.

Oropharyngeal carcinomas turned out to have the highest HPV incidence, with 9 out of 22 cases (41%) positive for HPV16. The oropharyngeal SCC positive for both HPV16 and HPV33 was localized to a tonsil. The tenth case of HPV16 infection was located to the oral cavity, and was the only one of the 22 cases of carcinoma arising in the oral cavity to be positive for HPV-positivity (4.5%). In carcinomas of the larynx, HPV was not detected in any of the 27 cases analyzed.

The viral load in all 10 HPV-positive cases was quantifiable and showed great variability (ranging between $7.4*10^{-4}$ and 4.56 HPV16 copies per cell), with the highest value detected in an oropharyngeal carcinoma involving the tonsil and the base of tongue, and the lowest in a carcinoma of the oral cavity (tongue). No correlation was found between gender, age at diagnosis, TNM classification, grading of differentiation, or HPV infection. A significant correlation was observed between the smoking history and the presence of HPV-DNA: in the cohort of heavy smokers (defined as smoking more than 20 cigarettes a day), no case of HPV infection was found. Only in patients with a smoking history of less than 20 cigarettes a day (7 cases) and in non-smokers (3 cases) was HPV DNA found (p=0.0518). The history of alcohol consumption did not seem to be related to the presence of HPV DNA in tumors. All data are reported in Table 1.

p53 codon 72 polymorphism

The tumor suppressor protein p53 can be bound, degraded and inactivated by the human HPV16 E6 oncoprotein (Scheffner *et al.*, 1990). The susceptibility of p53 to the HPV16 oncoprotein may be influenced by the p53 codon 72 polymorphism (Ji *et al.*, 2008). To analyze the p53 status in our cohort of patients, p53 RFLP analysis was performed on all 71 tumor specimens; the results are displayed in Table 2. The homozygous allele for p53 Arg was the most commonly detected (32 cases: 45.1%); 30 tumors (42.3%) possessed the heterozygous genotype (p53 Arg/Pro) and 9 cases (12.6%) were homozygous for p53 Pro. No correlations were found between p53 polymorphism genotype and tumor site, grading, TNM staging or any other clinical-pathological feature (Table 2). Nevertheless, a significant association was observed between p53 codon 72 polymorphism and the presence of HPV. As shown in Table 3, the majority of HPV-positive tumors presented a Pro/Arg p53 polymorphism at codon 72 (8 out of 10 HPV-positive tumors, p=0.0278). RFLP analysis revealed that 8 out of 9 HPV16-positive oropharyngeal carcinomas possessed the p53 Pro/Arg allele; with the ninth case being Pro/Pro (p=0.0217). This association between HVP-positivity and p53 polymorphisms in oropharyngeal carcinomas was maintained when we compared the HPV profiles of Pro/Pro and Pro/Arg patients against those of Arg/Arg patients: collectively, 9 of the 17 PP/PR patients turned out to be HPV16

TABLE 2 - *p53 codon 72 polymorphisms according to clinical and histopathological parameters of the HNSCC patients.*

			p53 codon 72 polymorphism			
		*patients no. (%)**	*Pro/Pro no. (%)**	*Pro/Arg no. (%)**	*Arg/Arg no. (%)**	*p value*
Gender	female	12 (16.9)	0	5 (7)	7 (9.9)	
	male	59 (83.1)	9 (12.7)	25 (35.2)	25 (35.2)	0.3019
Smoking	no smoking	10 (14.1)	1 (1.4)	4 (5.6)	5 (7)	
	<10 cigs/day	9 (12.7)	1 (1.4)	2 (2.8)	6 (8.5)	
	10-20 cigs/day	25 (35.2)	4 (5.6)	15 (21.1)	6 (8.5)	
	>20 cigs/day	27 (38)	3 (4.2)	9 (12.7)	15 (21.1)	0.261
Alcohol consumption	no	16 (22.5)	2 (2.8)	8 (11.3)	6 (8.5)	
	moderate	46 (64.8)	6 (8.5)	19 (26.8)	21 (29.6)	
	heavy	9 (12.7)	1 (1.4)	3 (4.2)	5 (7)	0.9308
Tumor site	oral cavity	22 (31)	1 (1.4)	7 (9.9)	14 (19.7)	
	oropharynx	22 (31)	5 (7)	12 (16.9)	5 (7)	
	larynx	27 (38)	3 (4.2)	11 (15.5)	13 (18.3)	0.0750
Grading of differentiation	g1	12 (16.9)	1 (1.4)	3 (4.2)	8 (11.3)	
	g2	42 (59.2)	5 (7)	18 (25.4)	19 (26.8)	
	g3	17 (23.9)	3 (4.2)	9 (12.7)	5 (7)	0.4053
T classification	T1	15 (21.1)	5 (7)	5 (7)	5 (7)	
	T2	29 (39.4)	3 (4.2)	13 (18.3)	13 (18.3)	
	T3	13 (18.3)	1 (1.4)	7 (9.9)	5 (7)	
	T4	14 (19.7)	0	5 (7)	9 (12.7)	0.1325
N classification	N0	32 (45.1)	4 (5.6)	11 (15.5)	17 (23.9)	
	N1	10 (14.1)	2 (2.8)	4 (5.6)	4 (5.6)	
	N2	29 (40.8)	3 (4.2)	15 (21.1)	11 (15.5)	0.6461
M classification	M0	71 (100)	9 (12.7)	30 (42.3)	32 (45)	
	M1	0	0	0	0	NA
Age at diagnosis (mean)			61.7 yrs	61.7 yrs	61.4 yrs	0.9943

* Number of patients (percentage of patients).

TABLE 3 - *Distribution of p53 codon 72 polymorphisms according to HPV status in the three anatomic sites.*

	p53 codon 72 polymorphism			
	*Pro/Pro no. (%)**	*Pro/Arg no. (%)**	*Arg/Arg no. (%)**	*p value*
Oropharynx carcinoma				
HPV neg	4 (18.2)	4 (18.2)	5 (22.7)	
HPV pos	1 (4.5)	8 (36.4)	0	0.0217
Oral cavity carcinoma				
HPV neg	1 (4.5)	7 (31.8)	13 (59.1)	
HPV pos	0	0	1 (4.5)	0.7413
Larynx carcinoma				
HPV neg	3 (11.1)	11 (40.7)	13 (48.2)	
HPV pos	0	0	0	NA

*Number of patients (percentage of patients).

positive, whereas none of the 5 Arg/Arg cases were HPV positive (p=0.0343, data not reported).

Immunohistochemical analysis

Many studies report the presence of tumor-infiltrating lymphocytes (TILs) as representing a survival factor in HNSCC (Fridman *et al.*, 2011). Moreover, FoxP3+CD4+ T-cells positively influence locoregional control in patients with head and neck cancers by down-regulating harmful inflammatory reactions known to favor tumor progression (Badoual *et al.*, 2006). In an attempt to verify the role of HPV infection on TIL frequency and phenotype, IHC analysis was performed on the 22 cases of oropharyngeal SCC. The results of the immunohistochemical assays are illustrated in Figure 1. No difference was found in the extent of tumor infiltration by CD25+, FoxP3+, CD3+, C68+, CD20+, CD8+, or CD4+ cells in HPV-positive versus HPV-negative tumors. The results are summarized in Table 4. Moreover, no significant correlations were found between the TIL subpopulations present in the tumors and their pathological classifications, as determined by grading and TNM status (data not shown).

Viral loads were determined in the HPV16-positive oropharyngeal carcinomas in an attempt to verify whether they were correlated with the phenotype of TILs present. HPV16 load values equal or greater than 10^{-1} copies per cell were associated with higher densities of tumor infiltrating CD3+ and FoxP3+ cells. The mean density of FoxP3+ cells was 52.5% for carcinomas with HPV16 viral loads $\geq 10^{-1}$ copies/cell, and 27.5% in carcinomas with HPV16 viral loads $<10^{-1}$ copies/cell (p=0.0469). A similar correlation was found for CD3+ tumor infiltrating cells, with mean densities of 80% and 37.6% for carcinomas with HPV16 viral loads $\geq 10^{-1}$ and $<10^{-1}$ copies/cell, respectively (p=0.0352). Data are summarized in Table 5.

Prognosis

Among the patients presenting oropharyngeal carcinomas, those with tumors positive for HPV16 turned out to have better clinical outcomes than those negative for HPV. Kaplan-Meier analysis showed that patients with HPV16-positive tumors had significantly better overall survival rates than patients with HPV-negative tumors (p=0.0468). The median survival time was

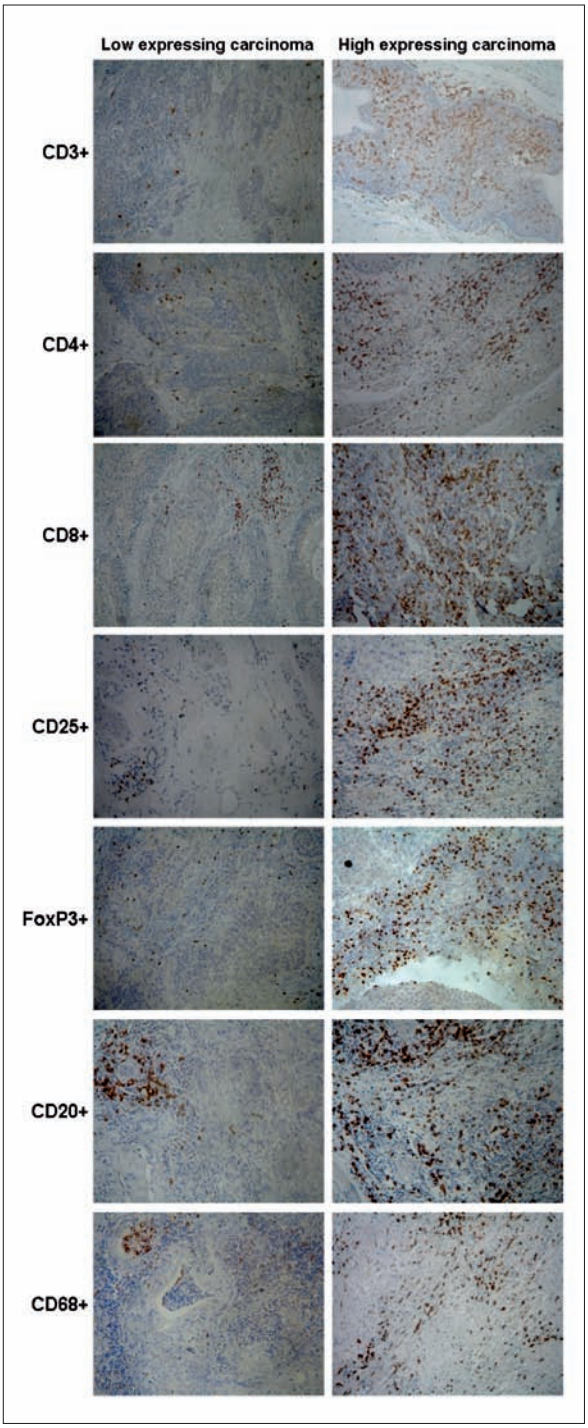

FIGURE 1 - *Representative immunohistochemical staining of CD3+, CD4+, CD8+, CD25+, FoxP3+, CD20+, and CD68+ tumor-infiltrating cells in low-expressing (left panels) and high-expressing (right panels) HNSCC. Immunohistochemical staining shows positive cells in brown, counterstained with hematoxylin (original magnification 200X).*

TABLE 4 - *Differences between HPV-positive and -negative oropharyngeal carcinomas with respect to tumor infiltrates.*

	*HPV+ oropharynx carcinomas (mean density)**	*HPV- oropharynx carcinomas (mean density)**	*p value*
CD3+	49.3%	35%	0.3198
CD4+	55%	37.1%	0.2932
CD8+	41.7%	26.8%	0.3012
CD25+	50.1%	34.8%	0.1977
FoxP3+	40%	31.7%	0.5540
CD20+	29.5%	28.8%	0.9558
CD68+	64.5%	58.5%	0.7232

*Mean density of tumor infiltrating leukocytes.

46 months in the HPV-negative group, whereas it was undefined in the HPV-positive group (Figure 2). Focusing on the HPV-positive oropharyngeal carcinomas, the HPV16 viral load values (as determined by quantitative RT-PCR) did not turn out to represent a significant predictor of clinical outcome (data not shown). A survival analysis was also performed with respect to the p53 codon 72 polymorphism results. The genetic polymorphisms were not associated with any clinical outcomes in any carcinoma subgroup (p=0.7548, p=0.2612, and p=0.7491 for carcinomas located to the oropharynx, oral cavity, and larynx respectively).

DISCUSSION

Increasing epidemiological evidence posits the existence of a subgroup of head and neck cancers localized to the oropharynx that are causally associated with HPV infection, and that are char-

TABLE 5 - *Mean density of tumor infiltrates in low- and high-HPV16 viral load oropharyngeal carcinomas.*

	*Low-HPV16 viral load carcinomas (<10^{-1} copies/cell) (mean density)**	*High-HPV16 viral load carcinomas (≥10^{-1} copies/cell) (mean density)**	*p value*
CD3+	37.6%	80%	0.0352
CD4+	51.3%	61%	0.4037
CD8+	40%	45%	0.8640
CD25+	48%	58.2%	0.2249
FoxP3+	27.5%	52.5%	0.0469
CD20+	17.3%	54%	0.0742
CD68+	59.3%	75%	0.5625

*Mean density of tumor infiltrating leukocytes.

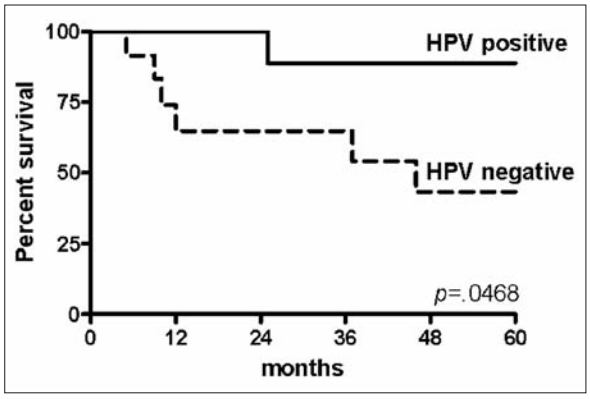

FIGURE 2 - *Cumulative prognostic value of human papillomavirus (HPV) positivity for patients with squamous cell carcinoma of the oropharynx, expressed as probability of overall survival.*

acterized by a markedly better prognosis than that for HPV-negative tumors arising in the same anatomical area (Gillespie *et al.*, 2009). In an attempt to disclose, at least in part, the mechanisms responsible for this favorable response, the present study investigated the potential correlations between the presence of HPV and the tumor profiles of infiltrating leukocytes and p53 codon 72 polymorphisms. Both of these parameters have been shown to correlate with favorable prognosis of the oropharynx carcinoma (Li *et al.,* 2010). To avoid possible artifacts due to sample processing we decided to use fresh instead of archive paraffin-embedded samples. As a consequence, the number of samples available was lower, but still suitable for a correct statistical analysis.

The results obtained with fresh frozen samples showed how patients with oropharyngeal carcinoma harboring HPV16 exhibited a better overall survival (5 year follow-up) than those negative for HPV (p=0.0468). This finding is in line with recent reports (Gillison. 2006, Hansson *et al.*, 2005, Mannarini *et al.*, 2009, Mork *et al.*, 2001) showing that HPV infection of oropharyngeal carcinomas is associated with a reduced risk of relapse and second tumors compared to HPV-negative tumors. The mechanisms underlying the improved clinical outcome of HPV-positive SCC are still unclear. A few studies have suggested that the better prognosis could be related to an enhanced radiosensitivity of HPV-positive SCC compared to HPV-negative ones. Furthermore, HPV16 positive HNSCC are more likely to carry wild type TP53 and express p16. The presence of functional TP53 in HPV-positive HNSCC may render the tumors susceptible to radiation-induced apoptosis. Another reason appears to be the absence of carcinogen-induced early genetic changes in the epithelium and the development of multifocal tumors (the so-called "field cancerization") (Mannarini *et al.*, 2009, Slaughter *et al.*, 1953).

Recent studies have shown that patients with HPV16-positive HNSCC expressing wild-type TP53 or p16 have improved disease-free survival, supporting the notion that the improved prognosis may in fact be related to HPV infection. We found a significant association between the presence of the p53 Pro/Pro or Pro/Arg polymorphism and the presence of the HPV16 genome in the subgroup of oropharyngeal carcinomas. Indeed, all of the 9 HPV-positive tumors arising in the oropharynx presented the p53 Pro allele (p=0.0343). This finding is in agreement with the hypothesis that the Pro allele may enhance the carcinogenetic potential of head and neck HPV-positive cells, which may also be related to the reduced capacity of the Pro72 form of p53 to induce apoptosis compared to Arg72 form. Consistent with our results, Perrone *et al.* reported a greater frequency of p53 Pro homozygosity in their HPV-positive tumor group, and postulated the presence of the p53 Pro allele as being a risk factor for the development of HPV-associated oropharyngeal SCC (Perrone *et al.*, 2007). Moreover, in line with our finding, Ji *et al.* (*Ji et al.*, 2008) demonstrated that HPV16 seropositivity was associated with an increased risk of squamous cell carcinoma of the oropharynx, especially among never-smokers and subjects with the p53 codon 72 Arg/Pro and Pro/Pro genotypes. Altogether, these results suggest that the p53 codon 72 variant genotypes modify the risk of HPV16-associated SCC of the oropharynx, especially among never-smokers.

In humans, regulatory CD4+CD25+ T-cells have been found to be increased in tumor-infiltrating lymphocytes and in the peripheral circulation of patients with various malignancies, including head and neck cancer (Badoual *et al.*, 2006, Schaefer *et al.*, 2005). In non-small cell lung cancer (NSCLC) there is a marked infiltration of different types of immune cells, and the distribution, tissue localization, and cell types are significantly associated with cancer progression and

patient survival (Bremnes *et al.*, 2011). However, the accumulation of T-regs in various human carcinomas is generally associated with a poor prognosis, as they are expected to inhibit anti-tumor immune responses (Ladoire *et al.*, 2011). In hepatocellular carcinoma, it has indeed been reported that the prevalence of tumor-infiltrating T-regs significantly increases in a stepwise manner during the progression of hepatocarcinogenesis, whereas the prevalence of tumor infiltrating CD8+ T lymphocytes decreases significantly with disease progression (Hiraoka 2010). This relationship has also been reported in a wide range of localized or metastatic human carcinomas, including breast (Bates *et al.*, 2006), ovarian (Curiel *et al.,* 2004), lung (Petersen *et al.*, 2006), renal cell (Li *et al.*, 2009), pancreatic (Hiraoka *et al.*, 2006), and gastric carcinomas (Perrone *et al.*, 2008). However, conflicting data have accumulated suggesting that high tumor infiltration by FoxP3+ T-regs is not always associated with a poor prognosis, but, on the contrary, can be associated with an improved prognosis in some cancer types. Indeed, in the context of colorectal carcinoma, various studies have demonstrated the favorable prognostic importance of CD8+ and CD45R0+ T-cell densities, the associated high degree of tumor infiltration with FoxP3+ T-cells, and better patient survival (Ladoire *et al.*, 2011). The presence of FoxP3+ CD4+ regulatory T-cells may also be linked with better locoregional control in HNSCC, most likely involving the down-regulation of an inflammatory reaction, but which could also favor tumor progression (Badoual *et al.*, 2006, Balkwill *et al.*, 2004, Schaefer *et al.*, 2005). In our study, no correlation between CD3+ and FoxP3+ T-cells and a favorable patient follow-up was observed. However, a significantly higher level of CD3+ and FoxP3+ T-cell infiltration was observed in oropharyngeal tumors displaying high HPV16 copy numbers. As oropharyngeal carcinomas harboring HPV16 exhibit a better overall survival, it is conceivable that the presence of HPV may trigger a stronger immune response responsible for a more favorable prognosis of the tumors arising in this anatomical site.

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