Head and neck squamous cell carcinoma: role of the human papillomavirus in tumour progression

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SUMMARY

High risk human papilloma viruses (HPVs) have been shown to be independent risk factors for anogenital tract cancers, and have also been detected in head and neck squamous cell carcinomas (HNSCC). The aim of our study was to determine the prevalence of HPV DNA in a group of 47 squamous cell carcinomas of the oropharynx and the oral cavity, and to compare the clinical behaviour of HPV positive and negative tumours. We also assessed the proliferation index, as evaluated by Ki67 immunohistochemistry positivity, and the level of p53 reactivity. HPV DNA was found in 50% of carcinomas of the oropharynx and 36% in those of the oral cavity, the only genotype detected being HPV 16. Patients with HPV-positive carcinomas had a better overall survival than those with HPV-negative carcinomas. Our data suggest that HPV-positive oropharyngeal cancers comprise a distinct disease entity with an improved prognosis.

KEY WORDS: HPV, HNSCC, p53, Ki67, immunohistochemistry, PCR, prognosis.

ABBREVIATIONS: HPV, human papilloma virus; HNSCC, head and neck squamous cell cancer; PCR, polymerase chain reaction

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INTRODUCTION

Squamous cell carcinoma of the head and neck (HNSCC) is the eighth most common malignancy in the world, with approximately 620,000 patients diagnosed with cancer of the oral cavity, nasopharynx, oropharynx and larynx each year (Li *et al.*, 2003). The disease is characterised by local tumour aggressiveness, early recurrence and high frequency of second primary tumours (Chen *et al.,* 2005). It is generally accepted that oral carcinogenesis is a multi-step process of accumulated genetic damage leading to cell dysregulation, with disruption in cell signalling, DNA repair and cell cycle, all of which are fundamental for homeostasis (Bettendorf *et al.*, 2004). Established etiological factors include cigarette, pipe and cigar smoking, heavy alcohol abuse, betel nut chewing, and smokeless tobacco use. However, 15-20% of HNSCC patients have no known tobacco or alcohol history; this group may include a large proportion of young adults and women with other possible contributing exposures. Viruses, mainly HPV and Epstein-Barr virus (EBV), have been associated

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with head and neck cancer development (Ringstrom *et al.*, 2002; Chen *et al.*, 2005).
Mucosotropic high-risk papillomaviruses (HPVs) known to cause cervical and anogenital cancers are thought to play a role in the development of some head and neck squamous cell carcinomas. In 1995, the International Agency for Research on Cancer (IARC) classified HPV types 16 and 18 as human carcinogens, although an association between HPV and a subset of head and neck cancers had been noted since the 1980s (Chen *et al.*, 2005). Molecular studies have shown that by expressing viral oncoproteins E6 and E7, high-risk HPVs dysregulate crucial cellular mechanisms, such as the cell cycle and the apoptotic pathway, by inhibiting the activity of cellular p53 and pRb tumour suppressor proteins, respectively (Azzimonti *et al.*, 2004). These viral oncoproteins have been shown to be capable of transforming primary human keratinocytes from either genital or upper respiratory tract epithelia and disrupting cell-cycle regulatory pathways, thus playing a part in the genetic progression of cancer (HNSCC) (Gillison *et al.*, 2000). The p53 gene is mutated in approximately 45% of cases of HNSCC (Sisk *et al.*, 2002), and the expression of upstream regulators of pRb function such as p16 and cyclin D is also commonly altered in HNSCC. HPV infection may represent an alternative molecular pathway for HNSCC tumorigenesis (Gillison *et* al., 2000). In particular, HPV 16 infection may be a risk factor for oropharyngeal HNSCC, especially for tonsillar carcinomas (Niedobitek *et al.*, 1990). According to published studies, the frequency of HPV DNA in HNSCC varies widely depending on tumour site, material and methods applied, averaging 15% to 60%. HPV 16 has been identified in 20-90% of HPV positive HNSCCs, and HPV types 18, 31, and 33 in the remainder. Notably, low-risk HPV types 6 and 11 have also been identified in some oral carcinomas and laryngeal carcinomas (Chen *et al.*, 2005).
A challenge to current management strategies for HNSCC is tumour heterogeneity, which limits the reliable use of currently available prognostic factors (Quon *et al.*, 2001). Assessment of specific genetic changes as biological markers in HNSCCs has generated conflicting data, and no particular marker has emerged as a reliable guide to clinical outcome (Li *et al.*, 2003). There is no consensus on the prognostic significance of HPV in HNSCCs generally, but some recent studies have shown an association between improved clinical outcome and HPV positivity in tonsillar cancer (Li *et al.*, 2003; Gillison *et al.*, 2000).
The aim of our study was to correlate the tumour proliferation index (as assessed by Ki67 expression), grade of differentiation, expression of a well-known tumour suppressor gene (p53) to the presence of HPV, with clinical outcome in a group of 47 patients diagnosed with cancer of the head and neck district.

MATERIALS AND METHODS

Patients and tumour specimens
Investigations were carried out on 47 patients with a diagnosis of SCC of the oral cavity or oropharynx treated at the Department of Clinical Oncology and the Department of Otorhinolaryngology, San Giovanni Battista Hospital, Turin, between 1995 and 2004. The patients were identified through a search of the surgical pathology records and tumour registry. Clinical information was collected by medical chart review. Clinical or pathologic staging and identification of the anatomical site of the lesion were based on the International Union Against Cancer TNM classification of malignant tumours (2003). The distribution of the 47 lesions included 25 (53.2%) cases of SCC of the oral cavity and 22 (46.8%) SCCs of the oropharynx. The mean age of patients was 59.15 years (range, 38-75); the male to female ratio was 36:11; the follow-up interval was 5 to 166 months.
The diagnosis and assessment of the grade of differentiation were made according to Wiernik *et al.* on haematoxylin and eosin-stained sections from 10% buffered formalin-fixed paraffin-embedded tissues.

Immunohistochemistry
Cell-cycle marker expression was analyzed by performing immunohistochemistry on 5-µm sections of paraffin-embedded tumour on silane-coated glass slides. Briefly, sections were deparaffinized in xylene (2 x 10 min), rehydrated through a graded series of ethanols (1 x 5 min in 100%, 95%, 90% and 70%). For antigen retrieval, after permeabilization with 0.2% Triton X100, the slides were incubated in a 10-mM trisodium citrate solution at pH 6.0, and heated for 21 min at 650 W and

then for 15 min at 280 W in a conventional pressure cooker, undergoing one cycle in a microwave oven. Endogenous peroxidase activity was blocked using 3% H_2O_2 in 1X PBS for 10 min. Afterwards, slides were incubated in blocking solution (5% donor serum plus 0.2% Triton X100 in PBS 1X) to prevent non-specific binding, and then with an optimal dilution of the primary antibody. After washing in 1X PBS, the slides were incubated with the appropriate biotinylated secondary antibody followed by streptavidin-horseradish peroxidase complex (Immunotech, Marseille, France). Immunostaining was performed by incubating the slides in diaminobenzidine solution (DAB) (Roche, Mannheim, Germany) and monitoring the enzymatic reaction under a conventional microscope. The slides were then counterstained with Mayer's haematoxylin for 15 s, dehydrated and mounted with EUKITT (Bioptica, Milan, Italy). Negative controls included omitting the primary antibody and substitution of the primary antibody with normal serum. Sections from a sample of squamous cell carcinoma known to have strong immunoreactivity for the markers were used as positive controls. Immunostaining was evaluated semiquantitatively by three observers unaware of clinical data or HPV status.

Mouse monoclonal antibodies raised against the following antigens were used: Ki67 (MIB-1 clone, DAKO Cytomation, Denmark); Cyclin E (HE12) and p53 (DO-1) provided by Santa Cruz Biotechnology, Santa Cruz, CA, USA. Semi-quantitative scoring of immune reactivity in SCC was performed according to the percentage of nuclear positivity in the tumour cells, dividing the staining into two categories: less than or greater than 30% or 40% for Ki67 and Cyclin E in oral cavity or oropharyngeal SCCs, respectively, and less than or greater than 20% for p53 expression in the tumours from both anatomical sites.

DNA extraction

A modified version of the Wright and Manops method (PCR protocols: a guide to methods and applications. New York: Academic Press, 1990) was used for DNA extraction. One to three 10-µm sections were taken from the paraffin blocks, placed in a 1.5 ml plastic tube containing 150 µL of digestion buffer (50 mM Tris HCl, 1mM EDTA, 0.5% Tween 20, 0.2 mg/mL Proteinase K). Samples were incubated at 65°C; proteases were destroyed by incubation for 5 minutes at 95°C, and the samples were immediately centrifuged for 5 minutes at 11,000 g. The concentration of the extracted DNA was spectrophotometrically evaluated at 260 nm.

HPV detection and analysis

Polymerase chain reaction (PCR) analysis was carried out on crude extracts of ~10-µm sections of paraffin-embedded tumour tissues. DNA integrity was confirmed by amplification of β-globin from the DNA samples. Positive and negative controls were used in each HPV detection analysis. To increase the sensitivity of HPV detection, nested PCR assays were performed using MY09-MY11 as the outer and GP5+-GP6+ as the inner primers (Table 1). The outer primer pair is specific for a conserved approximately 450-base sequence contained within the L1 gene, and the inner primers amplify a 140-base sequence within the sequence amplified by the outer primer pair. A 50-µl reaction mixture consisted of 1 µM of each primer, 300-500 ng of the extracted sample, 1 X Taq Buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 11 mM $MgCl_2$ and 0.1% gelatin), 200 µM of each of the four dNTPs and 1 unit of Taq DNA Polymerase (Sigma, St. Louis, MO., USA). Initial denaturation occurred at 95°C for 5 min, followed by 40 cycles as described in Table 1. A final extension was done at 72°C for 7 min. The negative controls were samples with water replacing target DNA in the reaction mixture. DNA samples were reamplified in a nested PCR using a GP5+/GP6+ primer pair. The amplification parameters were modified as described in Table 1, using a 2.5 µL template from the first-step amplification products. Subsequently, 10 µl of the PCR reaction mixture were electrophoresed through a 2% agarose gel containing 0.5 µg/mL ethidium bromide and then visualized under an ultraviolet transilluminator. To avoid contamination of specimens with viral DNA, standard precautions concerning spatial separation of pre- and post-PCR steps, aliquoting of reagents and single use of scalpels for processing tissue specimens were strictly followed. A set of negative controls was included during all steps of the DNA isolation and amplification procedures. None were positive for HPV DNA. The PCR products were verified by direct sequencing with the GP5+ primer on a DNA sequencer (PRIMM, Milan,

TABLE 1 - *Primers and PCR amplification conditions used for HPV detection*

		Primer Sequence	*Amplicon length (bp)*	*PCR Cycling Conditions*
β-globin	PC04	[+]5'CAACTTCATCCACGTTCACC-3'	268	35 cycles: 60 s at 94°C, 60 s at 55°C, 120 s at 72°C
	GH20	[+]5'GAAGAGCCAAGGACAGGTAC-3'		
L1 degener mHPV	MY09	[+]5'CGTCCMARRGGAWACTGATC-3'[-]	450	35 cycles: 60 s at 94°C, 60 s at 55°C, 60 s at 72°C
	MY11	[+]GCMCAGGGWCATAAYAATGG-3'[-]		
L1	GP5+	[+]5'TTTGTTACTGTGGTAGATACTAC-3'[-]	140	35 cycles: 60 s at 94°C, 120 s at 45°C, 90 s at 72°C
	GP6+	[+]5'GAAAAATAAACTGTAAATCATATT3'[-]		

Primer sequence abbreviation. M=A or C; W=A or T; R=A or G; Y= C or T.

Italy). HPV type was determined on the basis of >90% homology with HPV sequences deposited in GenBank using BLAST software.

Statistical analysis

Correlations between immunohistochemical scoring results and various clinicopathological features were assessed using the χ^2 test. Kaplan-Meier survival analysis was used to estimate the actuarial survival of subjects stratified by HPV and cell-cycle markers. All P values were considered statistically significant if $\leq$0.05. All data were processed with BMDP software.

RESULTS

The study population consisted of 47 patients (36 men, 11 women) with histologically confirmed HNSCC treated between 1995 and 2004. The mean age at diagnosis was 59.15 years (range, 38-75). The follow-up interval was 5-166 months (mean, 35.5). Distribution by site included 25 SCCs of the oral cavity and 22 SCCs of the oropharynx. Classified by pathologic grade, 17 (36.2%) tumours were moderately differentiated, 4 (8.5%) well differentiated, 21 (44.7%) poorly differentiated and 5 (10.6%) unclassified. Lymph node involvement was present in 26 patients; none had distant metastases.

Derangement of the p53 tumour suppressor system is one of the most common molecular aberrations in human malignancies, with a rate of mutation in HNSCCs of roughly 45% (Bettendorf *et al.*, 2004). In our study, staining of p53 was often of high intensity and confined to tumour cells. In a few cases, weak staining was noted in the normal cells of tumour-containing sections. Proliferation activity was assessed by immunostaining for Ki67. In the normal squamous epithelia, Ki67-positive cells were located mostly in the basal cell layers, whereas within the tumour mass they were distributed at all levels, and nearly all cells of the invading

TABLE 2 - *Distribution of patients according to the presence of HPV 16 DNA and the main clinical and pathologic features*

No. of patients (%)	*HPV 16 (+) (n=20)*	*HPV 16 (-) (n=24)*
CLINICAL FEATURE		
Tumour site		
Oral cavity	9 (36)	14 (56)
Oropharynx	11 (50)	10 (45.5)
T status		
T1	3	3
T2	6	4
T3	2	3
T4	6	7
Unclassified	3	7
Lymph nodes		
N0	3	5
N1	2	4
N2	9	8
N3	1	-
Unclassified	5	7
Grade		
G1	2	2
G2	7	9
G3	8	12
Unclassified	3	1

TABLE 3 - *Correlation between HPV DNA status and Ki 67 and p53 expression*

		Ki 67 (MIB-1)[1]		***p 53***[2]	
		positive	*negative*	*positive*	*negative*
HPV positive	Oral cavity	5	3	5	4
	Oropharynx	5	6	5	5
HPV negative	Oral cavity	7	7	6	8
	Oropharynx	7	3	5	4

[1]The cut off was set at ≤ or > 40% in carcinomas of the oropharynx and at ≤ or > than 30% in carcinomas of the oral cavity.
[2]The cut off was set at ≤ or > 20% for both oral cavity and oropharyngeal carcinomas.

front were positive. Remarkably, Ki67 staining was more diffuse in the high-grade than the low-grade cancers. p53 immunostaining was considered positive when p53 expression was 20% or more in both anatomical sites. In Ki67 immunostaining, a cut-off of less than or equal to or greater than 40% was set for oropharyngeal carcinomas and a cut-off of less than or equal to or greater than 30% was set for oral cavity carcinomas. According to the selected staining cut-off value, 12 of 22 (55%) oropharyngeal carcinomas were positive for Ki67 and 10 of 22 (45%) were negative. In p53 staining, 10 of 22 (45.5%) carcinomas turned out to be positive, 10 of 22 were negative and 2 samples were unclassifiable. The immunohistochemical analysis of the proliferative marker Ki67 showed that 13 of 25 (52%) oral cavity carcinomas were positive and 11 of 25 (44%) were negative. p53 immunostaining was positive in 12 of 25 (48%) oropharyngeal tumours and negative in 13 of 25 (52%) (tab.4). The overall prevalence of HPV DNA detected in the samples was 42.5% (36% of oral cavity and 50% of the oropharyngeal tumours). All the detected HPVs were genotype 16 at sequencing analysis. No coinfections were found. HPV positivity did not correlate with tumour differentiation, node status or with the other clinical features examined (Table 2).

Association between presence/absence of HPV and expression of cell-cycle markers

HPV-positive carcinomas from both anatomical sites had a lower proliferative index, as shown by percentage of Ki67-positive cells, than those negative for HPV DNA (P=0.08 for the oropharynx, P=0.09 for the oral cavity). No significant correlation was found between p53 positivity and HPV status (Tables 3, 4).

Association between HPV status, expression of cell-cycle markers and clinicopathologic variables

There was a significant association between HPV-positive tumours and younger patient age (P=0.05); the mean age of patients with HPV-positive carcinoma was 52 years versus 62 years for patients with HPV-negative carcinoma. No correlation was found between HPV DNA status and patient gender, TNM status or histopathological grade of differentiation.

Survival analysis

Patients with oropharyngeal carcinomas harbouring HPV-16 turned out to have a better clinical outcome than those testing HPV-negative. The Kaplan-Meier analysis showed that patients with HPV 16-positive tumours had significantly improved disease specific survival when compared with those with HPV-negative tumours (P=0.02) (Fig. 1). Ki67 expression in oropharyngeal carcinomas tended to be inversely correlated with prognosis, i.e. patients with cancer expressing Ki67 at a level ≤40% were found to have a better clinical outcome than those with Ki67 expression at higher lev-

TABLE 4 - *Characteristics of the study population grouped by HPV status*

	HPV positive (carcinomas)	*HPV negative (carcinomas)*	*P value*
Oropharynx	*(n=11)*	*(n=10)*	
Ki67+ cells (mean)	41.8%	58.5%	0.08
P53+ cells (mean)	34.4%	30.2%	0.7
Oral cavity	*(n=9)*	*(n=14)*	
Ki67+ cells (mean)	28.22%	37.86%	0.09
P53+ cells (mean)	38.8%	29%	0.4

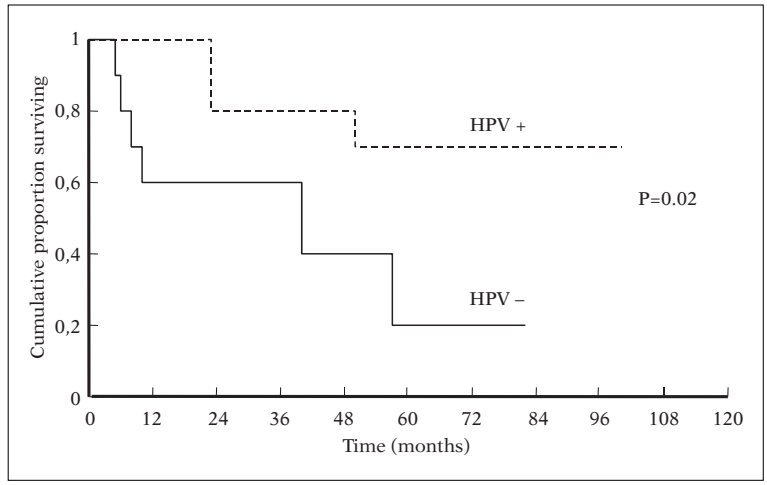

FIGURE 1 - *Cumulative prognostic value of HPV positivity for patients with squamous cell carcinomas of the oropharynx, expressed as probability of overall survival.*

els. (P=0.04) (Fig. 2). In carcinoma of the oral cavity, HPV positivity seemed to be a negative prognostic factor, although the significance of the Kaplan-Meier analysis was low (P=0.1) (Fig. 3). Both Ki67 positivity and p53 expression showed a strong correlation with worse prognosis (P=0.02 for Ki67, and P=0.001 for p53) (Fig. 4, 5) (Table 5).

DISCUSSION

The incidence of head and neck cancer is strongly linked to alcohol abuse and smoking, however, increasing epidemiologic evidence posits the existence of a subgroup of these cancers with a different oncogenesis based on HPV infection (12). HPV-positive oropharyngeal cancers, in particular, may comprise a distinct molecular and pathologic disease entity that is causally associated with HPV infection and has a markedly improved prognosis (4). Some studies found HPV in oropharyngeal cancers three times as often as other HNSCCs primaries, and 50-60% of tonsillar carcinomas (a subset of oropharyngeal tumours) were shown to be HPV-positive versus only 6-10% of tumours at other anatomic sites (P <0.001) (4, 13-15).

We examined the prevalence of HPV in carcinoma of the oropharynx and the oral cavity carcinoma. In addition, the clinical outcome of the patients was reviewed and correlated to the presence of HPV, the proliferation index of tumour cells and p53 expression rate. The overall fre-

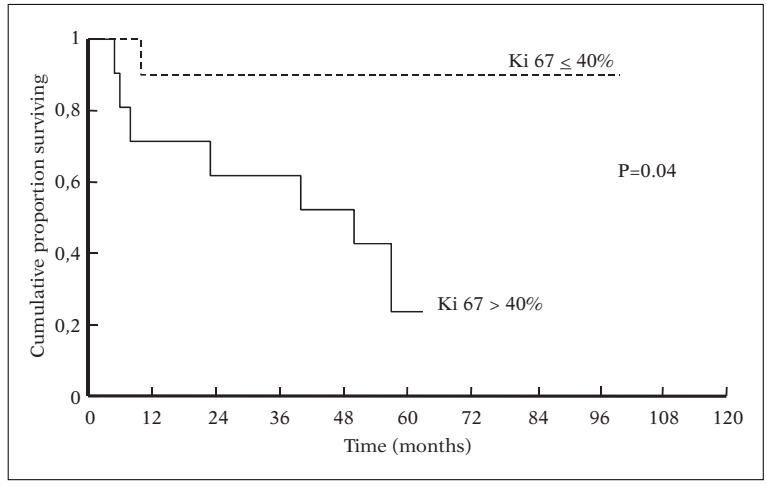

FIGURE 2 - *Patients with oropharyngeal SCC with low Ki67 tumour expression had a better 5-year survival than those with highly proliferating tumours (P=0.04).*

TABLE 5 - *Survival analysis of patients stratified by HPV, Ki67 and p53 status*

	Patients (no.)	*Median of Survival (mo.)*	*12 months (%)*	*36 months (%)*	*60 months (%)*	*P value*
Oropharynx	22	/	81	75	54	
Ki67 ≤ 40%	10	/	90	90	90	P=0.04
Ki67 > 40%	11	40	73	62	28	
P53 ≤ 20%	9	/	89	89	71	P=0.3
P53 > 20%	10	40	70	60	50	
HPV16+	10	/	100	89	78	P=0.02
HPV -	10	40	60	60	22	
Oral cavity	25	24	81	48	42	
Ki67 ≤ 30%	12	/	91	69	58	P=0.02
Ki67 > 30%	10	12	50	26		
P53 ≤ 20%	12	/	100	64	64	P=0.001
P53 > 20%	11	12	50	16	/	
HPV16+	9	11	52	26	26	P=0.1
HPV -	12	24	100	50	50	

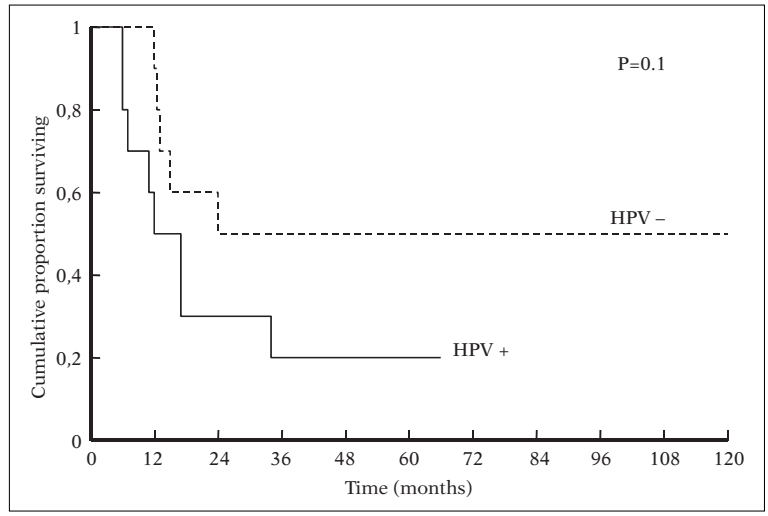

FIGURE 3 - *Kaplan Meier cumulative survival curves of patients with oral SCCs with regard to HPV status.*

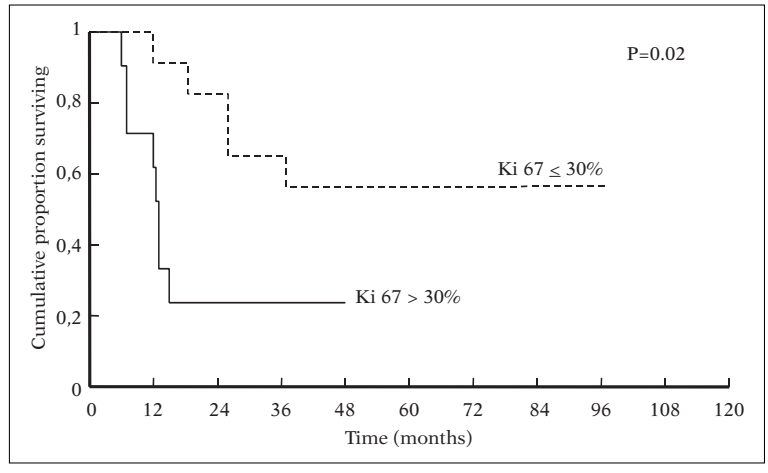

FIGURE 4 - *Kaplan-Meier curves showing the overall survival of oral cavity carcinoma patients with Ki67 low-expressing tumours versus those with high-expressing tumours. Patients with high proliferating tumours had a worse overall survival (P=0.02).*

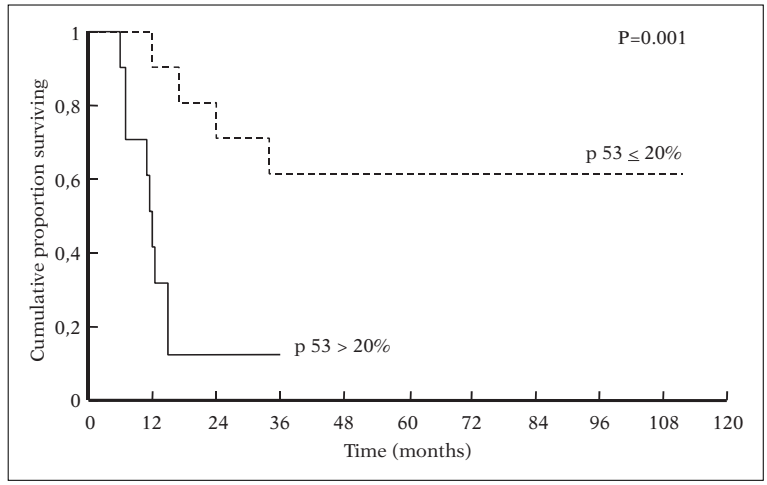

FIGURE 5 - *Kaplan-Meier curves showing overall survival of oral cavity carcinoma patients with regard to p53 expression. Low p53 expression in the tumour mass correlated with a better overall survival (P=0.001).*

quency of HPV was 50% in carcinoma of the oropharynx and 36% in that of the oral cavity; these rates are within the range reported by previous studies (Gillison *et al*., 2000; Fouret *et al*., 1997; Haraf *et al*., 1996). The only genotype detected was HPV 16, demonstrated by several investigators to be the one most commonly associated with HNSCC (Syrjanen S., 2005). A high proliferation rate has been correlated with aggressive behaviour of tumours from various sites. In HNSCCs, several data suggest that cell proliferation indices are reliable and reproducible indicators of tumour aggressiveness (Azzimonti *et al*., 2004; Munk-Winkland *et al*., 1994; Valente G *et al*., 1999). In both anatomical sites, carcinomas with a low proliferation index, as shown by Ki67 immunostaining, were found to have a better overall survival (P=0.04 and P=0.02 for the oropharynx and the oral cavity, respectively). Low p53 expression in oral cavity tumours was found to be a predictive factor for good prognosis. At 60 months, 64% patients with p53 expression ≤20% were alive, whereas none of those with high p53 expression were alive (P=0.001).

The Kaplan-Meier analysis showed that patients with HPV-positive oropharyngeal carcinoma had a better overall survival than those with HPV-negative carcinomas. Interestingly, the proliferation index was lower in HPV-positive than in HPV-negative tumours. The mechanisms underlying the apparently better clinical outcome remain unexplained. An association between HPV positivity in oropharyngeal tumours and radiosensitivity has been demonstrated. It has been hypothesised that radiation and other toxic agents may decrease the capacity of HPV E6/E7 to interfere with p53 or pRb and other host proteins, rendering the cancer more susceptible to therapy (Li *et al*., 2003; Mellin *et al*., 2000).

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