

High prevalence of p16 genetic alterations in head and neck tumours

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Summary Inactivation of the p16 gene is believed to contribute to the tumorigenic process of several neoplasms, including head and neck tumours. In the present study, DNA samples from paired tumour and adjacent normal tissue from 47 patients with squamous cell carcinoma of the head and neck were investigated for the occurrence of p16 genetic alterations. Single-strand conformation polymorphism and direct DNA sequence analysis led to the identification of p16 mutations in six cases (13%). Southern blot analysis showed that homozygous deletion is a rare event in the group of tumours analysed. Loss of heterozygosity (LOH) analysis was performed by polymerase chain reaction (PCR) using two microsatellite markers (IFNA and D9S171) from the 9p21 region. Taking into account only the informative cases, 17 of 32 tumours (53%) showed LOH for at least one of the markers analysed. The methylation status of the CpG sites in the exon 1 of the p16 gene was analysed using methylation-sensitive restriction enzymes and PCR amplification. Hypermethylation was observed in 22 (47%) of the head and neck tumours analysed. In our series of head and neck tumours, evidence for inactivation of both p16 alleles was observed in 13 cases with hypermethylation and LOH, two cases with hypermethylation and mutation, four cases with mutation and LOH and one case with homozygous deletion. These findings provide further evidence that genetic alterations, especially hypermethylation and LOH, leading to the inactivation of the p16 tumour suppressor gene are common in primary head and neck tumours. © 1999 Cancer Research Campaign

Keywords: p16; head and neck tumours; hypermethylation; LOH

The *p16* tumour suppressor gene located on chromosome 9p21, encodes a 16 kDa protein that acts as a cyclin-dependent kinase (cdk) 4/6 inhibitor (Serrano et al, 1993). This gene, whose locus is denominated CDKN2A, has also been named *MTS1* and *p16^{ink4a}* (Kamb et al, 1994a; Ranade et al, 1995). The CDKN2A gene utilizes alternative first exons and common downstream exons to encode two structurally unrelated proteins, *p16^{ink4a}* and *p19^{arf}*, which mediate cell cycle arrest through different mechanisms (Quelle et al, 1995; Stone et al, 1995; Chin et al, 1998).

The progression of proliferating cells through the different phases of the cell cycle is highly regulated by activators and inhibitors (Hunter and Pines, 1994). p16 belongs to an important group of proteins that includes the *p15^{ink4b}*, *p21^{waf1}* and *p27^{kip1}*, which negatively regulate the G1 phase of the cell cycle (Serrano et al, 1993). The p16 gene product binds to cdk4 and cdk6 inhibiting their association with cyclin D1. The inhibition of the cyclin D1–cdk4/6 complex activity prevents retinoblastoma protein (pRB) phosphorylation and the release of E2F, leading to the inhibition of the cell cycle in the G1/S transition (Serrano et al, 1993; Tam et al, 1994; Yeundall and Jakus, 1995). Genetic abnormalities inactivating the *p16* gene might confer cell growth advantages contributing to the tumorigenic process.

Genetic alterations involving the chromosomal region 9p21–22, such as translocations, insertions, heterozygous and homozygous deletions are frequently observed in human cancer. The *p16* gene is considered to be the deletion target in this region (Kamb et al, 1994b; Williamson et al, 1995). High frequencies of homozygous deletion and mutations of this gene have been detected in cell lines derived from different types of tumours (glioma, breast cancer, melanoma, lung, bladder, leukaemia) (Kamb et al, 1994a; Nobori et al, 1994), suggesting that *p16* may play an important role in the regulation of cellular growth in the majority of cells. However, in primary tumours, *p16* genetic alterations occur frequently in only a subset of tumour types (Koh et al, 1995; Pollock et al, 1996). The highest frequencies of *p16* inactivation by mutations and homozygous deletions are observed in carcinomas of the pancreas, oesophagus, renal cell, head and neck and in melanoma (Caldas et al, 1994; Mori et al, 1994; Cairns et al, 1995; Flores et al, 1996; Reed et al, 1996). Furthermore, germline p16 mutations predispose to familial melanoma (Hussussian et al, 1994; Kamb et al, 1994b).

Several studies have demonstrated high frequencies of loss of heterozygosity (LOH) on the short arm of chromosome 9 compared to the p16 mutations found in primary tumours. In addition, a complex pattern of LOH on 9p21–22 has been observed in different types of tumours, suggesting that this region may harbour other tumour suppressor genes associated with the tumorigenic process (Puig et al, 1995; Farrell et al, 1997; Kim et al, 1997). On the other hand, de novo methylation has been proposed to be an important alternative mechanism of *p16* gene inactivation. Merlo et al (1995), studying cell lines and primary solid tumours

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(carcinomas of the lung, head and neck and gliomas), demonstrated that *p16* hypermethylation is a common event in those tumours. Subsequent studies have confirmed that *p16* is hypermethylated in carcinomas of the breast (31%), colon (40%), gliomas (44%), oesophageal adenocarcinomas (38%) and multiple myeloma (75%) (Herman et al, 1995; Fueyo et al, 1996; Lo et al, 1996; Ng et al, 1997; Wong et al, 1997).

Chromosome 9p deletions are considered to play a role in the early stages of the tumorigenic process of the head and neck (Califano et al, 1996). High frequencies of LOH of the 9p21–22 chromosomal region have been reported in squamous cell carcinomas of the head and neck, including dysplasia and carcinoma in situ (Nawroz et al, 1994; van der Reijt et al, 1994). Analysis of *p16* mutations, hypermethylation and homozygous deletions showed that 7–79% of squamous cell carcinomas of the head and neck had at least one of those genetic events (Cairns et al, 1994; Zhang et al, 1994; Lydiatt et al, 1995; Reed et al, 1996; Jares et al, 1997); however, none of these studies have examined the biallelic inactivation of the *p16* and its relationship with the patients clinicopathological characteristics or survival.

In this report, to investigate the role of the *p16* genetic alterations in head and neck tumours, we performed a comprehensive analysis of the mechanisms involved in *p16* inactivation, such as mutations, hypermethylation, homozygous and heterozygous deletions. We further investigated whether there was a relationship between *p16* inactivation and clinicopathological characteristics and survival of the patients.

MATERIALS AND METHODS

Tissue samples

Paired tumour and normal tissue were obtained from 47 patients with primary head and neck squamous cell carcinoma, before any treatment, at the AC Camargo Hospital, São Paulo, Brazil. Tumours consisted of squamous cell carcinomas localized to the oral cavity ($n = 25$), oropharynx ($n = 8$), hypopharynx ($n = 7$) and larynx ($n = 7$). Tumour samples were dissected to remove residual normal tissue before freezing and storage in liquid nitrogen. To determine the amount of residual normal tissue, sections of tumour were stained with haematoxylin and eosin for histopathological examination. The amount of normal cell contamination in each tumour sample was estimated by the pathologist to not exceed 25%. The age of the patient at the time of operation ranged from 27 to 80 years (median 61). The study included a total of 40 males and seven females. Information on smoking history and alcohol intake was available from 36 and 31 patients respectively. Regular alcohol intake was declared by 83% of the smokers. The clinical stage of the patients was determined according to the UICC TNM staging system and histopathological grade based on the WHO classification.

DNA extraction

Tissue was ground to a powder using a Frozen Tissue Pulverizer (Termovac), the powder was resuspended in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA and 0.6% sodium dodecyl sulphate (SDS) and 100 $\mu\text{g ml}^{-1}$ proteinase K, and incubated at 37° overnight. High molecular weight DNA was extracted with phenol-chloroform and precipitated with ethanol.

LOH analysis

LOH for the chromosomal region 9p21–22 was analysed using two polymerase chain reaction (PCR)-based polymorphic markers, as described previously (Kwiatkowsky and Diaz, 1993; Gyapay et al, 1994). Allelic losses were determined by densitometric scan (UltraScan XL; Pharmacia) as complete or partial if the intensity of one allele was reduced by at least 40% in tumour DNA as compared with normal DNA of the same patient. LOH was scored for informative (heterozygous) patients only.

PCR – single-strand conformation polymorphism analysis

Two sets of oligonucleotide primers were used to amplify exons 1 and 2 of the *p16* gene, the primers used were the same as those described by Okamoto et al. (1994) and Sun et al (1995). PCR reactions were performed in 25- μl volumes using 50–100 ng of genomic DNA template, 1 μM of each primer, 1.5 mM magnesium chloride, 200 μM of each deoxynucleotide triphosphate, 0.1 μCi of [$\alpha^{32}\text{P}$ -dCTP] (Amersham, specific activity, 3000 Ci mmol^{-1}), 50 mM potassium chloride, 10 mM Tris-HCl pH 8.0, and 0.5 unit of *Taq* DNA polymerase (Pharmacia, NJ, USA). Samples were overlaid with mineral oil and amplified for 35 cycles of denaturation, annealing and extension optimized for each primer set. The reactions were performed with an automated Thermal Cycler (Perkin-Elmer 580). Amplification products (1 μl) were diluted tenfold in a buffer containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol, heated at 83°C for 5 min and applied (3 μl per lane) on two 6% polyacrylamide non-denaturing gel, one containing 5% glycerol and the other 10% glycerol.

Electrophoresis was performed at 6 W for 14–16 h at room temperature with two cooling fans. Band shift mobility was detected by autoradiography of dried gels using Kodak X-Omat XAR film with an intensifying screen for 12–48 h at –70°C.

Direct DNA sequencing

DNA samples with suspected *p16* mutations as judged by single-strand conformation polymorphism (SSCP) gels were amplified using the same primers. The PCR products obtained were purified using Wizard PCR Preps kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's procedure. Three to 5 μl of ten out of the purified DNA was subjected to a dideoxy chain termination reaction using a double-stranded DNA Cycle Sequencing kit (Pharmacia) for both sense and antisense primers. Sequencing reaction products were denatured and resolved on 6% denaturing urea/polyacrylamide gels. Gels were fixed for 15 min in a 10% methanol/ 10% acetic acid solution, dried and exposed to X-ray film overnight.

Homozygous deletion analysis

By Southern blot: high molecular weight DNAs (10 μg) were digested with *EcoRI* restriction endonuclease according to manufacturer's specification. Digested DNA samples were electrophoresed in 0.8% agarose gels with ethidium bromide and transferred to nylon membranes, which were hybridized with the PE1 probe described by Merlo et al (1995) labelled with [^{32}P]dCTP by random priming. Membrane hybridizations and

Table 1 *p16* genetic alterations observed in head and neck tumours

Analyses		Number of cases analysed	Alterations observed
PCR-SSCP	Exon 1	47	2/47 (6%)
	Exon 2	47	4/47 (8%)
Methylation assay	Exon 1	47	22/47 (47%)
LOH	IFNA	47	11/28 (39%)
	D9S171	47	14/27 (52%)
Southern blot		47	1 case

washing were performed as described previously (Nagai et al, 1993). Southern blots were stripped of probe by sodium hydroxide treatment and re-probed with a β -microglobulin probe to evaluate the amount of DNA loaded onto each lane. Scanning densitometry of the autoradiographies was carried out to quantify the signal intensity of the hybridized bands using an Ultrascan XL (Pharmacia). By PCR, the same primers used for the SSCP analysis were used to investigate the occurrence of homozygous deletions. PCR reactions were performed using 100 ng of genomic DNA in the same conditions described for the SSCP analysis but with 24 cycles. Genomic DNA from the breast cancer cell line MCF-10F was used as positive control for homozygous deletion. PCR products were analysed on a 2% agarose gel.

PCR-methylation assay

p16 methylation status was examined using the combination of digestion of genomic DNA with methylation-sensitive restriction enzymes and PCR amplification. Genomic DNA (1 μ g) was digested with 10 units of *HpaII*, *CfoI* or *SmaI* overnight according to manufacturer's instructions. In order to ensure complete digestion this step was repeated. Digested DNA samples were amplified by PCR using primers specific for exon 1 of the *p16* gene (Kamb et al, 1994a) and for a microsatellite marker (D9S145, 9q13–21.2), used as PCR control (Furlong et al, 1992). PCR was performed under the same conditions described for the SSCP analysis, without [α^{32} P-dCTP], for 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were analysed by electrophoresis on 2% agarose gels.

Statistical methods

Analyses of statistical significance between the *p16* genetic alterations, and the clinicopathological characteristics of the patients were performed by the χ^2 test and Fisher exact test for frequency data in contingency tables. Disease-free survival and overall survival probabilities were calculated based on the Kaplan–Meier product limit technique (Kaplan and Meier, 1958).

RESULTS

Paired normal and tumour DNA from 47 patients with head and neck cancer were examined for the occurrence of *p16* genetic alterations (Table 1). Exons 1 and 2 of *p16* were analysed for mutations by PCR-SSCP. Seven out of the 47 cases analysed showed evidence for *p16* mutations (exon 1, three cases; exon 2, four cases). DNA samples showing electrophoretic band shift mobility were re-amplified and the product purified and used

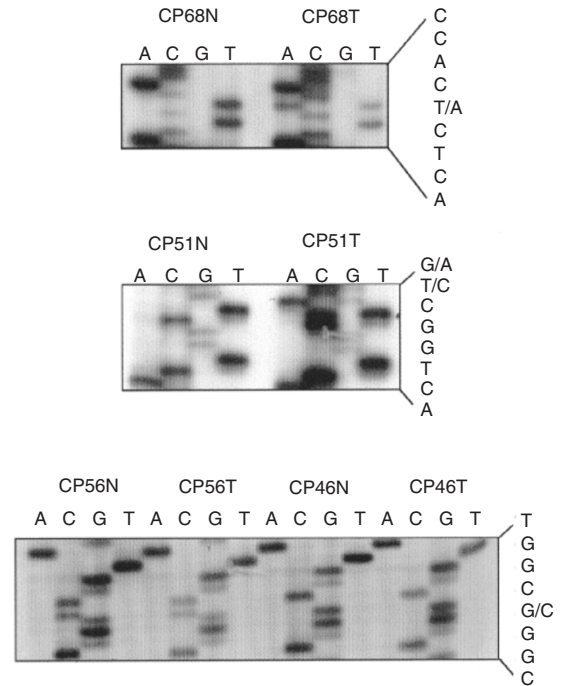


Figure 1 Sequencing analysis of *p16* exons 1 and 2 mutations in head and neck tumours. Case CP68 and CP51 showed a mis-sense mutation in exon 2 (codon 78, CTC→CAC, Leu→His) and in exon 1 (codon 16, CTG→CCA, Leu→Pro) respectively. Case CP56 shows an intronic polymorphism

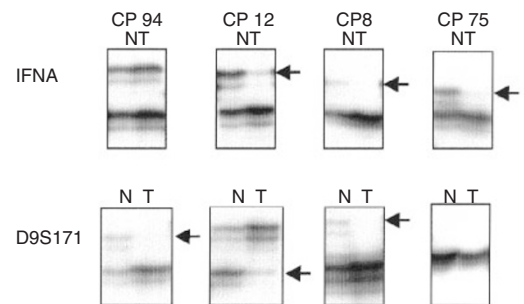


Figure 2 Representative autoradiographs from loss of heterozygosity analysis of chromosome 9p in head and neck tumours. DNAs extracted from tumour (T) and corresponding normal (N) tissues were analysed using microsatellite markers IFNA and D9S171 as indicated on the left of the autoradiographs. Top, case numbers; arrow, allele showing reduction in intensity

directly for sequencing. Sequencing revealed the presence of six mutations and one intronic polymorphism. Figure 1 shows representative example of the sequencing analysis. Sequencing results are summarized in Table 2. The *p16* mutations observed included three mis-sense mutations (exon 1, codon 16, CTG→CCA, Leu→Pro; exon 2, codon 78, CTC→CAC, Leu→His; and exon 2, codon 114, CCC→CTC, Pro→Leu), one frameshift mutation (exon 2, codon 85, 1 bp insertion), one non-sense mutation (exon 2, codon 80, CGA→TGA, Arg→Stop) and one intronic mutation (intron 1, G→T; splicing alteration). All tumours with mutations were advanced stage tumours (two stage III and four stage IV), two in the oral cavity, two in the larynx and two in the hypopharynx.

Table 2 Summary of the p16 mutations observed in head and neck tumours

Case	Exon	Codon	Mutation	Effect
CP 1	Intron1		G→T	Splicing alteration
CP 16	2	80	CGA→TGA	Arg→Stop
CP 28	2	85	1 bp insertion	Frameshift
CP 30	2	114	CCC→CTC	Pro→Leu
CP 51	1	16	CTG→CCA	Leu→Pro
CP 68	2	78	CTC→CAC	Leu→His

The occurrence of homozygous deletions was investigated by Southern blotting and PCR. Only one tumour DNA sample showed reduction (40%) in the intensity of the bands in the autoradiograms when compared with the corresponding normal DNA (data not shown), suggesting the occurrence of homozygous deletion.

LOH was analysed by PCR using two microsatellite markers, IFNA and D9S171, flanking the p16 locus (CDKN2). IFNA and D9S171 showed allelic loss in 11/28 (39%) and 14/27 (52%) informative cases respectively. Of the 32 informative tumours examined 17 (53%) showed LOH for at least one of the markers analysed. Representative results of the LOH analysis are shown in Figure 2.

Methylation status of the CpG sites in exon 1 of the p16 gene was examined using methylation-sensitive enzymes (*HpaII*, *SmaI* and *CfoI*) and PCR amplification. Hypermethylation was detected in 22 of 47 cases analysed (47%). Tumours with different patterns of DNA methylation are shown in Figure 3. The absence of a 310 bp PCR product for exon 1 of the p16 gene indicates that the *HpaII*, *SmaI* and/or *CfoI* restriction sites were unmethylated and had been cleaved (case CP44T). However, the presence of the 310 bp PCR product resistant to digestion with methylation-sensitive enzymes indicates the occurrence of de novo methylation (cases CP13T and CP88T).

In the present study, taking in account only the informative patients, p16 biallelic inactivation was found in 59% (19/32) of the

Table 3 Associations between p16 biallelic inactivation and the clinicopathological characteristics of 47 patients with head and neck tumours

Characteristics	Categories	biallelic inactivation			P-value ^a
		N	No	Yes	
Age	≤ 50 years	9	5	4	0.57
	> 50 years	38	25	13	
Gender	Male	40	25	15	0.65
	Female	7	5	2	
Tumour site	Oral cavity	24	16	8	0.75
	Oropharynx	8	4	4	
	Hypopharynx	7	4	3	
	Larynx	7	5	2	
Lymph-node status	Negative	22	13	9	0.53
	Positive	25	17	8	
Histological grade ^b	I	31	19	12	0.87
	II	10	7	3	
	III	6	4	2	
Tumour stage ^c	I	1	–	1	0.31
	II	5	3	2	
	III	20	15	5	
	IV	21	12	9	
Tobacco consumption	Smoker	36	23	13	0.33
	Non-smoker	6	5	1	
Alcohol consumption	Drinker	31	20	11	0.35
	Non-drinker	10	8	2	

^aChi-square test; ^bUICC TNM staging system; ^cWHO classification.

head and neck tumours analysed; 13 cases with hypermethylation and LOH; four cases with mutation and LOH; and two informative cases with retention of heterozygosity showing concomitant hypermethylation and mutation. In addition, homozygous deletion was observed in one case.

In the series of tumours examined no correlations were found between p16 genetic alterations (mutation, hypermethylation and LOH together or alone) and age, tumour site, TNM staging,

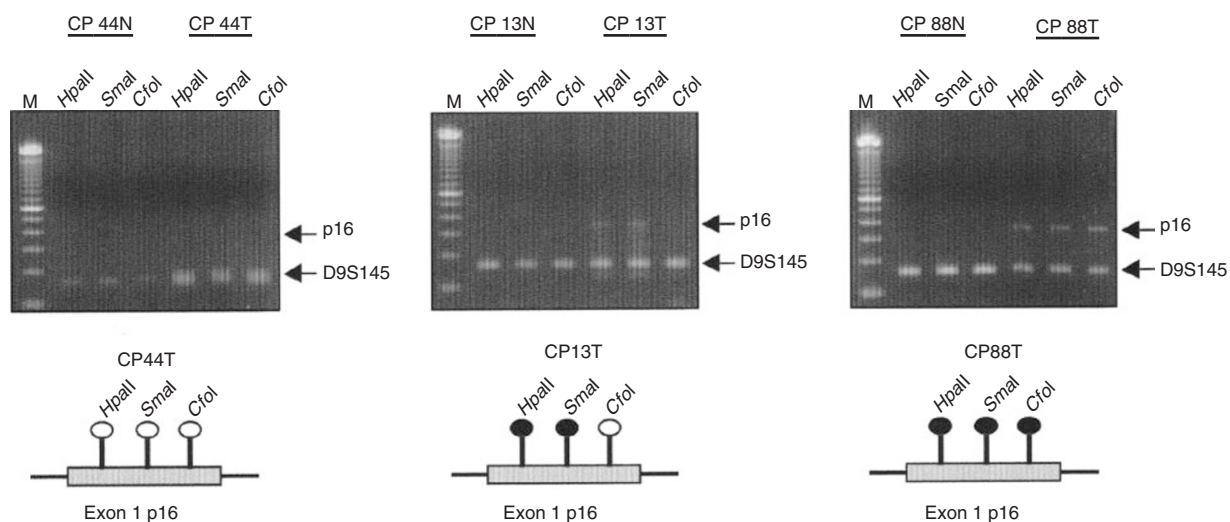


Figure 3 Analysis of methylation status of the CpG island in exon 1 of the p16 gene in head and neck tumours. The presence of a 310 bp PCR product after digestion with *HpaII*, *SmaI* or *CfoI* indicates de novo methylation. A representation of the methylation status of the restriction sites in each case is shown on the right. Case CP13T showed methylation at the *HpaII* and *SmaI* sites; Case CP88T showed methylation at *HpaII*, *SmaI* and *CfoI* sites; and Case CP44T showed complete digestion at all restriction sites examined indicating absence of methylation

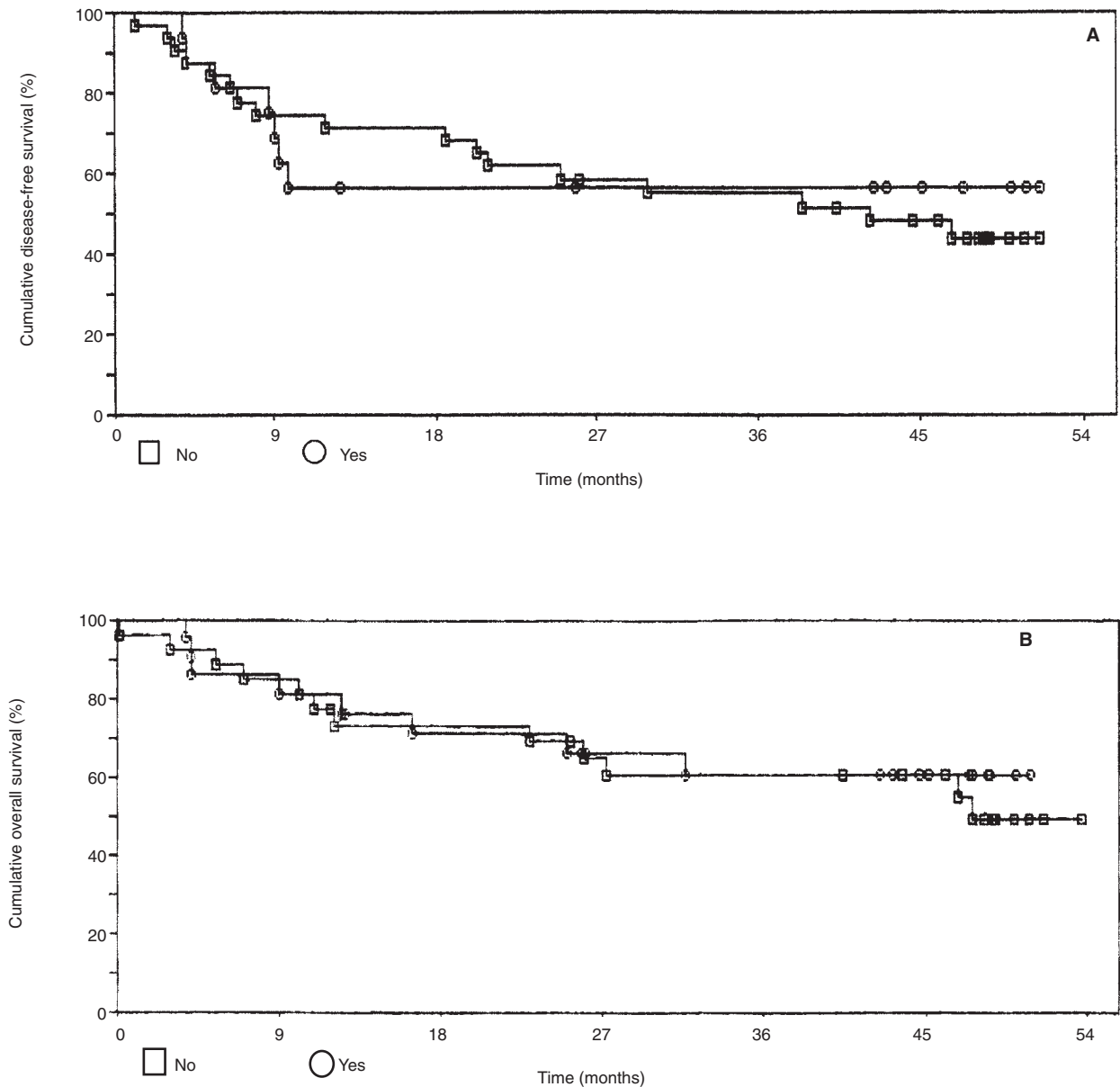


Figure 4 Kaplan–Meier estimates of disease-free (A) and overall (B) survival in head and neck patients stratified according to the *p16* biallelic inactivation. □, patients with tumours showing *p16* biallelic inactivation; ○, patients with tumours without *p16* biallelic inactivation (A, *P* = 0.74; B, *P* = 0.73)

histological differentiation, positive lymph nodes or tobacco and alcohol consumption of the patients (Table 3). In addition, no differences in survival were found between patients stratified for *p16* hypermethylation or biallelic inactivation (median survival 36.71 months) (Figure 4).

DISCUSSION

Mutations, homozygous and heterozygous deletions and hypermethylation are the most common genetic events associated with the *p16* tumour suppressor gene inactivation. In the present study, we found evidence of *p16* inactivation in a high proportion (59%) of the head and neck tumours examined.

Thirteen per cent of the tumours analysed were found to have *p16* mutations. The base substitutions in exon 2 were at codons 78,

80 and 114 located within the ankyrin domains (Serrano et al, 1993). Mutations at codon 114 involved a highly conserved proline (P114) in the fourth ankyrin domain. Hence, the affected codons involve amino acids in domains which are likely to be essential for *p16* biological activity. Although, the exon 2 is shared by *p16^{ink4a}* and *p19^{arf}* (Serrano et al, 1996; Chin et al, 1998) experimental evidence indicates that mutations at exon 2 of the *CDKN2A* affects *p16^{ink4a}* only (Arap et al, 1997). In addition, exon 1β of the *CDKN2A* appears to be critical for *p19^{arf}* function, both binding of *p19^{arf}* to p53 and cell cycle arrest requires exon 1β but not exon 2 (Quelle et al, 1997; Kamijo et al, 1998). Furthermore, in the present study we have observed a point mutation in the acceptor site of intron 1 and a missense mutation in exon 1α (codon 16) implicating *p16^{ink4a}* as the major target of inactivation in the head and neck tumours analysed.

Although homozygous deletions has been reported to be an important mechanism of *p16* inactivation in several human cancers, including head and neck tumours (Reed et al, 1996), in our series of tumours only one tumour showed evidence for homozygous deletion. Despite the fact that Southern blot and PCR analyses were used to examine the occurrence of homozygous deletions, we cannot rule out the possibility that normal cell contamination could account for the exquisitely low frequency of homozygous deletion found in the present study.

LOH and DNA hypermethylation were observed in 53% and 47% of the cases analysed respectively, representing the major mechanisms which may lead to *p16* inactivation. In total 59% (19/32) of the informative patients with head and neck tumours examined showed evidence of *p16* biallelic inactivation. Overall, 20% (4/20) of the cases with biallelic inactivation showed mutation and hypermethylation, 10% (2/20) showed mutations and LOH, 65% (13/20) showed hypermethylation and LOH and 5% (1/20) showed homozygous deletion. These observations indicate that LOH and hypermethylation leading to *p16* inactivation is common in head and neck tumours. These results are similar to those of Wong et al (1997) in oesophageal adenocarcinomas and corroborate with previous studies that have demonstrated high incidence of *p16* hypermethylation in head and neck tumours (El-Naggar et al, 1997; Gonz ales et al, 1997).

To assess the prognostic potential of *p16* inactivation in the development of head and neck tumours the genetic alterations observed (alone and in combination) were correlated with the clinicopathological characteristics (such as age, tumour size, lymph node status, clinical stage, histological grade) and patient outcome. Our study failed to demonstrate any correlation between *p16* inactivation and these clinicopathological characteristics or survival of the patients. LOH of chromosomal region 9p21 has been postulated to be an early event in head and neck cancer (Califano et al, 1996) and *p16* inactivation has been detected in preneoplastic lesions of the larynx and oral cavity (Gallo et al, 1997; Papadimitrakopoulou et al, 1997). Our failure to find prognostic significance for the *p16* genetic alterations might suggest that *p16* inactivation is an early event in carcinogenesis in a subgroup of head and neck tumours but with little or no influence on further tumour progression. The high frequency of tumours with *p16* biallelic inactivation observed here provides further support to previous report (Reed et al, 1996) that *p16* tumour suppressor gene does play an important role in the tumorigenic process of the head and neck. This hypothesis is also supported by observations that *p16* expression inhibits growth in cell lines derived from squamous cell carcinomas of the head and neck (Liggett et al, 1996). However, whether *p16* inactivation is an important predictor for prognosis and disease outcome needs to be clarified by further molecular epidemiological studies.

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