Deletion and methylation of the tumour suppressor gene p16/CDKN2 in primary head and neck squamous cell carcinoma

M Victoria González, Marta F Pello, Carlos López-Larrea, Carlos Suárez, Maria J Menéndez, Eliecer Coto

Abstract

Aims—To study the homozygous deletion and methylation status of the 5′ CpG island of the p16 and p15 genes (9p21) in a set of primary advanced head and neck squamous cell carcinomas (SCC) and to test whether inactivation of these genes by these mechanisms contributes to head and neck SCC development.

Methods—DNA was extracted from fresh tumours. Homozygous deletion was determined by the polymerase chain reaction (PCR) followed by hybridisation with the corresponding probe, radioactively labelled by the random priming method. Methylation status of the CpG island of the 5′ region of these genes was assessed by digestion with the appropriate restriction enzymes followed by PCR and subsequent hybridisation with the corresponding probe. The presence of point mutations was determined by PCR-SSCP (single strand conformation polymorphism).

Results—The p16 and p15 genes were homozygously deleted in 20% and 10% of the tumours, respectively. No point mutations were found at p16 and p15. The 5′ CpG island at the p16 gene was methylated in 20% of the cases.

Conclusions—The tumour suppressor gene p16 is inactivated through homozygous deletion or methylation in a significant proportion of cases of head and neck SCC.

(J Clin Pathol 1997;50:509–512)

Keywords: head and neck squamous cell carcinoma; tumour suppressor genes; homozygous deletion; methylation; CpG island; single strand conformation; polymorphism (p16, p15)

Head and neck squamous cell carcinoma (SCC) is a common type of cancer strongly associated with tobacco and alcohol consumption. Several genetic alterations have been reported to contribute to head and neck SCC development. Mutations in the oncogene Ha-Ras and overexpression of cyclin D have been described. Loss of heterozygosity studies identified several chromosome regions that are frequently deleted. Loss of heterozygosity and mutations at the p53 gene are frequent in head and neck SCC, as well as loss of heterozygosity at 9p21. However, the p16 gene, a candidate tumour suppressor gene from the 9p21 region, has a very low rate of point mutations.

The tumour suppressor gene p16/INK4A encodes a protein that belongs to the INK4 family of cdk inhibitors. It binds specifically to cyclin dependent kinase (CDK) 4/6 and prevents it from forming a complex with cyclin D1, thus blocking the entrance into the G1 phase of the cell cycle. The cyclin D1/CDK4 complex phosphorylates the retinoblastoma protein (pRB), thus releasing cells from the growth inhibitory effect of unphosphorylated pRB. The lack of p16 or the expression of a mutated form of the p16 protein results in abnormal cell cycling and growth, the features that characterise tumour development. The role of p16 as a tumour suppressor gene in head and neck SCC has recently been demonstrated by transfection of p16 into head and neck SCC cell lines that lacked p16 function, resulting in cell cycle arrest.

In accordance with its central role in cell growth inhibition, the p16 gene is frequently mutated in most human cancers, following the two hits model for the inactivation of tumour suppressor genes—one allele is deleted and the other has a point mutation or is deleted. Cell lines and primary tumours from most human cancer types show a high frequency of loss of heterozygosity at chromosome 9p21, the region where the p16 locus is located. However, they differ in the nature of the second mutation—either a point mutation, resulting in a stop codon, or an amino acid change or a second deletion, resulting in the homozygous deletion of the p16 gene. More recently, several authors have established an alternative mechanism for p16 inactivation through methylation of a 5′ CpG island, resulting in the transcriptional silencing of the p16 gene. Analysis of several head and neck SCC derived cell lines and primary tumours indicated that homozygous deletion and transcriptional silencing through methylation of the 5′ CpG island would be a major mechanism for p16 inactivation in this type of cancer.

The p15/INK4B gene is related to p16/INK4A and is located 28 kilobases centromeric to the p16 gene on 9p21. Transforming growth factor β (TGFβ) induces the expression of p15, which in turn inhibits the cell cycle, thus mediating the inhibitory function of TGFβ.

We describe the homozygous deletion and methylation of p16/INK4A in a significant number of primary head and neck SCC,
indicating that inactivation of this tumour suppressor gene through these two genetic mechanisms would be a basic event in the progression of this type of cancer.

**Methods**

**PATIENTS AND DNA EXTRACTION**

Tumour samples were collected from 20 patients. The fresh tumour tissue was microdissected. Specimens containing more than 70% neoplastic cells were placed in lysis solution containing 1% sodium dodecyl sulphate (SDS) and proteinase K for 24 hours at 37°C, and DNA was obtained by phenol–chloroform extraction and ethanol precipitation.

**HOMOZYGOUS DELETION OF THE P16 GENE**

A 340 base pair sequence encompassing the first exon of the p16/INK4A gene was amplified with primers p16-1: 5′GAGAGAAGAGGAGGGCTGGGCTGGTCACCA3′ and p16-2: 5′GCAGCTCCTGATTCCTCCCTGCAAC3′. Reactions consisted of 100 ng of genomic DNA from tumour samples, 20 pmol of each primer, 200 μM of each dNTP, 2.5 mM MgCl₂, 1 × reaction buffer and one unit of Taq polymerase in a total volume of 20 μl. PCR conditions consisted of 30 cycles of 30 seconds at 95°C, one minute at 62°C (annealing) and one minute at 72°C (extension). The reactions (5 μl each) were electrophoresed overnight on a 1.5% agarose gel and blotted on to a nylon membrane. Hybridisation was at 42°C with a formamide hybridisation solution containing the p16 (first exon) probe radioactively labelled by the random priming method. After three washes of 30 minutes at 65°C with 0.1 × SSC/0.5% SDS, membranes were autoradiographed at −80°C for 2–12 hours.

To test the quality of the DNA, an 1125 base pair sequence corresponding to nucleotides 755–1880 of the N-acetyl transferase-2 (NAT-2) gene was amplified with primers 5′GGGGATCATTGACATTGGAGAG3′ and 5′CTTCCACAAGATAATCAACGGC3′ (EMBL accession number X14672). Southern blot analysis, including hybridisation with a NAT-2 radiolabelled probe, was performed as described for the analysis of the p16 gene.

**HOMOZYGOUS DELETION OF THE P15 GENE**

The first exon of p15/1NK4B was amplified with primers p15-1: 5′GCAAGCTTGGGAAAGGAG3′ and p15-2: 5′GCCCTTGGGCCCCAGCTA3′, yielding a fragment of 260 base pairs. Annealing temperature was 65°C and PCR was performed under the same conditions as the amplification of the p16 gene. The reactions were electrophoresed (10 μl each), blotted on to a nylon membrane and hybridised with a p15 exon 1 probe.

**METHYLATION ASSAY**

We examined the methylation status of two HpaII sites (CCGG) at the 5′ CpG island of the p16 and p15 genes. One Smal site (CCCGGG), coincident with one HpaII site on the 5′ CpG region of p16 was also studied (fig 1). Tumour DNA (1 μg) was digested with 20 units of HpaII and 20 units of Smal (Boehringer Mannheim, Germany). The digested DNA (50 ng) was subjected to 20 cycles of PCR with the primers used for the analysis of homozygous deletion of the p16 gene. The amount of amplified DNA was quantified after Southern blot analysis of 10 μl of the PCR product with a p16 exon 1 probe, as described above. The same protocol was followed for the exon 1 of p15, amplified with the same primers that were used for the homogeneity of p16.

**PCR-SSCP (SINGLE STRAND CONFORMATION POLYMORPHISM)**

Tumour DNA was amplified with primers for p16 exon 1 and p15 exon 1. SSCP analysis was adapted from the original method of Orita et al.[9] and PCR was performed as previously described.[9] Formamide denaturing loading buffer (100 μl) was added to the reaction mixture and heated to 98°C for five minutes after which 5 μl was immediately loaded on to a 6% polyacrylamide gel containing 10% glycerol. Electrophoresis was carried out at 10 W for 12 hours.

**Results**

**HOMOZYGOUS DELETION OF THE P16 AND P15 GENES**

Tumour DNA samples from 20 patients were subjected to PCR for the assessment of homozygous deletion (table 1). PCR performed over 25–30 cycles rendered an amplification product in all samples checked. How-

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**Table 1** Homozygous deletion and methylation of the 5′ CpG island in the p16 and p15 genes in 20 cases of head and neck squamous cell carcinoma

<table>
<thead>
<tr>
<th>Case</th>
<th>LOH 9p21*</th>
<th>p16 del†</th>
<th>p16 met‡</th>
<th>p15 del†</th>
<th>p15 met‡</th>
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<tr>
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*LOH 9p21, + allelic loss; − heterozygosity retained; NI not informative.
†p15/p16 met, + methylation of the 5′ CpG island; − no methylation.
‡p15/p16 del, + deletion of the first exon; − no deletion.
ever, when fewer than 20 cycles were used some samples did not yield any product, indicating that this was the ideal number of cycles to minimise the contaminating effect of non-tumour cells. All cases showed amplification with the primers for the NAT-2 gene (fig 2). This was taken as a control for the quality of DNA. Four of 20 cases (20%) did not amplify the first exon of the p16 gene (fig 2). The p15 gene (first exon) was homozygously deleted in two cases (10%) that were also deleted for the p16 gene (see fig 2).

METHYLATION STATUS OF THE P16 AND P15 GENES
All the samples were digested with HpaII and Smal and subjected to amplification of the first exon of the p16 and p15 genes. Hybridisation with the corresponding probes and subsequent autoradiography showed the methylation of p16 and p15 in four cases (20%) and one case (5%), respectively (table 1, fig 2).

POINT MUTATIONS IN THE P16 AND P15 GENES
The 20 cases were subjected to PCR-SSCP analysis for the detection of point mutations in the first exon of the p16 and p15 genes. None of them showed any band shift. We had previously studied the second exon with the same approach and no abnormalities were found.

Discussion
Allelic loss of the chromosome 9p21 region is one of the most frequent genetic alterations in head and neck SCC. This region contains the gene that encodes the inhibitor of cyclin D/CDK4 complexes. This gene, p16/INK4A, is the target of the 9p21 loss of heterozygosity observed in many human tumours. The p15/INK4B gene maps to the same region, 28 kilobases centromeric to p16. However, p15 mutations are a rare event in human tumours. Some cases harboured p16 homozygous deletion that did not involve p15. Moreover, in the rare cases where p15 was deleted, this deletion also included the first exon of p16.

We analysed the p16 and p15 genes in a set of 20 cases of primary advanced head and neck SCC. According to Knudson's two hits model for tumour suppressor genes, p16 would be inactivated in human tumours by allele loss and point mutation. Previous studies using tumour cell lines showed a high incidence of p16 mutations. However, this was not consistent with the results obtained from fresh tumour samples. p16 is frequently mutated in hereditary melanoma and pancreatic carcinoma but not in many other human cancers. We did not find any evidence of p16 mutation in the first and second exons of p16 in the 20 head and neck SCC studied. Some authors have described the presence of p16 mutations in 10% of head and neck SCC, while others have found a much lower incidence (one in 29 cases).

We used PCR to identify the cases displaying homozygous deletions, another known mechanism of p16 inactivation. We failed to amplify the first exon of p16 in four cases and the first exon of p15 in two cases, both also showing p16 homozygous deletion. These tumours would not express any p16 protein and would lack its inhibitory effect on the cell cycle, allowing it to progress in some situations that would otherwise lead to a cell cycle arrest. Reed et al have reported a higher incidence (67%) of homozygous deletion. They used microsatellite analysis for the assessment of this alteration. The different experimental approach might account for the discrepancy in the results, since the presence of contaminating DNA from normal tissue could have made us miss some cases harbouring homozygous deletions. However, this is unlikely, as we reduced the number of PCR cycles to 20 to minimise this masking effect.

Hypermethylation of the 5' CpG island of the p16 gene correlates with gene silencing. This hypermethylation is frequently present in many types of cancers. Our results indicate that p16 was inactivated by this mechanism in 20% of the cases studied. The p16 gene is the major target of inactivation by methylation in head and neck SCC as the incidence of p15 methylation is much lower. Thus, p15 methylation would not be a specific event contributing to progression of the disease. This is in line with the finding that hypermethylation of p16 and p15 is a selective phenomenon depending on the tissue of origin. Thus, in most epithelial derived tumours (lung, head and neck SCC, breast, prostate, and colon) methylation would affect p16 exclusively, while p15 would be the
main target of inactivation in gliomas and leukaemias.1

In 40% of our patients the p16 gene was inactivated either by homozygous deletion or methylation. The importance of p16 alterations in the development of head and neck SCC indicates that this tumour suppressor gene could be a major target of antitumour treatments. In accordance with this, it has been seen that transfection of head and neck SCC lines with the p16 gene has reverted the tumour phenotype.12

This work was supported by Grant FISS 94/1421 and 96/1418 (EC). MVG is the recipient of a fellowship of fomento en Asturias de la Investigacion Cientifica aplicada y la Tecnologia.


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*J Clin Pathol* 1997 50: 509-512
doi: 10.1136/jcp.50.6.509

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