Loss of Heterozygosity and Mutation Analysis of the \textit{p16} (9p21) and \textit{p53} (17p13) Genes in Squamous Cell Carcinoma of the Head and Neck\textsuperscript{1}

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ABSTRACT

We analyzed allelic loss at the \textit{p53} gene (17p13) and at chromosome region 9p21 in 35 primary head and neck squamous cell carcinomas. Loss of heterozygosity (LOH) at \textit{p53} and 9p21 was found in 50 and 75\% of informative cases, respectively. LOH at \textit{p53} did not increase significantly with tumor stage, but was more frequent in moderately and poorly differentiated tumors than in well-differentiated tumors. LOH plus mutation or homozygous deletion of \textit{p53} was limited to advanced stage and poorly differentiated tumors. Allelic loss at 9p21 is frequent in early stage head and neck squamous cell carcinoma and is not significantly associated with LOH at \textit{p53}. The second exon of the \textit{p16/MTSI/CDKN2} gene was found to be homozygously deleted in 1 of 19 cases showing LOH at 9p21, but direct sequencing did not show mutations in the remaining 18 cases. This suggests that \textit{p16} plays a limited role in the development of head and neck squamous cell carcinoma.

INTRODUCTION

HNSCC\textsuperscript{4} is one of the most common types of cancer in the world. More than 10,000 Americans die each year from HNSCC (1). Its 5-year survival rate has not changed in recent years, and is still one of the lowest among the prevalent cancers. HNSCC is strongly associated with tobacco and alcohol consumption (2).

Little is known about the genetic events involved in its progression, although some genes have been reported to contribute significantly (3). Mutations have been found in the oncogene \textit{Ha-ras} (4). Overexpression of cyclin D has been reported to be associated with aggressive behavior of HNSCC (5–7). Allelotyping using DNA microsatellite markers representative of all human chromosomes have found regions of common LOH (8, 9), an event associated with the inactivation of TSGs. To date, the highest mutation rate for any gene in the HNSCC is that of \textit{p53} (chromosome 17p13; Refs. 10–13), which is also mutated in most human cancers (14). Mutations of the \textit{p53} gene in HNSCC resembles those found in the squamous cell lung carcinoma. \textit{p53} is known to be a TSG (15) that encodes a nuclear protein of 53,000, which acts as a transcription factor (16–19) through sequence-specific DNA binding (20, 21). Mutations in \textit{p53} abrogate its transactivating activity, leading to uncontrolled cell growth (22). Approximately 90\% of the \textit{p53} mutations are located in exons 5–9. These exons encode the surface of the protein, and amino acids at this region are directly involved in DNA binding (23).

Recent studies have described the presence of homozygous deletions in multiple tumor-derived cell lines (24) and in primary melanoma (25, 26), pancreatic carcinoma (27), and esophageal squamous cell carcinoma (28) on another TSG, \textit{p16/MTSI/CDKN2}, mapped to chromosome 9p21. The protein binds to cdk-4, preventing it from forming a complex with cyclin D. The cyclin D/cdk-4 complex allows the cell cycle to progress through G\textsubscript{1}, thus permitting cell division. If cdk-4 is overexpressed or expressed at the wrong moment, then \textit{p16} would exert its role as a TSG by binding cdk-4 and inhibiting cell growth (29).

TSGs are generally inactivated by the loss of one copy and inactivation of the remaining copy by point mutation or deletion. In an attempt to determine the role of \textit{p53} and \textit{p16}, we have analyzed several microsatellite markers that are intragenic or closely linked to these genes, and searched for mutations at these genes in 35 unselected HNSCCs.

MATERIALS AND METHODS

Patients and DNA Extraction. Paired tumor and blood samples were collected from 35 patients in the operating room immediately after operation. For tumor DNA extraction, the fresh tumor tissue was isolated by microdissection, and specimens containing more than 70\% neoplastic cells were placed at 37°C for 24 h in lysis solution containing SDS and proteinase K. DNA was obtained after phenol-chloroform extraction and ethanol precipitation (30). DNA from WBCs was obtained in accordance with a previously described protocol (31).

The 35 samples were from the pharynx (20 samples), larynx (13 samples), and mouth floor (2 samples). Histologically, 13 were well-differentiated, 12 were moderately differentiated, and 10 were poorly differentiated tumors. In reference to the tumor stage, 1 neoplasm was stage I, 9 were stage II, 15 were stage III, and 10 were stage IV tumors.
Mutation Analysis of the p53 and p16/CDKN2 Genes in HNSCC

Microsatellite LOH Analysis. Microsatellite markers, D9S157 and D9S171, were analyzed to determine the LOH at chromosome region 9p21. One dinucleotide repeat and one VTR polymorphism, both of which are intragenic to p53, were also analyzed. Primers for these polymorphic markers were as previously described (32–34). PCR was performed in 15-μl reaction volumes containing 100 ng genomic DNA, 10 pmol of each primer, 200 μM each dATP, dGTP, and dTTP, 100 μM dCTP, 2 mm MgCl₂, 0.10 μCi [³²P]dCTP, 1 X Taq buffer, and 1 unit Taq polymerase (Promega). Conditions consisted of an initial denaturing step of 5 min at 98°C, followed by 30 cycles of 1 min at 98°C and 1 min at 55°C and a final extension of 5 min at 75°C. Taq polymerase was added after the initial denaturing step. PCR products were mixed with 1 volume of denaturing loading buffer (95% formamide, 1% xylene cyanol), heated at 98°C for 5 min, and electrophoresed on a 6% denaturing polyacrylamide gel (45% urea, 5.7% acrylamide, 0.3% bis-acrylamide). After electrophoresis the gels were vacuum dried and autoradiographed at −80°C for 2 to 24 h.

After visual inspection of bands of the two matched samples, LOH was defined as a reduction of at least 50% in the allelic intensity in tumor DNA.

PCR Amplification of p16 and p53 Genes. Genomic DNA from primary tumors was amplified for exons 5 and 6 and 7–9 of p53 with the primers and under the conditions previously described (35, 36). Exon 2 of p16 was amplified with primers 42F (GGAAATGGGAAAAGAGT) and 551R (TCT GAGCTTGGAAAGCTCT) as described previously (24). Reactions consisted of 200 ng genomic DNA, 50 pmol of each primer, 0.2 mm each dNTP, 2 mm MgCl₂, 1 X reaction buffer, and 1 unit Taq polymerase (Promega) in a final volume of 50 μl. Amplification conditions were as described for microsatellite LOH analysis, with an extension step of 1 min at 72°C. Aliquots of each reaction mixture were electrophoresed on a 1% agarose

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**Fig. 1** LOH for microsatellites p53-AC/GT and p53-VTR, both intragenic to the p53 gene and D9S171 and D9S157. Arrows, deleted alleles, characterized by a significant reduction of the signal in tumor (T) DNA relative to normal (L) DNA.
gel, and the gels were ethidium bromide stained and photographed.

Multiplex PCR consisted of the same reaction conditions and contained two sets of primer pairs, permitting the simultaneous amplification of two gene sequences.

SSCP Analysis of p53 and p16. Primers and annealing temperatures for amplification of exons 5–8 of p53 have been described previously (35, 36). Exon 2 of p16 was split into two fragments. The first half of p16 was amplified with primers 42F (see above) and 16.6 (CCAGGTCCACGGGCAGA, annealing temperature 55°C), and the second half with primers 551R (see above) and 16.3 (ACTCTCACC/GACCCCAGT, annealing temperature 52°C). SSCP analysis was adapted from the original method of Orita et al. (37). PCR was done as described for microsatellite analysis. Formamide-denaturing loading buffer (100 µl) was added to the reaction mixture and heated at 98°C for 5 min. Five µl were immediately loaded on a 6% polyacrylamide gel containing 10% glycerol. Electrophoresis was at 10 W for 12 h. Tumors showing band shifts were reamplified to confirm the presence of shifts.

Direct Sequencing of the p16 and p53 Genes. PCR products were purified from agarose gels with the Sephaglas Band Prep kit (Pharmacia). Subsequently, the double-stranded DNA was directly sequenced by the dye-exchange method with 35S-labeled ATP using the Sequenase Version 2.0 kit following the manufacturer’s protocol (USB). PCR primers and other internal oligonucleotides derived from the p53 and p16 genes were used as sequencing primers. Sequenase reaction products were separated on a 6% polyacrylamide sequencing gel and autoradiographed. Mutations were confirmed by sequencing both strands from two different amplifications.

RESULTS

Deletion and Mutation Analysis of p53 (17p13) and p16 (9p21) Genes. To determine the relative incidence of LOH at 17p13 and 9p21, we compared the microsatellite patterns of DNA samples from 35 HNSCCs with their normal tissue (lymphocytes) counterparts (Fig. 1). Two 17p13 microsatellites were studied, both of them intragenic to the p53 gene. Thus, LOH at this region is due to the loss of one of the p53 alleles. A total of 32 cases were informative for at least one of the two p53 markers, and LOH was found in the tumor tissue of 14 patients. A very faint band was obtained for the tumor DNA alleles in two cases. In these two cases a PCR product was not obtained for exons 5 and 6 to 7 to 9, suggesting that deletions have eliminated both p53 alleles (Fig. 2). To determine the mutational status of the remaining allele, we amplified exons 5–9 in the 14 cases showing LOH. SSCP and direct-sequencing analysis showed the presence of at least one mutation in 4 cases (Fig. 3). Three were missense mutations (codon 210, AAC:Asn—>AGC:Ser; codon 154, GGC:Gly—>GTC:Val; codon 173, GTG:Val—>ATG:Met) and one was a stop mutation at codon 196 (CGA:Arg—>TGA:stop) (Fig. 4).

The chromosomal region 9p21 is frequently deleted in HNSCC and other tumors, and homozygous deletion for markers mapped to this region has been found in bladder, melanoma, and lung cancers. We analyzed 2 microsatellites (D9S171 and D9S157) at 9p21 in the 35 HNSCC tumors. A total of 31 cases were informative for at least one of the markers, and LOH was found in 19 of them (Fig. 1).

A gene at 9p21 with the properties of a TSG has been recently described (29). This gene, p16/MTS1/CDKN2, is frequently mutated in human cancer cell lines (24), and somatic mutations have been demonstrated in primary melanomas and esophageal and pancreatic carcinomas. To determine the mutational status of the p16 gene in the HNSCC, we amplified the second exon, where previous studies have located most p16 mutations. We failed to obtain a PCR product in one of the tumors that showed LOH at 9p21 (Fig. 2). This case gave a normal size p53 product (exons 5 and 6 and 7–9), but we also failed to amplify two regions encompassing the second exon of p16 in a SSCP experiment (Fig. 3). These data suggest the homozygous deletion of a 9p21 region that included the p16 locus. In four cases a p16 PCR product was obtained, but with

![Fig. 2 Amplification of sequences of the p53 and p16 genes. A, multiplex PCR (p16-exon 2 + p53-exons 5 and 6) of normal (Lane 1) and tumor (Lane 2) DNAs from case 51. Multiplex PCR (p16-exon 2 + p53-exons 7–9) of normal (Lane 3) and tumor (Lane 4) DNAs from the same case. Lanes 5–7, amplification of p16-exon 2, p53-exons 5 and 6, and p53-exons 7–9, respectively, in a control DNA. M, marker. B, multiplex PCR of p16 (exon 2) and p53 (exons 5 and 6) in five cases, two of them showing homozygous deletion of p53. C, multiplex PCR of p16 (exon 2) and p53 (exons 7–9) of seven cases showing LOH at 9p21. Lane 8, homozygous deletion of p16. Lanes 1, 4, and 6, reduced amplification of p16.](https://clincancerres.aacrjournals.org/content/10/4/1120/F1.large.jpg)
a reduced yield (Fig. 2). We suspect the homozygous deletion of this gene in these tumors, with the amplification observed corresponding to ‘‘contaminating’’ nontumor cells. The presence of normal tissue DNA in these cases was indicated by the fact that a significant reduction in the autoradiographic intensity of the deleted allele was obtained instead of the total absence of a signal that characterizes the LOH in the tumor free of normal ‘‘contaminant’’ tissue. SSCP and direct-sequencing analysis of the cases showing LOH at 9p21 failed to detect any difference relative to the normal p16 sequence (Fig. 3).

A significant difference was obtained for p53 LOH in tumor grade ($\chi^2 = 6.8, P < 0.025$), using $\chi^2$ test for independence). LOH at this locus is more frequent in poorly and moderately differentiated tumors than in well-differentiated tumors. When considering the tumor stage, LOH at both regions occurred at the earliest stages (I and II) at a frequency that did not differ significantly with respect to the advanced stages (III and IV). In 10 cases we found LOH at p53 and 9p21 but no significant association between them was found, suggesting that they are independent genetic events in HNSCC.

DISCUSSION

We have analyzed the role of mutations at the p53 and p16 genes in the progression of HNSCC. These two loci map at regions showing a high frequency of LOH (8, 9, 38). Several authors have previously described mutations at the p53 gene in the HNSCC (10–13, 39–42). Mutations at this gene have also been found in normal epithelia from smokers, which supports the hypothesis of field cancerization in the development of HNSCC (40). Chung et al. (41) found p53 mutations in 47% and 37% of the patients with stage I or II tumors and stage III or IV tumors, respectively. Boyle et al. (42) described a frequency of 19% in noninvasive lesions, increasing to 43% in invasive carcinomas. We have found p53 LOH in 50% of our tumors, a frequency similar to that described by other authors. LOH at the p53 gene is not limited to advanced stages: it occurs in 33% of the early stage (I/II) tumors and in 55% of the advanced stage (III/IV) tumors. Our work suggests that p53 LOH is an early event that does not increase significantly with tumor progression. However, a second mutation, either deletion or point mutation, would be more frequent in advanced stages.

LOH at the p53 gene is significantly associated with moderately and poorly differentiated tumors. Several authors have described that moderately and poorly differentiated tumors are frequently aneuploid (43–45), and the loss of the wild-type p53 protein has been correlated with aneuploidy in several cancer types. Our work suggests that the hypothesis of p53 as a guardian that controls the genome stability, previously suggested for other cancers (46, 47), could also apply to HNSCC.

LOH at 9p21 occurs at a high frequency in several human cancers, including HNSCC (36). This genomic region contains the p16 gene that encodes an inhibitor of the cell cycle, thus having the property of a TSG. Initial studies demonstrated a high frequency of p16 mutations, either microdeletions and point mutations, in most human cancer cell lines (24, 48). The name of multiple tumor suppressor 1 (MTS-1) was then given to this gene. Analysis of tissue from melanomas, pancreatic adenocarcinomas, and squamous esophageal carcinomas demonstrated the presence of p16 mutations at a high frequency (25–28). However, recent studies on primary carcinomas of bladder, lung, and head and neck found gene mutations in a limited number of cases (49, 50). All of these tumors show LOH at 9p21. Our study did not find any point mutation at p16 in those cases showing LOH at 9p21. However, homozygous deletion of p16 was found in one case, suggesting a role for this gene in the development of this tumor. We suspect the homozygous deletion of p16 in three additional HNSCC cases that showed a reduced amount of the amplified product, as determined by the reduced ethidium bromide staining and autoradiographic (SSCP) signals of the PCR products. Several tumors contained a significant amount of contaminating non-neoplastic tissue (infiltrating cells and normal mucosa). The presence of DNA from nontumor tissue contaminating the DNA from neoplastic tissue is also a problem faced by all of the previous...
Fig. 4 Mutations in the p53 gene in four tumors showing LOH at this gene. Missense mutations occurred at codons 210, 154, and 173 in three cases. In one case, the nucleotide change at codon 196 was a non-sense mutation.

studies on HNSCC and other cancer types, as demonstrated by the fact that in most of the cases that showed LOH at any polymorphic marker the deleted allele is characterized by a reduced signal rather than by the total absence of the band. This general problem makes it difficult to identify homozygous deletion affecting any locus. In our study, homozygous deletions at the p53 and p16 genes could not be confirmed because of insufficient DNA for Southern blot analysis.

An alternative interpretation of LOH could be allelic imbalance, whereby a chromosome region is amplified, increasing the yield of PCR product from the allele amplified. However, duplication/amplification would not account for the alterations seen on chromosomes 9p and 17p. Previous works have tested the p53 and p16 genes with Southern blot analysis, and no amplification of these genes was described (49).

In a recent report, the homozygous deletion of p16 has been described in 19% of 31 bladder primary tumors, while no point mutations at the second exon of p16 were found in the same set of tumors (50). It is possible that some mutations affecting p16 on exons other than the second exon (e.g., mutations at exons 1 and 3, or at the regulatory sequences) have not been detected. Recently, Zhang et al. (51) analyzed the p16/CDKN2 gene in 68
primary tumors of the head and neck. None of these tumors showed homozygous deletion, and seven had missense or nonsense base changes (51). These along with our own work, showing a reduced frequency of homozygous deletion or base changes of p16 in the primary tumors, suggest that this gene is not the only target of 9p21 LOH in the HNSCC. Alternatively, loss of one copy could affect cell growth and tumor development.

LOH at 9p21 was not found to be significantly associated with tumor stage or tumor grade in the HNSCC. It occurs in early stages (I/II) at a higher frequency (75%) than LOH at the p53 gene (33%). Interestingly, the case showing homozygous deletion of p16 was an early (I) stage tumor, while the two cases with homozygous p53 deletion were advanced (III) stage tumors. A high frequency of loss of chromosome 9p in preinvasive lesions, suggesting that this is an early event in HNSCC progression, has been described previously (38).

Cancer progression requires several genetic events, either mutation at proto-oncogenes or tumor suppressor genes. It has been suggested that HNSCC arises following 6–10 independent genetic events (52). Our work suggests that loss of the p16 gene through homozygous deletion, although at a low frequency, could contribute to HNSCC development.

REFERENCES


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