Three Discrete Regions of Deletion at 3p in Head and Neck Cancers

Roberta Maestro, Daniela Gasparotto, Tamara Vukosavljevic, Luigi Barzan, Sandro Sulfaro, and Mauro Boiocchi

ABSTRACT

Alteration of the short arm of chromosome 3 is one of the most consistent cytogenetic abnormalities found in human head and neck cancers. These alterations, composed of translocations and deletions, have been associated with the presence of a tumor suppressor gene(s), but no clear evidence of the location of this presumptive gene(s) was available. We performed a molecular analysis of the 3p region using a polymerase chain reaction-based approach. Twenty-eight of the 38 cases analyzed (74%) showed the presence of single or multiple areas of allelic loss. Three commonly deleted regions, tentatively mapped to 3p24-ter, 3p21.3, and 3p14-cen, were identified. Our results suggest that at least three oncogenic regions of 3p may be involved in head and neck cancer development and support a common oncogenic pathway with squamous cell lung cancer, for which a similar pattern of 3p deletion has been described recently.

INTRODUCTION

Cancer is considered to be a multistage process involving a number of aberrant molecular events culminating in malignant transformation. Considerable evidence demonstrates that genetic alterations in both dominant oncogenes and oncosuppressor genes are responsible for human cancer development. The identification of such phenomena may be useful in deepening the knowledge of the biological features of neoplastic cells and, in particular, may allow the identification of new diagnostic and prognostic markers, thus providing a basis for more appropriate therapeutic approaches.

With regard to HNSCC,4 basic studies have been largely neglected mainly because of the relatively limited frequency of this neoplasm. However, the incidence of this type of tumor is expected to rise as a result of the increasing number of female and adolescent smokers and as a consequence of environmental pollution (1, 2). Moreover, despite the great emphasis on early diagnosis and the efforts to improve surgical, pharmacological, and radiation treatment management, about 50% of HNSCC patients do not survive for more than 5 years after diagnosis (3). The growing epidemiological problem and the lack of progress in head and neck oncology emphasizes the need for basic studies on the molecular biology of HNSCC.

Cyto genetic studies have revealed that the short arm of chromosome 3 (3p) is frequently affected by chromosomal rearrangements and deletions in HNSCC (4–6). In addition, HNSCC cell lines show some 3 (3p) is frequently affected by chromosomal rearrangements. Analysis of 3p allelic deletions was performed using a PCR-based approach. Polymorphic loci and relative amplification primers are listed in Table 1. Chromosomal position and sequential order on the chromosome are according to the criteria of Jones and Nakamura (9), Jones et al. (10), and Yokoyama et al. (11).

RESULTS

Samples. Matched tumor and corresponding normal mucosa were obtained from 38 patients with primary HNSCC. No patient had been treated with chemo- or radiotherapy prior to surgery. All tissues were frozen in liquid nitrogen immediately after surgery and stored at −80°C until extraction of DNA.

Frozen tissues were powdered with a cell dismembrator. Genomic DNA extraction was performed with a DNA extractor according to the manufacturer’s instructions (Applied Biosystem, Inc., Foster City, CA).

Analysis of LOH Using RFLP and Microsatellite Polymorphisms. Analysis of 3p allelic deletions was performed using a PCR-based approach. Polymorphic loci and relative amplification primers are listed in Table 1. Chromosomal position and sequential order on the chromosome are according to the criteria of Jones and Nakamura (9), Jones et al. (10), and Yokoyama et al. (11).

LOH for the loci THRB, DNF1552, DSS32, DSS32, and DSS30 was evaluated with a PCR-based RFLP approach using the primers and restriction enzymes listed in Table 1. PCR reactions were performed in a 50-μl reaction volume as described previously (12). Reactions were heated to 90°C for 3 min and then cycled 40 times. Each cycle consisted of denaturation at 94°C for 1 min, annealing at the appropriate temperature for 1 min (see Table 1), strand elongation at 72°C for 1 min, and final elongation at 72°C for 5 min. Following PCR, 25 μl of reaction were digested with 10 units of the appropriate restriction enzyme and then electrophoresed in a 4% agarose gel.

Microsatellite polymorphic loci CI3-946, CI3-830, CI3-771, CI3-373, and GLUT2 were tested for LOH according to Jones and Nakamura (9). In detail, PCR products were analyzed by blotting to nylon membranes and hybridized with specific probes (13). After hybridization, the blots were washed and exposed to X-ray film. Samples were considered informative for CA-dinucleotide repeat microsatellite polymorphisms for alleles separated by more than 4 base pairs.

Ascertainment of LOH was performed by densitometric analysis comparing the ratio between the signal intensity of the two alleles in the neoplastic DNA with the same ratio in the corresponding normal DNA. LOH was defined as reduction in the allelic ratio >50%.

RESULTS

Squamous cell carcinomas from 38 patients with head and neck cancer were examined for LOH on the short arm of chromosome 3 using 13 polymorphic markers mapped to 3p. In addition, the status of the chromosome 3 long arm was tested by analysis of the GLUT2 polymorphic locus (Table 1). The use of highly variable microsatellite polymorphisms greatly increased the informativeness of the cases and allowed a precise definition of the extent of the deleted region.

All of the patients included in this study showed constitutional heterozygosity for one or more loci. In 28 cases (74%), LOH was
detected for at least 1 of the 13 polymorphisms of 3p tested. No preferential allelic loss was observed. Four cases (HN58, HN26, HN11, and TC43), together with the LOH for all the polymorphic loci of the short arm, carried a deletion for the 3q marker (GLUT2), suggesting the entire loss of chromosome 3. Fourteen tumors carried a partial or interstitial deletion at 3p, thus providing information on the minimal deleted regions (Fig. 1). All of the data are summarized in Table 1. All of the tumors of the upper aerodigestive tract. In particular, it provides evidence that at least three distinct loci, tentatively mapped to 3p14-cen, 3p21.3, and 3p24-ter, are involved in HNSCC.

### DISCUSSION

Deletions of the short arm of chromosome 3 is a frequent finding in HNSCC. In fact, about 74% of the cases analyzed displayed the presence of single or multiple areas of 3p allelic loss. A previous study by Latif et al. (7) suggested the involvement of 3p deletions in HNSCC development. However, such a study was performed on a series of cell lines for which no matched normal tissue was available, thus providing only a presumptive and statistical ascertainment of LOH and identifying an area broadly distal to 3p14 and proximal to 3p26 as the commonly deleted region. Our report is the first to describe clearly the role of 3p allelic deletions in the genesis of the tumors of the upper aerodigestive tract. In particular, it provides evidence that at least three distinct loci, tentatively mapped to 3p14-cen, 3p21.3, and 3p24-ter, are involved in HNSCC.

The use of highly polymorphic microsatellite CA repeat markers greatly increased the number of informative cases and allowed a better definition of the minimal deleted regions.

### Table 1 Polymorphic DNA markers used for LOH analysis

<table>
<thead>
<tr>
<th>Marker</th>
<th>Chromosome location</th>
<th>Polymorphism</th>
<th>Primers (5' to 3')</th>
<th>Alleles*</th>
<th>Annealing temperature</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C13-946</td>
<td>3p25</td>
<td>CA repeat</td>
<td>TCCAGTTAAGGGCTTCTCTAG</td>
<td>AAGGTCCTCCAAGAAACTCTG</td>
<td>50°C</td>
<td>9, 10</td>
</tr>
<tr>
<td>THR-B</td>
<td>3p24</td>
<td>HindIII</td>
<td>AGCTTTAAGGGCTCATAAGG</td>
<td>TCACTGAGGATTTGACCAAAG</td>
<td>432</td>
<td>221 + 211</td>
</tr>
<tr>
<td>THR-B</td>
<td>3p24</td>
<td>BanHI</td>
<td>AACTTCAAGGGCTGGAGGTT</td>
<td>CCGCAGGAGGAGATCTCTCTT</td>
<td>364</td>
<td>182 + 182</td>
</tr>
<tr>
<td>THR-B</td>
<td>3p24</td>
<td>MspI</td>
<td>AAGGTTGGACCTCAAAGCCCAT</td>
<td>CAGGTTTCCTTTATTAACCATG</td>
<td>681</td>
<td>560 + 123</td>
</tr>
<tr>
<td>THR-B</td>
<td>3p24</td>
<td>DraI</td>
<td>AAGGTTGGACCTCAAAGCCCAT</td>
<td>CAGGTTTCCTTTATTAACCATG</td>
<td>657</td>
<td>405 + 252</td>
</tr>
<tr>
<td>C13-830</td>
<td>3p21.3-p22</td>
<td>CA repeat</td>
<td>GAGGGCTACTGAGCTAGGAA</td>
<td>ATATACTTCACTGACCATGTG</td>
<td>535</td>
<td>383 + 152</td>
</tr>
<tr>
<td>C13-771</td>
<td>3p21.3</td>
<td>CA repeat</td>
<td>TAGACTTCTGAGGCTGGGCTGGCCACGGAA</td>
<td>GAGGTTTCACGAGCAGGAGGAGTTGACCATG</td>
<td>421</td>
<td>211 + 210</td>
</tr>
<tr>
<td>DNF15S2</td>
<td>3p21.3</td>
<td>HindIII</td>
<td>TACCTCTGAGGCTGAGGGGAA</td>
<td>TATGCTCTCAAATGGTGGGAG</td>
<td>473</td>
<td>237 + 236</td>
</tr>
<tr>
<td>C13-917</td>
<td>3p21.2-p21.3</td>
<td>CA repeat</td>
<td>ATCTTCTGAGGCAAGGATGCTTCAG</td>
<td>TATGCTCTCAAATGGTGGGAG</td>
<td>693</td>
<td>224 + 69</td>
</tr>
<tr>
<td>DSS2</td>
<td>3p21.2-p21.3</td>
<td>RsaI</td>
<td>TGGCGGTGGGACCTGAGGAATGTG</td>
<td>TGGCGGTGGGACCTGAGGAATGTG</td>
<td>693</td>
<td>224 + 69</td>
</tr>
<tr>
<td>DSS2</td>
<td>3p21.1</td>
<td>MspI</td>
<td>CCAAGTGGCCAGGATCTTCTTA</td>
<td>ACATCTATCTGGTCCCTAGACCA</td>
<td>473</td>
<td>237 + 236</td>
</tr>
<tr>
<td>DSS30</td>
<td>3p13-p14</td>
<td>MspI</td>
<td>AAAGCTCTTCCCTTCTGAGATGG</td>
<td>CAGGTTTCCTTTGTTGCTGTTCCC</td>
<td>50</td>
<td>224 + 69</td>
</tr>
<tr>
<td>C13-373</td>
<td>3p13</td>
<td>CA repeat</td>
<td>CTGCAAGGGTTTTAACACAG</td>
<td>ATTCGAGGGAGCAAGCTCCC</td>
<td>224 + 210</td>
<td>55</td>
</tr>
<tr>
<td>GLUT2</td>
<td>3q26</td>
<td>CA repeat</td>
<td>TGGCAAGGTTCTTTAACAG</td>
<td>ATTCGAGGGAGCAAGCTCCC</td>
<td>657</td>
<td>405 + 252</td>
</tr>
</tbody>
</table>

*Allele A1, undigested amplification product.

b The PCR product of 683 base pairs contains a constant DraI site 26 base pairs from one end.

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The analysis of LOH in cases HN47, HN14, and HN12 defined a centromeric area of deletion proximal to 3p21.1 band. The constant loss for the CI3-373 marker in all the cases carrying allelic deletions at 3p and the selective LOH for this locus in case HN32 suggest the localization of the centromeric hot spot of deletion in HNSCC at 3p14–cen.

A very high frequency of LOH was observed for the 3p21.3 band (DNF15S2, 79% of LOH; CI3-771, 72% of LOH). In particular, like CI3-373, DNF15S2 polymorphic locus was constantly deleted in all the informative cases involved in 3p deletion phenomena. The presence in cases TC40, HN47, and HN12 of a defined middle area of chromosomal loss, demarcated proximally by the marker CI3-917 (3p21.2–p21.3) and telomerically by THR-B (3p24), together with the reduction in the frequency of LOH for the distal marker CI3-830 (3p21.3–p22), support the presence of a hot spot of deletion at 3p21.3.

In addition to the centromeric and middle region of LOH, a specific telomeric region of deletion could also be identified in HNSCC. Cases TC47, TC37, and HN12, in fact, lost the heterozygosity for the distal marker CI3-946 but retained both the alleles of locus THR-B (3p24). These data suggest the existence of a defined region of deletion at 3p24–ter.

The existence of multiple and distinct regions of 3p deletion in HNSCC was clearly confirmed by those cases carrying two or three distinct areas of LOH (cases HN15 to HN12; Table 2). Similar results describing three distinct regions of deletion at 3p (3p14–cen, 3p21.3, and 3p25) have been reported recently by Hibi et al. (13) for squamous cell lung carcinoma; this type of tumor shares with HNSCC histology and major etiological factors such as smoke and alcohol (14, 15). These data support the hypothesis of a common oncogenic pathway for these two neoplasms and suggest the existence in 3p of three oncosuppressor genes playing a crucial role in the neoplastic phenomena of the aerodigestive tract.

The three hot spots of 3p deletion for HNSCC identified in our study have been proposed to be involved, singularly or in combination, in various human cancers. The 3p14 band has been identified as the most common fragile site in humans (16). Alterations at 3p13–p14 have been described for several tumors such as lung cancer, hereditary renal carcinoma, mixed salivary gland tumor, breast carcinoma, acute leukemia, and rhabdomyosarcoma (17–21), but to date no solid candidate gene has been identified from this chromosomal region.

In renal cancer, in addition to 3p13–p14, a second common region of deletion has been identified at 3p21.3 (22). LOH for 3p21 markers
has been described in lung, testicular, breast, ovarian, and cervical cancers and an increasing amount of evidence supports the hypothesis of the presence of a tumor suppressor gene located at 3p21 (9, 11, 23–27). McNeil Killary et al. (28) have recently documented the reversion of tumorigenicity in cell hybrids transfected with a fragment of chromosomal material encompassing the region 3p21–p22. Furthermore, a series of putative oncosuppressor genes have been isolated from 3p21–p22 bands (29–33). In particular, the gene coding for tyrosine phosphatase \(\gamma (PTP\gamma)\), an enzyme presumably involved in the control of activation of membrane-associated receptors which has been surmised to function as an oncosuppressor gene, has been mapped to the 3p25–p26 band (35, 36) and at the band 3p24, it maps the gene for RAR\(\beta\), the product of which has putative oncosuppressor functions since it is involved in the control of epithelial cell growth and differentiation (37). A large number of squamous lung cancers carry deletions which involve the telomeric part of chromosome 3 and are associated with a reduced expression of the RAR\(\beta\) gene (38–40). Thus, RAR\(\beta\) could be the genetic target for the chromosomal rearrangements at 3p24–ter in HNSCC as well.

This study points out the role of 3p allelic deletions in HNSCC development and suggests that at least three distinct loci, tentatively mapped to 3p14–cen, 3p21.3, and 3p24–ter, are involved in the generation and/or progression of HNSCC. The functional role of these deletions will follow from the identification of the specific targets of these phenomena. To this end, the present study lays down the bases for an accurate positional cloning of the putative oncosuppressor genes in the three specific regions which have been identified. The deletion at the 3p24–ter region has been described originally by Sithananandam et al. (34) for lung cancer. Considerable evidence is in agreement with the existence of a third tumor suppressor gene in this region. The gene responsible for von Hippel-Lindau syndrome has been mapped to the 3p25–p26 band (35, 36) and at the band 3p24, it maps the gene for \(RARB\), the product of which has putative oncosuppressor functions since it is involved in the control of epithelial cell growth and differentiation (37). A large number of squamous lung cancers carry deletions which involve the telomeric part of chromosome 3 and are associated with a reduced expression of the \(RARB\) gene (38–40). Thus, \(RARB\) could be the genetic target for the chromosomal rearrangements at 3p24–ter in HNSCC as well.

### Table 2. Results of 3p LOH analysis

<table>
<thead>
<tr>
<th>Case</th>
<th>Tumor site</th>
<th>C13–946</th>
<th>THR–R</th>
<th>C13–830</th>
<th>CHROMOSOME 3 DELETIONS IN HEAD AND NECK CANCERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN6</td>
<td>P</td>
<td>□</td>
<td></td>
<td>□</td>
<td></td>
</tr>
<tr>
<td>HN17</td>
<td>SGL</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td></td>
</tr>
<tr>
<td>HN2</td>
<td>T</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td></td>
</tr>
<tr>
<td>HN3</td>
<td>T</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td></td>
</tr>
<tr>
<td>HN58</td>
<td>SGL</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td></td>
</tr>
<tr>
<td>HN40</td>
<td>SGL</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td></td>
</tr>
<tr>
<td>HN15</td>
<td>PS</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td></td>
</tr>
<tr>
<td>HN32</td>
<td>SGL</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td></td>
</tr>
<tr>
<td>TC47</td>
<td>SGL</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td></td>
</tr>
<tr>
<td>TC37</td>
<td>SGL</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td></td>
</tr>
<tr>
<td>TC40</td>
<td>GL</td>
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<tr>
<td>HN47</td>
<td>T</td>
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<tr>
<td>HN12</td>
<td>T</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td></td>
</tr>
</tbody>
</table>

Deletion at the 3p24–ter region has been described originally by Sithananandam et al. (34) for lung cancer. Considerable evidence is in agreement with the existence of a third tumor suppressor gene in this region. The gene responsible for von Hippel-Lindau syndrome has been mapped to the 3p25–p26 band (35, 36) and at the band 3p24, it maps the gene for \(RARB\), the product of which has putative oncosuppressor functions since it is involved in the control of epithelial cell growth and differentiation (37). A large number of squamous lung cancers carry deletions which involve the telomeric part of chromosome 3 and are associated with a reduced expression of the \(RARB\) gene (38–40). Thus, \(RARB\) could be the genetic target for the chromosomal rearrangements at 3p24–ter in HNSCC as well.

This study points out the role of 3p allelic deletions in HNSCC development and suggests that at least three distinct loci, tentatively mapped to 3p14–cen, 3p21.3, and 3p24–ter, are involved in the genesis and/or progression of HNSCC. The functional role of these deletions will follow from the identification of the specific targets of these phenomena. To this end, the present study lays down the bases for an accurate positional cloning of the putative oncosuppressor genes in the three specific regions which have been identified. The

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\(P\), pharynx; \(T\), tongue; \(SGL\), supraglottic larynx; \(PS\), pyriform sinus; GL, glottic larynx; OC, oral cavity.

\(\square\), retention of heterozygosity (no loss); \(\blacksquare\), LOH; \(\neg\), homozygosity in normal tissue (not informative; No entry, not done.)
identification of these target genes will increase our understanding of the biology of HNSCC and constitute a basis for improving the management of this neoplasm.

REFERENCES

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