Chromosome 3p Deletions in Head and Neck Carcinomas: Statistical Ascertainment of Allelic Loss

Farida Latif, Matthew Fivash, Gladys Glenn, Kalman Tory, Mary Lou Orcutt, Krista Hampsch, John Delisio, Michael Lerman, Janet Cowan, Michael Beckett, and Ralph Weichselbaum

ABSTRACT

Loss of function of tumor suppressor genes is important in the origin and progression of common adult tumors. Loss of heterozygosity indicating allelic loss has been used to detect chromosomal regions that harbor these genes. Using over 20 restriction fragment length polymorphism markers spaced throughout the entire length of chromosome 3p, we have generated 3p allelotypes for 18–26 head and neck squamous cell carcinoma cell lines. We then estimated the average heterozygosity over 19 loci for a random sample drawn from normal populations to be 0.780 and that for the tumor lines to be 0.65, indicating a gross reduction of heterozygosity, presumably due to allelic loss. Further comparison of per locus heterozygosity in normal and tumor DNAs showed which loci contributed to the general loss of heterozygosity. We showed that the commonly deleted region of 3p probably lies telomeric to D3S3 (3p14) and centromeric to RAP1 (3p25). This large region includes several putative tumor suppressor genes involved in multiple common tumor types of lung, breast, kidney, ovary, and cervix. The data demonstrate that chromosome 3p allelic loss is a common event in head and neck cancers and suggest that chromosome 3p tumor suppressor genes contribute to the pathogenesis of these tumors.

INTRODUCTION

Tumors of the head and neck are a heterogeneous group of neoplasms that manifest a wide range of clinical behaviors (1, 2). In Western countries they constitute up to 5% of all cancers and 90–95% of them are squamous cell carcinomas (1–3). Tobacco and alcohol, well established as attributable risk factors, and some industrial carcinogens are thought to be among etiological factors for head and neck cancers (1–4). Genetic predisposition has also been described in some families with multiple affected individuals (5). However, despite emphasis on early diagnosis, survival rates for patients with head and neck tumors have not improved dramatically (1).

Lack of progress in head and neck oncology emphasizes the importance of molecular studies of genetic changes associated with the origin, development, and malignant progression of these neoplasms. It is assumed that knowledge of such alterations may establish molecular correlates of tumor behavior. This will, in turn, benefit the patient with diagnostic and prognostic information, regarding the clinical course and outcome.

Human carcinogenesis is thought to be a multistep process driven by genetic changes that include inactivation of TSGs (3) on several chromosomes (6, 7). Inactivation of both copies of TSGs as required by the “two-hit” mutation model (6) occurs over a period of time. The first mutation is proposed to occur either in the germline or in the somatic cells, whereas the second hit occurs in somatic cells. This second event often results in reduction to homozygosity of a segment of the chromosome region that harbors the particular TSG. Allelic loss as detected by RFLP deletion analysis has been successfully used to identify TSG loci in a growing number of human cancers (7). A high density set of ordered RFLP markers is required to delineate further the candidate region for a particular TSG.

Deletions in the short arm of chromosome 3 are common in many human cancers (7, 8), including sporadic and hereditary renal carcinomas, small cell lung carcinoma, nonsmall cell lung carcinoma, and the uterine cervix. The chromosome 3p region involved in these malignancies have been mapped with sufficient precision: the von Hippel-Lindau TSG to a small region between RAP1 (3p25) and D3S18 (3p26) (9); the renal carcinomas and small cell lung carcinoma genes to 3p14-p26 (8); the non-small cell lung carcinoma gene to 3p14-p16 (8); and the uterine cervix gene to 3p14-p21 (10).

Previously, cytogenetic studies have been performed in a number of head and neck tumor cell lines (11–13). These studies showed complex karyotypes with frequent abnormalities of chromosomes 1, 3, 6, 9, and 11. Common findings for chromosome 3 included deletions in the short arm with breakpoints at 3p11, 3p12, and 3p14 (11–13).

To date there have been no reports describing allelic loss in HNSCC. The lack of such studies prompted us to initiate molecular mapping of chromosome 3p loci involved in head and neck cancers. This study was greatly facilitated by a high density set of RFLP markers for which order and location information was obtained (14, 15).

Herein we present an extensive allelotype analysis of the short arm of chromosome 3 using 18–26 head and neck tumor cell lines and over 20 RFLP markers spaced throughout the entire length of the short arm. Because of the absence of matching normal tissues, we ascertained allelic loss by a novel statistical method. We tentatively mapped the commonly deleted region distal to D3S3 (3p14) and proximal to D3S18 (3p26).

MATERIALS AND METHODS

HNSCC Cell Lines. The establishment of the HNSCC cell lines was reported earlier (16, 17). They were derived from patient tumors prior to treatment and were free of any nontumorous contaminants that could affect allelic deletion analyses. To avoid changes in long term cultures early passage cells were used for RFLP studies. DNA finger-
printing with a chromosome 3 hypervariable minisatellite probe (14) was performed to confirm that no cross-contamination occurred in our cell cultures and that all cell lines were unique isolates (not shown). The HNSCC cell lines and selected clinical characteristics of the patients from whom they were derived were described earlier (16). Over 80% of them belonged to the Caucasian population.

DNA Extraction and Southern Blot Analysis. DNA was prepared from monolayer cell cultures as described elsewhere (17). DNA (5–10 μg) was digested with a 10-fold excess of restriction enzymes (Bethesda Research Laboratories, Inc., Bethesda, MD) according to the manufacturer’s specifications in buffers supplemented with 5 mM spermidine. Fractionation of digested DNA was performed by electrophoresis in 1% agarose gel in recirculating 20 mM Tris HCl, pH 7.3–10 mM acetate–2 mM EDTA buffer for approximately 10–20 h at 2 V/cm. Following electrophoresis DNA was transferred to nylon membranes (Micron Separations, Inc., Westboro, MA) by using the capillary transfer method (18); afterwards the membranes were baked for 3 h at 80°C in a vacuum oven.

Radioactive DNA probes were prepared by random primer extension method (19) using [α-32P]dCTP (specific activity, >3000 μCi/mmole; Amersham, Arlington Heights, IL). Hybridization of DNA was done at 65°C for 12–18 h in a 5x standard saline-citrate solution containing 0.75 M sodium chloride, 0.075 M sodium citrate (pH 7.0), 1% sodium dodecyl sulfate, denatured salmon sperm DNA (100 μg/ml), and if necessary varying amount of human DNA and denatured labeled DNA probe. Following hybridization, filters were washed at 60°C in 0.1 x standard saline-citrate and 0.1% sodium dodecyl sulfate.

Cytogenetic Analysis. Standard cytogenetic procedures were used in these studies. Briefly, cultured cell lines were harvested for analyses as described earlier (20) with 1 h Colcemid treatment and hypotonon treatment with warmed potassium chloride/sodium citrate solution (0.06 M KCl/0.6% sodium citrate mixed 1:1 before use) for 14 min. Fifteen G-banded cells were karyotyped from each cell line. Cytogenetic analyses were done by one of us (J. C.) on the 14 of the cell lines.

Allelic Frequency Determinations. The frequency of alleles for each of the 18–20 loci analyzed in the present study was determined using information generated from DNA samples obtained from Centre d’Etude du Polymorphisme Humain and from the literature. The observed frequency of heterozygosity in the HNSCC cell lines was determined using information generated from corresponding DNA samples.

Statistical Methods. The method used to calculate the expected heterozygosity in a randomly chosen individual is provided in the “Appendix.” In general, the χ² goodness of fit test and tests assuming binomial or multinominal distributions (21) were used to calculate significance of differences between experimental groups of data (e.g., estimated and observed heterozygosity at 3p loci).

RESULTS

In the commonly used method, allelic loss is determined by comparing constitutional and tumor genotypes for RFLP marker loci for a specific chromosome or chromosome region or throughout the whole genome (recently termed allelotype or allelotyping) (22).

In the present work we used a novel method to ascertain allelic loss on chromosome 3p in HNSCC. First, we generate an allelotype of 3p using a number of HNSCC cell lines and set of 3p RFLP marker loci for which order and map position was established (Table 1). We then compared the average heterozygosity computed for these loci in a randomly chosen individual from natural populations with observed heterozygosity in tumor cell DNA. Further, after concluding a general loss of alleles in the tumor population we estimate the statistical significance of loss for each individual locus which allows us to delimit the commonly deleted region on 3p. Finally, to delineate further the deleted region, we again compare per locus heterozygosity in the natural population and tumor cell DNA.

Table 1 Chromosome 3p polymorphic loci

<table>
<thead>
<tr>
<th>Locus</th>
<th>Location</th>
<th>Probe(s)</th>
<th>Restriction enzyme</th>
<th>Size of alleles (kilobases)</th>
<th>Allelic frequency normal populations</th>
<th>Allelic proportions in head and neck tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3S215</td>
<td>3p26-pter</td>
<td>λLIB 11-31</td>
<td>MspI</td>
<td>10.5; 12.5</td>
<td>0.08; 0.12</td>
<td>0.03; 0.17</td>
</tr>
<tr>
<td>D3S338</td>
<td>3p26-pter</td>
<td>λLIB 48-95</td>
<td>TaqI</td>
<td>9.4; 3.8</td>
<td>0.39; 0.61</td>
<td>0.23; 0.77</td>
</tr>
<tr>
<td>D3S25</td>
<td>3p26-pter</td>
<td>λLIB 42-26</td>
<td>HindIII</td>
<td>9.8; 5.5</td>
<td>0.82; 0.18</td>
<td>0.73; 0.27</td>
</tr>
<tr>
<td>D3S197</td>
<td>3p26-pter</td>
<td>λLIB 21-14</td>
<td>HindIII</td>
<td>2.2; 1.7 + 0.5</td>
<td>0.20; 0.80</td>
<td>0.48; 0.52</td>
</tr>
<tr>
<td>D3S191</td>
<td>3p26-pter</td>
<td>λLIB 38-96</td>
<td>MspI</td>
<td>6.8; 4.7</td>
<td>0.27; 0.73</td>
<td>0.41; 0.59</td>
</tr>
<tr>
<td>D3S18</td>
<td>3p26.1</td>
<td>CRI L162</td>
<td>D21</td>
<td>15.1; 15.5</td>
<td>0.25; 0.75</td>
<td>0.17; 0.83</td>
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<tr>
<td>D3S18</td>
<td>3p26.1</td>
<td>clL162-1</td>
<td>BamHI</td>
<td>8.7; 4.7</td>
<td>0.69; 0.31</td>
<td>0.56; 0.44</td>
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<tr>
<td>D3S72</td>
<td>3p26.1</td>
<td>λChA4-52</td>
<td>HindIII</td>
<td>9.0; 7.5 + 1.5</td>
<td>0.78; 0.22</td>
<td>0.74; 0.26</td>
</tr>
<tr>
<td>D3S771</td>
<td>3p26.1</td>
<td>λChB-4-2</td>
<td>HindIII</td>
<td>2.9; 2.65</td>
<td>0.75; 0.25</td>
<td>0.62; 0.33</td>
</tr>
<tr>
<td>RA1F</td>
<td>3p25</td>
<td>p267</td>
<td>BglII</td>
<td>4.0; 3.3</td>
<td>0.78; 0.22</td>
<td>0.71; 0.29</td>
</tr>
<tr>
<td>D3S58R</td>
<td>3p24-p24</td>
<td>λLIB 12-69</td>
<td>HindIII</td>
<td>7.2; 6.5; 5.9; 5.0</td>
<td>0.07; 0.41; 0.36</td>
<td>0.04; 0.04; 0.56; 0.36</td>
</tr>
<tr>
<td>THRB</td>
<td>3p24</td>
<td>pHB 302</td>
<td>TaqI</td>
<td>7.0; 5.5</td>
<td>0.35; 0.65</td>
<td>0.26; 0.74</td>
</tr>
<tr>
<td>D3S74</td>
<td>3p21-24</td>
<td>λLIB 7-78</td>
<td>TaqI</td>
<td>11.5; 8.9; 7.6</td>
<td>0.02; 0.75; 0.23</td>
<td>0.04; 0.80; 0.08</td>
</tr>
<tr>
<td>D3S62</td>
<td>3p21-24</td>
<td>λLIB 45-82</td>
<td>MspI</td>
<td>4.5; 3.5</td>
<td>0.61; 0.39</td>
<td>0.47; 0.53</td>
</tr>
<tr>
<td>D3F1552</td>
<td>3p21</td>
<td>pH3H2</td>
<td>HindIII</td>
<td>2.0; 2.3</td>
<td>0.50; 0.5</td>
<td>0.25; 0.48</td>
</tr>
<tr>
<td>D3S2</td>
<td>3p14-p21</td>
<td>pEFD145.1</td>
<td>TaqI</td>
<td>9.0; 4.0</td>
<td>0.47; 0.53</td>
<td>0.27; 0.63</td>
</tr>
<tr>
<td>D3S21</td>
<td>3p14.2-21</td>
<td>pH12-32</td>
<td>MspI</td>
<td>2.9; 1.3 + 1.6</td>
<td>0.74; 0.26</td>
<td>0.59; 0.41</td>
</tr>
<tr>
<td>D3S642</td>
<td>3p13</td>
<td>λCh 4A-7</td>
<td>MspI</td>
<td>3.8; 3.4; 2.8</td>
<td>0.34; 0.58; 0.08</td>
<td>0.44; 0.44; 0.12</td>
</tr>
<tr>
<td>D3S3</td>
<td>3p13</td>
<td>pMS 1-37</td>
<td>MspI</td>
<td>4.9; 3.7 + 1.2</td>
<td>0.06; 0.94</td>
<td>0.12; 0.88</td>
</tr>
<tr>
<td>D3S4</td>
<td>3p11-p12</td>
<td>λIB 267</td>
<td>TaqI</td>
<td>4.8; 1.6</td>
<td>0.16; 0.84</td>
<td>0.18; 0.82</td>
</tr>
<tr>
<td>D3S216</td>
<td>3p11</td>
<td>λIB 38-90</td>
<td>EcoRI</td>
<td>3.5; 3.0</td>
<td>0.68; 0.32</td>
<td>0.70; 0.30</td>
</tr>
<tr>
<td>D3S733</td>
<td>3p11</td>
<td>λLIB 17-78</td>
<td>HindIII</td>
<td>12.0; 7.5</td>
<td>0.20; 0.80</td>
<td>0.16; 0.84</td>
</tr>
<tr>
<td>D3S573</td>
<td>3p11</td>
<td>λLIB 3-23</td>
<td>EcoRI</td>
<td>7.3; 1.4</td>
<td>0.38; 0.62</td>
<td>0.43; 0.57</td>
</tr>
<tr>
<td>D3S42</td>
<td>3p11</td>
<td>pEFD 64.1</td>
<td>HindIII</td>
<td>1.08; 5.5</td>
<td>0.06; 0.42</td>
<td>0.23; 0.77</td>
</tr>
</tbody>
</table>

* Polymorphic probes and the loci they represent are listed in the order they occur on the chromosome. The order was established by physical mapping and genetic linkage (Refs. 9, 14, 15; K. Tory, F. Latif, W. Modi, L. Schmidt, M. H. Wei, H. Li, P. Cobler, M. L. Orcutt, J. Delisio, L. Geil, B. Zbar, and M. I. Lerman. A genetic linkage map of 96 loci on the short arm of human chromosome 3, Genomics, in press, 1992.)
Table 2 presents results of allelotyping of 18 HNSCC cell lines tested with 19–22 markers and an additional 8 lines tested with only 7 critical marker loci. A brief empirical inspection of the data clearly showed very low levels of heterozygosity for all tested loci suggesting possible allelic losses. Every locus distal to D3S2 retained heterozygosity at least in one or more tumor genotypes, suggesting that the critical region must be proximal to this locus. Interestingly, a variable number of tandem repeats type 3q marker retained heterozygosity in 18 of the 26 cell lines indicating that mostly the short arm suffered allelic losses and that a total loss of one of the chromosome 3s did not occur.

To precisely ascertain loss of heterozygosity on 3p the data were subjected to statistical analysis. Fig. 1 presents the expected distribution of the number of heterozygous loci in a random sample of individuals assayed for the 22 loci with varying per locus heterozygosities (Table 1). Assuming that both the normal population (unrelated individuals provided by the Centre d’Etude du Polymorphisme Humain reference families) and the tumor-bearing subpopulation are in Hardy-Weinberg equilibrium, these distributions can be calculated from per locus FOH, (see “Appendix” for the derivation of the probability function). FOH for the normal population was generated using Centre d’Etude du Polymorphisme Humain-provided DNAs and, for some loci, the Centre d’Etude du Polymorphisme Humain database. FOH for the tumor-bearing subpopulation was derived from an estimate based on the tumor allelotypes (Table 2) using allele representation at each locus in the tumor DNA. A χ² goodness of fit test of the hypothesis that both distributions are the same yielded P = 0.99. Since these two distributions are statistically the same, the normal population may be used as a reference for comparison with the tumor samples.

The tumor subpopulation may be examined for an overall loss of heterozygosity by comparing the observed number of heterozygous loci per individual with the expected number of heterozygous loci. This comparison may be made in three
different ways: for 10 cell lines on 22 loci; 17 cell lines on 19 loci; and 28 cell lines on 5 loci. For each comparison a distribution for the number of heterozygous loci per individual was determined (see "Appendix"), using the per locus FOH for the normal population and the tumors. These distributions were compared with a $\chi^2$ goodness of fit test to ensure the validity of using the normal population as a reference. Table 3 shows the results of comparing the expected and observed number of heterozygous loci per individual for the three ways outlined above. In each case it is clear that the tumors show a significant loss of heterozygosity. Fig. 2 illustrates this conclusion for the case 17 cell lines on 19 loci.

The per locus allele proportions in both the normal population and the tumors were found to be statistically the same (Table 1). This fact allows per locus comparisons of the expected and observed number of heterozygotes in the tumor panel. Individual loci which show significant loss of heterozygosity are identified at the 0.0023 significance level (to control type 1 errors). Table 4 shows which loci have lost significant heterozygosity.

### DISCUSSION

Loss of heterozygosity at TSG loci occurs frequently in human cancers and correlates with tumor stage and clinical behavior (7). Previously, cytogenetic analyses of HNSCC have revealed complex karyotypes suggestive of consistent deletions at TSG sites including chromosome 3p. However, to date there have been no molecular studies describing allelic loss at TSG loci in head and neck cancers. The present paper examines allelic loss at chromosome 3p TSG sites in HNSCC. We used 19–22 HNSCC cell lines and a high density set of RFLP markers (over 20) on chromosome 3p to generate 3p allelotypes of tumor cells. The high-resolution RFLP allelotypes were subjected to a novel statistical method to ascertain allelic loss and to define the commonly deleted region. This paper describes the experimental data and the empirical and statistical analyses, while the "Appendix" provides the derivation of the probability function for distribution of heterozygous loci in a random individual. Using these novel approaches, we demonstrated for the first time allelic losses from a large area of chromosome 3p in 18–28 HNSCC cell lines. We defined this area broadly as distal to 3p14 and proximal to 3p25 in agreement with our (not shown) and published cytogenetic observations (11–13). This large portion of chromosome 3p is known to harbor TSGs important in the development of common human cancers of the kidney, lung, breast, ovary, and cervix (7, 8), and the von Hippel-Lindau TSG (9). The dramatic loss of heterozygosity on 3p from this large region first shown in the present work suggests that TSGs located in this region of 3p may be important in the pathogenesis of HNSCC.

In relation to tumor stage, it should be emphasized that the
null

HNSCC cell lines used in our study were derived from late stage I and early stage II disease at which point there are no discernible nodal (N) or distant metastases (M) (Ti_2, N0, M0). This would suggest that 3p TSGs might be most important to the initiation of the tumorigenic process and/or early development of HNSCC. Obviously, analysis of a greater number of early (and late stage) forms of HNSCC is required to confirm 3p involvement in initiation and progression of head and neck cancers.

The present paper outlines a novel statistical method to ascertain allelic losses in tumor DNA samples when the absence of normal matching tissues precludes the use of previous methods that utilized comparisons of normal and tumor genotypes of RFLP marker loci. Chromosome-specific allelic loss for a high density markerallelotype can easily be computed using this approach (see "Appendix"). We believe that this method will prove important in analyzing allelotypes of tumor cell lines that have been established earlier. However, further work is necessary to examine the robustness of the method, especially with regard to high resolution mapping.

In summary, we have detected 3p allelic loss in a large sample (18-26) of HNSCC cell lines using a novel statistical approach to ascertain loss of heterozygosity. A high density set of 3p RFLP markers allows us to define the commonly deleted region as 3p14 to p26. Further work will be aimed at mapping the deleted TSG sites with better precision and trying to find correlates with tumor stage and behavior.

ACKNOWLEDGMENTS

We are grateful to Drs. B. Zbar and G. Alvord for support and Dr. J. Rowley's critical reading of the manuscript.

APPENDIX: A Probability Function for the Expected Number of Heterozygous Loci per Individual

M. Fivash

The probability function for the number of heterozygous loci per individual (of those used in this analysis) is calculated from the per locus heterozygosity with the assumptions that all the loci are in Hardy Weinberg equilibrium and there is random association between the loci. This problem has been examined by Mitton and Pierce (23) through a simulation study. The results of their simulation study were confirmed analytically by Chakraborty (24). Chakraborty presented a recursion for calculating the distribution function for the expected number of heterozygous loci per individual. The distribution function then yields the probability function through successive differences. Calculation of the distribution function and extraction of successive differences may, however, be avoided by a recursion which directly yields the probability function.

Denote by \( h_i \) frequency of heterozygosity of the genotype at the \( i \)th locus. Then the probability that none of the loci express a heterozygous genotype is

\[
Pr(x = 0) = \prod_{i=1}^{m} (1 - h_i)
\]

Denote the total number of loci by \( m \) and let \( x \) be the number of heterozygous loci. Define the following quantities used to calculate the recursion.

\[
p_i = Pr(x = i) \quad r_0 = p_0
\]

Then the probability that some number, \( i \), of the loci express heterozygous genotypes in an individual may be found through the recursion relations (21).

\[
p_{i+1} = \sum_{i} \frac{h_i}{1 - h_i} r_i
\]

The first sum in Equations C need not be actually calculated because all but one term of this sum is calculated for the first component of the vector \( r_{i+1} \). A PC program is available from the author which implements this recursion.

When all the \( h_i \) are equal the probability function reduces to the familiar binomial probability function. The distribution of the number of heterozygous loci per individual in a random sample was calculated for the normal population from the per locus FOH and for the tumor subpopulation from an estimate of the per locus FOH based on the allele representation at each locus in tumor DNA. A \( \chi^2 \) goodness of fit test showed that both distributions may be considered the same.

The expected number, \( x \), of heterozygous loci per individual and the variance of \( x \) may be determined from the probability function for the normal population by Equations D and C.

\[
E(X) = \sum_{i=0}^{m} p_i i
\]

\[
Var(X) = \sum_{i=0}^{m} p_i i^2 - E^2(X)
\]

The expected and observed number of heterozygous loci per individual may be compared by finding the probability that as few or fewer heterozygous loci per individual will be observed in the tumor panel by chance if it is assumed that the tumor panel has the same distribution of heterozygous loci per individual as the normal population. This probability can be calculated based on the distribution of the number of heterozygous loci per individual in the normal population and is given by

\[
Pr(X \leq x) = \prod_{i=1}^{m} \sum_{j=0}^{i} p_j
\]

where \( g_i \) is the number of heterozygous loci in the \( i \)th individual. This \( P \) value is \( 5.4 \times 10^{-28} \) for the tumor subpopulation (17 cell lines and 19 loci) considered here. Such a small probability for this overall test suggests that many tumor subpopulation loci have moved in the direction of reduced heterozygosity.
REFERENCES


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