Genetic Analysis of Head and Neck Squamous Cell Carcinoma and Surrounding Mucosa

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Objective: Comparative genomic hybridization was performed on head and neck squamous cell carcinoma and surrounding mucosa to determine whether common chromosomal aberrations could be detected that would predispose an individual to developing a second primary tumor.

Design: Biopsy specimens were taken from 19 patients with squamous cell carcinoma of the head and neck, 3 samples from each person: 1 specimen from the tumor site and 1 each from 1 and 5 cm from the macroscopic tumor margin. Samples were snap frozen in liquid nitrogen. A portion of each distant sample and tissue taken from immediately adjacent to the site of the tumor specimen were sectioned and stained with hematoxylin-eosin, either to search by light microscopy for tumor cells or signs of dysplasia in the distant samples, or to determine whether the tumor specimen had substantial non-tumor cell content. Tissue adjacent to the tumor biopsy site was used because the biopsy specimens were relatively small. Comparative genomic hybridization was performed on all samples.

Subjects: Nineteen patients with newly diagnosed carcinomas of the head and neck.

Results: The tumor biopsy specimens showed no substantial nontumor cell content, and the distant specimens were all histologically normal. The tumors showed multiple mutations: mean (SD) number of deletions, 5.4 (4.3); amplifications, 5.2 (4.6). Deletion of chromosome 3p was seen in 13 of 19 cases and was associated with amplification of 3q in 10 cases. No mutations were seen in the distant biopsy specimens.

Conclusions: Frequently occurring chromosomal aberrations were seen in the tumor cells, suggesting a key role for these mutations in tumor development. Screening histologically normal upper aerodigestive tract mucosa with comparative genomic hybridization does not provide information on early genetic events that predispose a patient to developing a second primary tumor.


Each year there are approximately 500,000 new cases of head and neck squamous cell carcinoma (HNSCC) globally, with HNSCC of the larynx composing about 1% of all malignancies. The overall survival rate for this disease group remains poor, largely due to the high incidence of recurrent disease at the primary site or in the regional lymph nodes. Patients presenting with HNSCC also demonstrate a high incidence of second primary tumors in the upper aerodigestive tract, which may be synchronous or metachronous.

Two theories, which are not mutually exclusive, have been advanced to explain the high frequency of second primary tumors: field of cancerization and micrometastatic spread. The first theory, proposed by Slaughter et al., hypothesizes that large tracts of the aerodigestive tissue are affected by carcinogens and that multifocal oral carcinomas occur as a result of independent mutations. This theory fits with the widely accepted model of Fearon and Vogelstein, who proposed a multistep theory of carcinogenesis; ie, there is a strong association between the number of genetic mutations and development of clinical malignancy. The second theory was proposed by Sidransky et al., who showed that some second primary tumors are derived from micrometastases and hence have the same clonal origin as the primary tumor. The hypothesis of Sidransky and his group was based on studies of X chromosome inactivation in a bladder tumor model. More recently, results from loss of heterozygosity studies on HNSCC, by the same group, have supported this theory. Ten independent loci were examined, and a progressive relationship was reported between the degree of chromosomal loss and histological abnormality in HNSCC.

A recent study on loss of heterozygosity has shown that deletions of 9p21, but not 3p14 or 3p21, occur in the absence of histological change in mucosa of the head and neck. Interestingly, this work showed that some patients had disparate...
PATIENTS, MATERIALS, AND METHODS

TISSUE SAMPLES

Tissue samples were collected from 19 patients undergoing composite resections for oral cavity and oropharyngeal tumors, or total laryngectomy with or without partial pharyngectomy for tumors of the laryngopharynx. Biopsy specimens of epithelial mucosa with the minimum of contaminating submucosal tissue were taken. Ethical committee approval was granted for this study, and informed consent was obtained from all patients. Three samples were taken in each case, 1 from the tumor site and 1 each from 1 cm and 5 cm away, respectively, from the superior macroscopic margin running superiorly from the tumor mass, as measured with a ruler. For the latter 2 samples, the mucosa was of normal macroscopic appearance. All samples were immediately snap frozen in liquid nitrogen and stored at −80°C. Table 1 gives clinical characteristics of the patients.

A representative section of the distant samples was removed and placed in 10% formal saline for histological analysis. The CGH tumor biopsy specimens were comparatively small, so histological examination of the tumor was undertaken on tissue immediately adjacent to the biopsy site. Extraction of genomic DNA from the remainder of the tissue was performed according to standard protocols9 (briefly, overnight digestion with proteinase K (0.5 mg/mL), followed by extraction with phenol, phenol chloroform, and chloroform isoamyl alcohol). The DNA quality and concentration were assessed by measuring absorbance at 260 nm and electrophoresis on a 1.2% agarose gel.

The samples sent for histopathological study were processed in the usual way and stained with hematoxylin-eosin, first, to detect signs of tumor or dysplasia in the 1- and 5-cm distant mucosa, and second, to determine whether the tumor biopsy specimen contained substantial nontumor cell infiltration. All sections were analyzed by one of us (A.W.M.).

COMPARATIVE GENOMIC HYBRIDIZATION

All reagents were purchased from Vysis UK Ltd, Richmond, England, unless otherwise indicated: 1 µg of test genomic DNA was nick labeled with fluorescein-12-d2'-deoxy-(uridine 5'-triphosphate) (Boehringer Mannheim, Lewes, England) according to the manufacturer’s protocol; 200 ng of the labeled DNA was combined with 100 ng of Spectrum Red–labeled normal reference genomic DNA and 10 µg of COT 1 blocking DNA, then ethanol-precipitated. A 2:1 ratio of test-reference DNA was recommended by the manufacturer’s protocol (Vysis UK Ltd). The probe was resuspended in 10 µL of hybridization buffer and hybridized to a normal male metaphase spread prepared from peripheral blood lymphocytes for 72 hours at 37°C in a humidified chamber. Nonspecifically bound probe was removed by washing the slides in 0.4 × saline sodium citrate/0.3% NP-40 (pH 7.5) at 74°C for 2 minutes followed by 2 × saline sodium citrate/0.1% nonidet P-40 (pH 7.5) at ambient temperature for 40 seconds. The slides were then air dried and the chromosomes counterstained with 30 µL of 125 ng/mL 4,6 diamidino-2-phenylindole in antifade.

IMAGE ANALYSIS

Slides were examined using a Nikon Diaphot-TMD inverted epifluorescent microscope (Nikon UK Ltd, Kingston, England) connected to a photometrics cooled CCD camera. Analysis was performed on a QUIPS XL genetic workstation (Vysis UK Ltd). For each metaphase, 3 fluorescent images were captured using filters specific for DAPI, fluorescein isothiocyanate, and tetra methyl rhodamine isothiocyanate. Images were analyzed through several steps: separation of chromosomes from the background, identification of chromosomes using the banding patterns of the DAPI image, and correction of background and normalization of the red and green fluorescent intensities. The ratio profiles were produced by the superimposition of the 3 fluorescent images and division of the green profile by the red profile along the axis of the chromosomes. For each hybridization, at least 10 metaphases were captured and averaged to produce a karyogram of mean ratio profiles. Copy number changes were defined as chromosomal regions for which the mean fluorescein–Spectrum Red ratio exceeded the thresholds determined by control experiments (0.85–1.15). High-level amplifications were defined as regions having a ratio greater than 2, as defined previously.14

deletions in preinvasive and invasive lesions, which supports the field-of-cancerization hypothesis. Conversely, other patients showed a pattern of mutations consistent with a micrometastases mechanism of spread, which would indicate that different mechanisms are responsible for the high incidence of second primary tumors in patients with HNSCC.

There has been little work on laryngopharyngeal cancer in relation to field change, which is surprising because it is the most common mucosal HNSCC in the United Kingdom and North America,4 and premalignant changes in the larynx are frequently observed. The long-term sequelae in patients with laryngeal epithelial changes range from mild dysplasia to carcinoma in situ. In a study of 98 patients with mild dysplasia, 2 subsequently developed squamous cell carcinoma, while 8 of 39 patients with severe dysplasia developed frank malignancies.7 Identifying tissues that show an increased risk of second primary tumors, whatever the cause, would be useful in planning treatment protocols for patients with HNSCC following surgery.

In the present study, comparative genomic hybridization (CGH) was performed on genomic DNA extracted from the tumor sites of 19 patients with HNSCC and on biopsy specimens taken from 1 and 5 cm away from each tumor site; 11 of these cases were laryngeal tumors. Comparative genomic hybridization is a powerful technique for identifying DNA copy number changes across the entire genome in a single hybridization.5,6 The aim of this study was to discover if mutations could be detected in the normal mucosa in addition to the tumor, complementing previous studies...
where a few specific genes and/or chromosomal loci were investigated.\(^4,5\) Previous CGH studies on head and neck tumors have identified common genetic aberrations,\(^10-14\) but to our knowledge, there has been no study of the surrounding normal mucosa using this technique.

### Table 1. Clinical Characteristics of Patients Undergoing Treatment for Head and Neck Squamous Cell Carcinoma

<table>
<thead>
<tr>
<th>Patient/Age, y</th>
<th>Smoker?</th>
<th>Radiotherapy</th>
<th>Primary Site</th>
<th>Staging</th>
<th>Differentiation</th>
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<tr>
<td>1/78</td>
<td>Ex-smoker</td>
<td>No</td>
<td>Larynx</td>
<td>T3 N1</td>
<td>Moderate</td>
</tr>
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<td>2/74</td>
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<td>Larynx</td>
<td>T2 N0</td>
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<tr>
<td>3/57</td>
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<td>Yes</td>
<td>Tongue</td>
<td>T3 N1</td>
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<td>4/56</td>
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<td>No</td>
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<td>5/51</td>
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<td>6/72</td>
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<td>7/59</td>
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<td>Tonsil</td>
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<td>Poor</td>
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<tr>
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<td>No</td>
<td>Tonsil/tongue base</td>
<td>T3 N1</td>
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<tr>
<td>9/64</td>
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<td>Yes</td>
<td>Larynx</td>
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<td>Moderate</td>
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<td>10/46</td>
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<td>Yes</td>
<td>Tonsil/retromolar trigone</td>
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<td>Post cricoid</td>
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<tr>
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<td>Oral cavity</td>
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<tr>
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<td>Yes</td>
<td>Larynx</td>
<td>T2 N0</td>
<td>Good/moderate</td>
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</table>

**Figure 1.** Comparative genomic hybridization profiles of biopsy specimens from the tumor taken from a patient with laryngeal squamous cell carcinoma. The comparative genomic hybridization ratio profile (yellow) with 95% confidence intervals (black) are shown for each chromosome. Vertical green bars on the right side of a chromosome indicate a gain of genetic material; vertical purple bars on the left side of a chromosome, a loss; n, the number of individual chromosomes analyzed.
RESULTS

HISTOLOGICAL FINDINGS

None of the tissue adjacent to the tumor biopsy specimens showed substantial nontumor cell infiltration, which indicated that the DNA extracted was suitable for CGH. Of the 19 samples taken from 1 cm away from the tumor margin, 1 contained an area of carcinoma in situ, although this area was less than 5% of the tissue sample. The remaining 1-cm, and all of the 5-cm-distant mucosa biopsy specimens were histologically normal. Some sections showed evidence of inflammation; however, contaminating “normal” DNA from the infiltrating leukocytes or from nonmucosal cells did not constitute a substantial proportion of the total DNA extracted from the sample and therefore would be unlikely to affect the final CGH profile.

CGH ANALYSIS OF SURROUNDING MUCOSA

The only regions that registered as amplifications or deletions on the 1- and 5-cm-distant samples occurred either at the centromeres or at the telomeres, regions from which little reliable data can be gained. Figures 1, 2, and 3 show the 3 CGH profiles for a representative patient.

CGH ANALYSIS OF TUMOR TISSUE

A summary of DNA copy number changes for all 19 tumors, highlighting common patterns of aberrations, is shown in Figure 4. The mean (SD) number of amplifications and deletions per tumor was very similar (5.2 [4.6] and 5.4 [4.3], respectively), although the deletions encompassed a larger portion of the chromosome than the amplifications (Figure 1, and Figure 4). No associations were found between tumor stage, degree of differentiation, previous radiotherapy, and genetic aberration (data not shown).

DELETIONS

The most common deletion was on chromosome 3. Thirteen of 19 tumors contained deletions on 3p, and in 10 of these cases the entire short arm was deleted. Deletions on 11q occurred in 9 of 19 cases with the region 11q23-qter deleted in all 9 cases. Other common regions of DNA loss were 9p2-pter (5 of 19), 18q21.2-qter.
(10 of 19), 8p21.1-qter (6 of 19), 13q32-qter (5 of 19), 4q31.3-qter (4 of 19), and 5q14-q23(4 of 19).

AMPLIFICATIONS

Gain on the 3q arm occurred in 11 of 19 cases, including a high-level amplification in 1 case of the region 3q24-qter. The common region of amplification on 3q was the locus 3q24-3q28. In 10 of 19 cases, amplification of the q arm occurred in tandem with deletion of the p arm. In 3 of these cases where gain and loss of the whole arm occurred, it is likely that a 3q isochromosome was formed. Increased copy numbers occurred on 11q for 6 of 19 tumors including 5 cases with amplification at the 11q13 locus. Amplification of 11q13 was accompanied by distal deletion of 11q in all 5 cases. Other regions of amplification were: 8q23-q24.2 (6 of 19), 8q12-13 (5 of 19), 9q13-23 (5 of 19), 5p11-p13 (5 of 19), and 2q31-q32 (5 of 19). High-level amplifications were found on 5p and 12p.

The identification of altered tissue with an increased risk of progression toward either synchronous or metachronous primary tumors would be invaluable for the clinician when monitoring patients with HNSCC for growth of a second primary or recurrent tumor. It is generally accepted that tumor development progresses via the accumulation of genetic change.\textsuperscript{2,15} In breast cancer, histologically normal tissue contains allelic imbalances,\textsuperscript{16} although this study used microsatellite analysis and, therefore, by definition was focused on particular genes. Other relevant studies include the demonstration that 9p21 mutations can be detected in normal mucosa\textsuperscript{5} and that gain of chromosome 3q marks the transition from severe dysplasia to invasive carcinoma in cancer of the uterine cervix.\textsuperscript{17} Therefore, we hypothesised that it would be possible to detect early genetic events using CGH to screen the entire genome in the histologically normal mucosa surrounding a primary tumor. Although laryngeal tumors do not metastasize as frequently as tumors of, for instance, the oral cavity, a relatively high occurrence of metachronous primary tumors has been reported for laryngopharyngeal tumors.\textsuperscript{18} Comparative genomic hybridization is an efficient and powerful method for molecular genetic analysis of solid tissues compared with traditional methods, which are labor intensive and yield results that are difficult to interpret.\textsuperscript{9}
Application of CGH to HNSCC has been limited to a handful of studies that have analyzed mixed groups of tumors, and it has not been used to examine mucosa surrounding the tumor site.10-14

Comparative genomic hybridization of the 19 HNSCCs highlighted common copy number changes. The most commonly affected chromosome was chromosome 3, with the presumed formation of the 3q isochromosome occurring in at least 3 cases. Several groups have reported tumor suppressor genes on the short arm of chromosome 3, in particular the regions 3p24-p25.119 and 3p25-2620; the latter harbors the von Hippel-Lindau tumor suppressor gene. These regions were deleted, as part of a much larger region, in 11 of 19 tumors in our study. Amplification of 11q13 has been reported in other studies and occurred in 5 of 19 tumors in this study.21 This region contains the PRAD-1 gene coding for cyclin D1 protein, a cell cycle regulatory protein involved in the transition of cells from G1 to S phase. Overexpression of cyclin D1 is thought to lead to biological aggressiveness and correlates with a poor prognosis for patients with HNSCC.22,23 All tumors with amplification of 11q13 had a simultaneous deletion of distal 11q, suggesting a connection between amplification and deletion at these regions. However, 4 of 19 tumors showed loss of distal 11q alone. No correlation was found, as it was by Bockmühl et al,12 between simultaneous amplification-deletion on 11q and poorly differentiated tumors, although the number of patients with poorly differentiated tumors in our study was low. The relatively low number of tumors studied prevents any meaningful associations between genetic events and clinical parameters. We are currently undertaking a larger study comparing known prognostic makers and clinical parameters with CGH data.

Interestingly, 2 of the 5 tumors with an amplification of 11q13 also had a deletion on 13q encapsulating region 13q14. This region contains the Rb gene, which codes for another cell cycle regulatory protein involved in the same pathway as cyclin D1. In addition, in 1 tumor the region 9p21, coding for a further component of this pathway, was also deleted. Bockmühl et al12 identified the same pattern but at a higher frequency (50%). Deletions of material distal to 13q14 occurred in 5 of 19 tumors, agreeing with other evidence for a putative tumor suppressor gene on 13q.24 In fact, the region 13q21 was common to all deletions of 13q, suggesting that this may be the site of additional tumor suppressor genes.

In laryngeal tumors there seems to be an association between amplifications of 5p and 8q24. A 5p amplification occurred only in laryngeal tumors (5 of 11), and 4 of these also had an amplification of 8q24. To our knowledge, neither of these facts has previously been reported. The biological and clinical relevance of this or any other association will require larger studies incorporating clinical follow-up data. A review of the published literature on CGH of head and neck tumors shows that different groups detect changes occurring with markedly different frequencies. Bockmühl and colleagues,11 working in Germany, reported deletion of 3p in 97% of cases (29 of 30). In contrast, Speicher et al,14 working in the United States, reported a 38% frequency (5 of 13); herein, we observed a 68% frequency (13 of 19). This and other apparent discrepancies are due either to differences in the CGH procedure and analysis or, more in-

**Figure 4.** Summary of the DNA copy number changes of all 19 tumors. Vertical bars on the right side of a chromosome indicate a gain of genetic material; vertical bars on the left side of a chromosome, a loss. The shading highlights aberrations found in at least 25% of tumors.
Interestingly, to genetic and environmental influences on the different groups of patients studied. This issue needs to be addressed because similar differences are reported in CGH data on breast cancer.25,26

One possible explanation for the failure of CGH to show chromosomal imbalances in the surrounding mucosa is that the transformed cells constitute only a small amount of the tissue. Using in situ hybridization, Choi and Chung27 found cells with polysomies of chromosomes 7 and 17 in normal mucosa adjacent to tumors in a variety of HNSCCs. Although the frequency of polysomic cells was relatively low for chromosomes 7 (6.61% [range, 2.5%-15%]) and 17 (7.13% [range, 2.0%-22.5%]), it must be remembered that because of nuclear truncation, in situ hybridization studies on tissue sections underrepresent the chromosome frequency. Nevertheless, altered genomes present at a low frequency would be masked by the majority of normal cells. One approach would be to use microdissection to ensure that DNA was extracted only from epithelial mucosa, as was done in a study of uterine dysplasia.17 but even this would not overcome the problem if the frequency of aberrant cells was low. Alternatively, the early genetic events responsible for a premalignant state may be subtle changes such as point mutations or relatively low copy number changes that cannot be detected by CGH. One of the limitations of CGH is that the resolution is limited to copy number changes of relatively large loci (>5 megabases), and it cannot provide information on DNA affected by other aberrations such as balanced translocations or point mutations.8

As no genetic aberrations were detected in the 38 distant biopsy specimens, we conclude that CGH is not suitable for screening histologically normal aerodigestive mucosa to detect genetic mutations predisposing a patient to a second primary tumor. However, CGH analysis has highlighted a number of important common regions of copy number changes in HNSCC. The frequency of the mutations differs substantially from other studies; the reasons for this need further investigation. Many of the loci identified by CGH are known to contain already characterized or putative oncogenes and tumor suppressor genes. In addition, several chromosomal regions have been identified that contain no tumor-associated genes (Table 2). These regions are worthy of further investigation using polymerase chain reaction–based techniques that can pinpoint the critical genetic loci responsible for the biological and clinical behavior of HNSCCs.

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REFERENCES


