Malignancy Detection by Molecular Cytogenetics in Clinically Normal Mucosa Adjacent to Head and Neck Tumors

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Objective: To identify the potential use of chromosome imbalances as biomarkers for tumorigenesis in head and neck squamous cell carcinoma (HNSCC) by fluorescence in situ hybridization (FISH).

Design: In this case-control study, chromosome copy numbers were assessed in dual-target, dual-color FISH assays using DNA probes specific for 14 human chromosomes (1, 2, 3, 6, 7, 8, 9, 10, 11, 12, 15, 17, X, and Y) applied to exfoliated epithelial cells.

Setting: University medical center.

Patients: We examined 20 cell brushings (from 10 primary tumors and 10 clinically normal margins) collected from 10 patients with HNSCC and compared these with cell brushings from the oral cavity of 10 non-smoker and 10 smoker control subjects.

Intervention: None.

Main Outcome Measure: Chromosomal aneuploidy.

Results: Specimens from nonsmokers displayed greater than 91% of cells with normal signals, indicating high analytical sensitivity for the probes. Specimens from smokers demonstrated large variability without significant imbalance (P<.05) compared with those from nonsmokers. Tumor specimens from patients with HNSCC displayed significant chromosomal imbalance (P<.05) for all probes except chromosome Y. Similar imbalance, although in lower frequency, was found in all clinically normal adjacent mucosa specimens.

Conclusions: Interphase FISH demonstrated great applicability in detecting chromosome imbalance associated with malignancy in HNSCC and clinically normal adjacent cells, thereby detecting subclinical tumorigenesis. A panel of chromosome probes (chromosomes 3, 8, 9, and 10) is proposed as an efficient and sensitive additional tool for future routine screening of tumor margins and potential diagnosis of residual disease in HNSCC.


HEAD AND NECK cancer is estimated to account for 30,300 new cases and 8000 deaths in 1998 in the United States.¹ The single most important prognostic factor for head and neck squamous cell carcinoma (HNSCC) is complete surgical removal of the neoplasm, and failure to eradicate the primary tumor is the leading cause of death for patients.²³ If microscopic cancer is present at a margin of resection, the rate of local recurrence increases substantially and the survival rate decreases.⁴ The presence of metastatic squamous cell cancer in cervical lymph nodes also increases the risk of local, regional, and distant metastatic spread, correlating with a 50% decrease in survival.⁵

Currently, head and neck surgeons rely heavily on the histopathological assessment of surgical margins to ensure total excision of the tumor in patients with head and neck cancer. However, conventional cytology techniques are subject to error in detecting small numbers of cancer cells at margins or in cervical lymph nodes. More so, subtle cellular changes that are known to exist in clinically and histopathologically normal tissue adjacent to tumors, likely representing the early genetic changes in carcinogenesis, are missed. Therefore, determining premalignant genetic abnormalities in histopathologically normal tissue would be useful. Potential biomarkers for tumorigenesis include indicators of the degree of generalized and specific genetic changes as well as the degree of cellular deregulation in the tissue at risk for tumor development. Several markers have shown promise for tumorigenesis, although each uti-
MATERIALS AND METHODS

SUBJECTS

After obtaining institutional review board approval and informed consent, cell specimens were collected from 10 healthy male nonsmokers and 10 healthy male smokers (≥1 pack-year history) selected as controls, and from 10 patients with a recently diagnosed, untreated HNSCC. Characteristics of the patients are summarized in Table 1. The mean age for nonsmokers was 28 years with a range between 19 and 36 years. The mean age for smokers was 39 years with a range between 18 and 61 years, and an average smoking exposure of 11.2 pack-years.

CELL COLLECTION

Selected control subjects were asked to rinse the mouth vigorously with 40 mL of antiseptic mouthwash solution. Cell sampling was done by swabbing the inside of cheek with a cytology brush (PurFybr, Munster, Ind), which was immediately washed in a preservative solution (0.01-mol/L Tris-hydrochloride, 0.02-mol/L sodium chloride, 0.1-mol/L EDTA [pH 7.0]). Cell suspensions were centrifuged for 10 minutes at 1000 rpm, resuspended in fresh fixative (3 parts methanol:1 part glacial acetic acid) for 20 minutes at room temperature, and subsequently pipetted onto microscope slides (Superfrost Plus, Erle Scientific, Portsmouth, NH). From patients with untreated HNSCC, following a similar procedure, cells were obtained from the tumor site and the clinically normal adjacent mucosa. Cells were brushed either before surgical resection for the oral lesions or from the surgical resections for other lesions.

DUAL TARGET, DUAL COLOR FISH ASSAYS

Slide and Probe Preparation

Cells were fixed onto the microscope slides by exposure to 65°C for 4 hours before being submitted for FISH. Cell debris and protein complexes were disrupted by washes in 70% acetic acid and digestion with 0.24% pepsin in 0.01-mol/L hydrochloric acid at 37°C for 10 minutes. Centromeric or pericentromeric satellite DNA sequences from 14 human chromosomes (1, 2, 3, 6, 7, 8, 9, 10, 11, 12, 15, 17, X, and Y) were utilized according to manufacturer’s instructions (Oncor, Gaithersburg, Md).

Hybridization and Detection

Two DNA probes (1 labeled with biotin and 1 labeled with digoxigenin) were combined, pipetted onto a target area of the slide, and covered with a 22 X 22-mm glass coverslip. The coverslip was sealed, the slide was incubated for 8 minutes at 72°C for codenaturation of chromosome and probe DNAs, and hybridization was performed overnight in a humidified chamber at 37°C. Unbound probe fragments were washed 3 times for 5 minutes at 43°C in 50% formamide/2xSSC (3-mol/L sodium chloride per 0.3-mol/L trisodium citrate) at 43°C and in 2xSSC (pH 7.0). Immunohistochemical detection of the biotinylated probes was performed with consecutive incubations of fluorescein isothiocyanate (FITC)-avidin and biotinylated anti-avidin (Vector, Burlingame, Calif). Digoxigenin-labeled probes were detected with incubations in mouse antidigoxigenin, sheep antimouse digoxigenin, and rhodamine sheep antidigoxigenin (Boehringer Mannheim, Indianapolis, Ind). Fluorescent probe DAPI (0.01 μg/mL) in antifade solution (DABCO, Boehringer Mannheim) was used as a counterstain.

Analysis

At least 200 nonoverlapping nuclei with intact morphologic features were evaluated per specimen by 2 investigators (H.A. and M.V.-G.) using fluorescence microscopes (BX-60, Olympus American, Lake Success, NY, and Axioskop, Carl Zeiss, Thornwood, NY) equipped with single- and triple-band pass filters for DAPI, FITC, and Texas red (Chroma Technology, Brattleboro, Vt). All cells in a field, except those with typical morphologic features of inflammatory and blood cells, were evaluated. In a normal diploid cell, 2 signals for the autosome and the sexual chromosomes should be seen. A finding other than 2 copies per cell for each autosome and X chromosome in females, or 1 X and 1 Y in males indicates chromosome imbalance. A mean proportion of signals per cell for each probe was calculated for the nonsmoker controls. All nonsmoker specimens displayed greater than 91% of cells with normal signals for every probe, indicating high analytical sensitivity for the probes. The 1-tailed 98% upper tolerance limit for signal imbalance was subsequently calculated for each probe. At this level of confidence, the specificity was 100% for all probes. These cutoff values were used to classify the data for smokers and patients; therefore, the resulting significance level relevant for this study was α = .02.
In this study, significant imbalance in chromosome copy numbers was found in untreated primary HNSCC. Part of these changes were also identified at the clinically normal cells sampled from sites adjacent to the tumors, supporting the use of this FISH assay as a complementary test to histopathological staging of tumors in patients with HNSCC.

The chromosome imbalances detected in cells of the 10 tumors and 10 clinically normal cells adjacent to tumors, and the pathological assessment of the adjacent margins are summarized by patient in Table 2. Tumor specimens exhibited high frequency of cells with multiple chromosomal imbalances (Figure 1). Greater than 70% of tumors displayed general instability involving chromosomes 2, 3, 6, 8, 9, 10, 15, and X. Numerical changes in chromosomes 1, 12, and 17 were evident in 60% of tumors, while chromosomes 7, 11, and Y were abnormal in 40% of tumors. Loss was the prevalent event for chromosomes 6, 8, 9, and 15, while gain was prevalent for chromosomes 1, 2, 3, 7, 9, 10, 12, X, and Y. Tumor specimens displayed significant imbalance (P<.05, Wilcoxon rank sum test) for all probes tested except chromosome Y. However, loss was not analyzed for chromosome Y. Several tumors were characterized as polyploid, although intratumoral heterogeneity was a common finding (Figure 2, A-F).

Interestingly, clinically normal cells adjacent to the tumors displayed part of the chromosomal imbalances present in their matched tumor cells, although in lower frequency of cells (Figure 1). More frequently involved in imbalances in these clinically normal cells were chromosomes 3 and 15 (≥40% of patients) and chromosomes 2 and X (30% of patients). Abnormalities detected in the clinically normal adjacent specimens were concordant with abnormalities observed in the matched tumors. Two of the 10 clinically normal adjacent specimens sampled by FISH (patients 1 and 10) were simultaneously examined by conventional cytology technique at tumor resection (Table 2). Both specimens displayed neoplasia by chromosomal aneuploidy despite negative cytological findings. Randomly selected adjacent sites taken during resection or endoscopy in 4 specimens (patients 2, 5, 6, and 8) corresponded with negative margins in the pathology report, but were found positive using FISH. Four additional specimens (patients 3, 4, 7, and 9), verified to be clinically normal although not tested by cytology technique, similarly demonstrated chromosomal aneuploidy. Figure 2, C and D, demonstrates identical results in tumor and adjacent margins for patient 8 by displaying +12 and −15, despite cytology-demonstrated negative margins. Specimens from the smokers displayed intraspecific variability involving chromosomes 8, 9, 10, and 15, although without significant imbalance (P>.05).

Each of the 14 probes used detected a mean proportion of balanced signals above 91% and exhibited small variability (<1.6%) in nonsmoker control samples. To provide a faster analysis, a set of probes was subse-

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**Table 1. Characteristics of Patients With Head and Neck Squamous Cell Carcinoma**

<table>
<thead>
<tr>
<th>Patient No./Sex/Age, y</th>
<th>No./Sex/ Age, y</th>
<th>Smoking, Pack-Years</th>
<th>Tumor Site</th>
<th>Clinical Stage*</th>
<th>Grade†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/61</td>
<td>30 Tongue</td>
<td>T1 N0 M0/I</td>
<td>G1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/M/41</td>
<td>0 Oropharynx</td>
<td>T3 N2 M0/IV</td>
<td>G3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/M/74</td>
<td>30 Epiglottis</td>
<td>T2 N1 M0/III</td>
<td>G3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/M/77</td>
<td>22 Floor of mouth</td>
<td>T3 N0 M0/III</td>
<td>G2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/M/54</td>
<td>60 Tongue</td>
<td>T4 N2 M0/IV</td>
<td>G2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/F/41</td>
<td>30 Floor of mouth</td>
<td>T2 N0 M0/II</td>
<td>G1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/M/47</td>
<td>120 Tongue/ pyriform sinus trigone</td>
<td>T4 N0 M0/IV</td>
<td>G2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/M/66</td>
<td>40 Retromolar trigone</td>
<td>T2 N2 M0/IV</td>
<td>G2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9/M/55</td>
<td>25 Maxilla/oral cavity</td>
<td>T2 N1 M0/II</td>
<td>G1-G2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10/M/55</td>
<td>26 True vocal cord</td>
<td>T1 N0 M0/I</td>
<td>G2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*According to American Joint Committee on Cancer.
†G1 indicates well differentiated; G2, moderately differentiated; and G3, poorly differentiated.

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**Table 2. Summary of Chromosomal Imbalance for Tumor and Clinically Normal Adjacent Specimens**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Tumor Chromosomal Gain</th>
<th>Tumor Chromosomal Loss</th>
<th>Specimen Collection</th>
<th>Surgical Margins*</th>
<th>Pathological Assessment</th>
<th>Adjacent Imbalance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+2, +3, +12, +X, +Y</td>
<td>-9</td>
<td>Operative</td>
<td>M1-M3</td>
<td>Negative</td>
<td>+3, +X</td>
</tr>
<tr>
<td>2</td>
<td>+10, +Y</td>
<td>-2</td>
<td>Operative</td>
<td>M1-M3</td>
<td>Negative</td>
<td>+10, -2</td>
</tr>
<tr>
<td>3</td>
<td>+1, +9, +10, +11, +12, +15, +X, +Y, +9, +10, +11, +12, +15, +17, +X</td>
<td>-6, -8, -9, -11, -15, -17</td>
<td>Preoperative Not tested</td>
<td>No result</td>
<td>+1, +X, -9, -15</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+1, +2, +3, +6, +7, +8, +9, +10, +11, +12, +15, +17, +X</td>
<td>-2, -6, -8, -10, -15, -17</td>
<td>Preoperative Not tested</td>
<td>No result</td>
<td>+7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>+3, +7, +X</td>
<td>-6, -8, -9, -11, -15, -17</td>
<td>Preoperative Not tested</td>
<td>M1-M7</td>
<td>Negative</td>
<td>+3, +X, -15, -17</td>
</tr>
<tr>
<td>6</td>
<td>+3, +9, +15</td>
<td>-6, -17, -X</td>
<td>Preoperative Not tested</td>
<td>M1-M2 M3</td>
<td>Positive</td>
<td>+3, +15</td>
</tr>
<tr>
<td>7</td>
<td>+1, +2, +6, +9, +10</td>
<td>-6, -8, -11, -15</td>
<td>Preoperative Not tested</td>
<td>M1-M2</td>
<td>Negative</td>
<td>-6</td>
</tr>
<tr>
<td>8</td>
<td>+1, +2, +12, +17, +Y</td>
<td>-3, -6, -8, -9, -15</td>
<td>Preoperative Not tested</td>
<td>M1-M2</td>
<td>Negative</td>
<td>+2, +12, -15</td>
</tr>
<tr>
<td>9</td>
<td>+1, +2, +3, +7, +9, +10</td>
<td>-6, -8</td>
<td>Preoperative Not tested</td>
<td>M1-M2</td>
<td>Negative</td>
<td>+2, +7, +15</td>
</tr>
<tr>
<td>10</td>
<td>+1, +3, +7, +9, +X</td>
<td>-2, -3, -6, -8, -9, -10, -11, -12, -15</td>
<td>Operative M1</td>
<td>Negative</td>
<td>+9, +10, -3, -6, -8, -9, -10, -12, -15</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates specimen taken during endoscopy or resection.
quently selected considering the hybridization efficiency, defined by high reproducibility (high mean of balanced signals) and low variability in normal cells (standard error), and the sensitivity for tumorigenesis. Among the 14 probes used, chromosomes 1, 3, 8, 9, 10, and X were the most efficient (mean, >95 signals; SE, < 1.00%), and chromosomes 3, 8, 9, and 10 were the most sensitive. Based on these properties, the best performing set of probes included chromosomes 3, 8, 9, and 10.

Using FISH, we have directly examined tumor cells and their normal adjacent mucosa cells of 10 patients with primary HNSCC, and buccal mucosa of 10 healthy nonsmokers and 10 healthy smokers used as control groups. Nonsmokers were used to establish upper tolerance limits for chromosomal instability and smokers were used to minimize bias and confounding with smoking. Our results provide a detailed view of the numerical chromosomal changes occurring in HNSCC at the tumor site and at clinically normal adjacent cells, thereby displaying the applicability of chromosomal aneuploidy as a biomarker for detection of malignancy.

All HNSCC samples exhibited a large degree of aneuploidy. Significant aneuploidy in tumor samples compared with control samples was evidenced by imbalance involving all chromosomes except the Y chromosome. Of interest was the detection of chromosomal aneuploidy in the clinically normal adjacent mucosa of tumors. Whereas cytological assessment yielded negative margins in 2 simultaneously sampled specimens and 4 randomly sampled specimens, 100% of clinically normal adjacent samples analyzed by FISH demonstrated chromosomal aneuploidy (Table 2). Similarly, Brennan et al identified tumor-specific TP53 mutations in the cleared surgical margins of 52% of patients with HNSCC after undergoing surgical resection, and these patients had a substantially increased risk of local recurrence. These results are encouraging, despite possible limitations of this study, including cross-contamination of the adjacent sites with tumor cells secondary to saliva transfer and lack of histopathological assessment in some samples analyzed by FISH. To minimize contamination with tumor cells, all patients with an oral lesion rinsed their mouths with an antiseptic solution twice before cell collection. In addition, all tumor-adjacent samples were verified to be clinically normal by more than 1 surgeon. However, future studies are being designed to simultaneously examine resected margins by FISH and cytology techniques.

![Figure 1](image1.png)

**Figure 1.** Proportion of head and neck squamous cell carcinoma specimens with chromosome imbalances in tumor cells and in clinically normal cells adjacent to the tumor.

![Figure 2](image2.png)

**Figure 2.** Results of fluorescence in situ hybridization in patient 9 (A-D) and patient 8 (E, F). Probes labeled with biotin were detected with fluorescein isothiocyanate (FITC) (green) and probes labeled with digoxigenin were detected with rhodamine (red). Tumor cells in patient 9 were largely found carrying trisomy for chromosomes 1 (red) and 2 (green) (A), and chromosomes 7 (red) and 10 (green) (B). However, distinct number of signals per cell were also detected for these 4 probes, indicating tumor heterogeneity, as illustrated in C and D, respectively. Tumor cells (E) and clinically normal cells adjacent to the tumor (F) were found with similar imbalances (+12 in red, −15 in green) in patient 8.
Complete surgical resection is the best treatment for advanced-staged HNSCC. After surgical resection, a primary lesion is normally sampled by intraoperative frozen sections to confirm staging and ensure a clear surgical margin. Negative margins should be truly negative. Cytological assessment detects morphologic changes in cells; however, early changes may go undetected. More so, this technique is subject to sampling error inherent in the examination of thin sections of a large piece of tissue and interpretive errors made by the pathologist. Intrathecal resections, another source of error, may not provide adequate tissue for sampling. Fluorescence in situ hybridization has distinct advantages. It allows the assessment of topographical distribution of the most prominent changes yielding information on tumor cell heterogeneity and progression. The FISH technique detects changes at the level of the individual cell, thereby describing the biological features of the tumor. Moreover, it is fast, requires only small biopsy specimens, and is suitable for screening of tumorigenesis.

The presence of microscopic cancer in surgical margins reduces local control of disease, decreases patient survival, and exposes the patient to additional morbidity if reoperation of the margin is undertaken. The presence of aneuploid cells serves as a bridge to study the clinical difference seen in patients with HNSCC and their genetic mechanism. Molecular recognition of tumor cells in apparently tumor-free margins may be an adjunctive measure in detecting preoperative abnormalities and residual disease. Fluorescence in situ hybridization can be applied preoperatively to determine the extent of invasion of a particular tumor. Postoperatively, FISH can aid in ensuring that surgical margins are clear. More so, FISH can be used as a screening tool in monitoring tumor regression months to years after resection. We propose the use of a set of 4 probes, from chromosomes 3, 8, 9 and 10, as an additional tool to complement pathological assays. This probe set was demonstrated as efficient and significantly sensitive for determining the involvement of adjacent margins and is potentially useful for screening high-risk patients for tumor initiation or recurrence.

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