Correlation of Numerical Aberrations of Chromosomes X and 11 and Poor Prognosis in Squamous Cell Carcinomas of the Head and Neck

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Objective: To investigate the prognostic significance of chromosomal aberrations of chromosomes X and 11 in relation to disease-specific survival in head and neck squamous cell carcinoma.

Setting: University hospital.

Design: A 10-year retrospective clinical study. Information about clinical findings, treatment, and follow-up has been recorded prospectively.

Patients: By means of the fluorescence in situ hybridization technique with centromeric probes for chromosomes X and 11, we analyzed 40 randomly selected patients before treatment for T1 to T4 head and neck squamous cell carcinoma. Numerical aberrations were scored and evaluated in frozen sections.

Main Outcome Measures: The significance of prognostic parameters was tested by the log-rank and Kaplan-Meier methods for the univariate analysis. The Cox proportional hazards regression model was used for multivariate analysis.

Results: Numerical aberrations of chromosome 11 correlated positively with T and N classification (P = .03 and P = .02, respectively) and with clinical stage (P = .02). Patients with higher frequencies of numerical aberrations for both chromosome X (>48%, mean) and chromosome 11 (>57%, mean) had shortened disease-specific survival compared with those with lower frequencies of numerical aberrations (P = .008 and P < .001, respectively). Of patients who died from disease within 3 years, 7 (50%) had a trisomic value of chromosome 11 of 35% or higher of nuclei (P < .001). Moreover, patients with a higher value (≥8%) of amplification of chromosome 11 (>4 signals) were associated with having poor prognosis compared with those with a lower value (P = .02).

Conclusion: Numerical aberrations of chromosomes X and 11 had prognostic value in head and neck squamous cell carcinoma, and higher frequencies of numerical aberrations correlated with poor prognosis.

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CHROMOSOMAL AND GENETIC aberrations occur in all human cancer types including squamous cell carcinoma (SCC) of the head and neck (HNSCC). Chromosomal and genetic aberrations disrupt normal cellular processes, such as cell signaling, cell cycle progression, and apoptosis and may thus result in an increased proliferation and/or reduction of apoptosis, thereby contributing to tumorigenesis and tumor development. Subsequent genetic changes in tumor cells could then contribute to other phenotypic changes that result in increased malignancy and a more aggressive behavior. Therefore, chromosomal and genetic aberrations in tumors may influence the clinical outcome and thus the prognosis of patients.

Chromosomal and genetic aberrations have been widely investigated in HNSCC. By means of different techniques, including cytogenetic analysis, molecular genetic analysis, comparative genomic hybridization, and fluorescence in situ hybridization, chromosomal and genetic aberrations have been reported within almost all chromosomes in HNSCC.

Chromosomal and genetic aberrations of chromosome 11 are common in a variety of malignant tumors including HNSCC. The most studied region in chromosome 11 is 11q13 because there are several important cancer-related genes in this region, including cyclin D1, hst-1, int2, EMS1, and Tao1. Genes in other regions of chromosome 11, such as 11q23 and 11q25, may also be associated with the development of HNSCC. Chromosomal abnormalities in 11q13, including amplification of the cyclin D1 gene (CCND1), have been suggested to correlate with poor prognosis in HNSCC.

Chromosomal and genetic aberrations of chromosome X have been investigated in several types of tumors such as breast carcinomas, prostate carcinomas, and uterine cervical cancer. Studies have indicated that genes located on chromosome X, such as the estrogen receptor (ER) gene, androgen receptor (AR) gene, and the testis can-
squamous cell carcinoma (HNSCC).

Table 1. Treatment of 40 Head and Neck Squamous Cell Carcinomas According to the Site of the Primary Tumor

<table>
<thead>
<tr>
<th>Site</th>
<th>Surgery</th>
<th>RT and Surgery</th>
<th>Surgery and RT</th>
<th>RT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>2</td>
<td>1</td>
<td>15</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>Larynx</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Nasal</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>3</td>
<td>18</td>
<td>17</td>
<td>40</td>
</tr>
</tbody>
</table>

Abbreviation: RT, radiotherapy.

In the present study we used 2 centromeric DNA probes for detection of chromosome X and chromosome 11 by means of fluorescence in situ hybridization technology to detect possible chromosomal aberrations in HNSCC. Correlations between chromosomal aberrations and clinical parameters were tested, as well as whether chromosomal aberrations might have prognostic value.

METHODS

From a total of approximately 200 patients with HNSCC admitted to the Department of Otolaryngology, Rikshospitalet, University of Oslo, Oslo, Norway, treated from 1988 through 1997, 20 T1 to T2 and 20 T3 to T4 tumors were randomly selected for the study. Tumor specimens and adjacent normal-appearing oral mucosa used for fluorescence in situ hybridization analysis were removed in surgery and frozen in liquid nitrogen and stored at −70°C until processed. There were 12 women and 28 men with a mean age of 63 years (range, 30-89 years). All the tumors were classified according to the International Union Against Cancer TNM classification of 1987. Of 40 tumors, 15 had metastasis (N1 [n=2], N2 [n=12], and N3 [n=1]). Clinical findings, treatment, and follow-up have been recorded prospectively. None of the patients was lost to follow-up.

Table 1 presents the treatment and the sites of the tumors. Patients treated with surgery followed by radiotherapy received 50 Gy toward the primary site and neck. For patients treated with radiotherapy followed by surgery, the radiation dose was 66 to 70 Gy for the primary tumor and 50 to 60 Gy for the neck. Fourteen patients died of disease.

HISTOPATHOLOGICAL ANALYSIS

Successive 5-µm sections were cut from the frozen tumor specimens and the normal oral mucosa and mounted on slides. One section stained with hematoxylin-eosin was used to verify the initial routine histopathological diagnosis of SCC.

FLUORESCENCE IN SITU HYBRIDIZATION

In brief, sections were placed on pollysine-coated slides. The slides were incubated in 4% formaldehyde in phosphate-buffered saline for 10 minutes at 4°C, then washed in phosphate-buffered saline 2 × 5 minutes. Dehydration was carried out in 70%, 80%, and 95% ethanol for 2 minutes each at room temperature, followed by air drying. The sections were incubated with 10-µL hybridization mixture (1-µL probe mixed with 20-µL of Hybrisol VI [Oncor, Gaithersburg, Md]) for chromosome X α-satellite (DXZ1) probe or for chromosome 11 α-satellite (D11Z1) probe (Oncor) and sealed with rubber cement around a coverslip. After denaturation at 80°C for 10 minutes, hybridization followed at 37°C overnight. The sections were then washed for 5 minutes in 2 × SSC (20 × SSC = 3M sodium chloride and 0.3M sodium citrate, pH7) at room temperature, washed twice in 50% formamide/2 × SSC for 15 minutes at 42°C, and rinsed twice in 2 × SSC for 7 minutes at 42°C, followed by incubation with 100 µL of 2% bovine serum albumin in phosphate-buffered saline for 15 minutes at 37°C. Sheep antidigoxigenin fluorescein isothiocyanate (1:100; Boehringer Ingelheim Pharmaceuticals Inc, Ridgefield, Conn) and rabbit anti-sheep fluorescein isothiocyanate (1:100; DAKO Corporation, Carpinteria, Calif) antibodies were used to...
visualize the signals after incubation for 30 minutes each at 37°C. The slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, Ore), and the signals were visualized with a Zeiss Axiosplan fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Fluorescent signals were counted in more than 200 nuclei with a ×100 oil immersion lens according to the criteria described by Hopman et al.²⁷ Only nonoverlapping nuclei with intact morphologic features were evaluated. Completely separated signals with more or less the same homogeneous staining intensity were counted. Minor hybridization spots, which could be recognized by a smaller size and lower intensity, were excluded. Paired or closely opposed spots were counted as 1 signal.

When 20% or more of nuclei in a tumor exhibited abnormal copy numbers for chromosome X or 11, it was classified as a numerical aberrant with respect to these chromosomes. Furthermore, signal numbers per nucleus more than double the normal copy number were considered as an amplification.

The assessments were performed without knowledge about the clinical outcome. Six months after completion of the evaluation, 8 sections were processed in the same way again to evaluate the reproducibility of the method.

STATISTICAL ANALYSIS

The data were analyzed using SAS 6.12 statistical software (SAS Institute, Cary, NC). The χ² test was used for comparison of chromosomal aberrations of chromosomes X and 11 in relation to survivors and nonsurvivors. The Pearson test was used to test the correlation between parameters. With the cutoff levels for each parameter having been decided, the log-rank test was used to test the prognostic significance for clinical parameters and parameters of chromosomal aberrations. A case was censored if death resulted from unrelated diseases or if the patient was alive with no evidence of the original tumor at the last follow-up consultation. Kaplan-Meier plots were used to illustrate the effect of selected variables in relation to disease-specific survival. The proportional hazards regression analysis (Cox regression model) was performed by the backward procedure. P < .05 was considered statistically significant. The reproducibility was tested by means of the least squares regression analysis.

RESULTS

In normal epithelium, more than 80% of the nuclei showed normal copy numbers for both chromosome X and chromosome 11 (ie, disomy for chromosome 11 and chromosome X in women and monosomy for chromosome X in men). All tumors showed numerical aberrations for chromosome 11, whereas 37 tumors (93%) showed numerical aberrations for chromosome X. The means of frequencies of numerical aberrations were 48% (range, 10%-86%) for chromosome X and 57% (range, 31%-77%) for chromosome 11. The means of frequency distributions of centromeric signals are shown in Figure 1 for chromosome X and in Figure 2 for chromosome 11, in normal oral epithelium and in HNSCC, respectively.

Numerical aberrations of chromosome 11 correlated positively with the T and N classification (P = .03 and P = .02, respectively) and with clinical stage (P = .02). No correlation was found between numerical aberrations of chromosome X and clinical variables or between numerical aberrations of chromosome X and numerical aberrations of chromosome 11.

Patients with a value above the mean value of numerical aberrations for both chromosome X (48%) and chromosome 11 (57%) had shortened disease-specific survival compared with those whose values were below the mean (P = .03 and P = .006, respectively). Using a plot diagram, we found that 46% for numerical aberrations of chromosome X and 38% for chromosome 11 gave the best cutoff level regarding patient clinical outcome (P = .008 and P < .001, respectively).

A better prognosis was associated in patients whose tumor had a trisomic value for chromosome 11 below the overall mean (23%, Figure 2) compared with those whose tumor had a trisomic value greater than the mean value (P = .045). Interestingly, 7 patients (50% of nonsurvivors) with trisomy values of chromosome 11 of 35% or higher all died from disease within 3 years (P < .001). Moreover, a poor prognosis was associated in patients with more than 8% of tumor nuclei amplified for chromosome 11 (>4 signals per nucleus) compared with those with values below 8% (P = .02). No correlation was found between clinical outcome and either loss of 1 arm of chromosome 11 (1 signal) or amplification of chromosome X (>4 signals for women or ≥3 signals for men, respectively).

Table 2 summarizes the results of the log-rank analyses for clinical parameters and parameters of chromo-

### Table 2. Log-Rank and Proportion Hazard Regression Analysis (Cox Method) for Clinical Parameters and Parameters of Chromosomal Aberrations for Chromosome X and Chromosome 11 in Relation to Disease-Specific Survival for 40 Patients With Head and Neck Squamous Cell Carcinomas

<table>
<thead>
<tr>
<th>Variable, Clinical Group/ Cutoff Level</th>
<th>Survivors, No.</th>
<th>Nonsurvivors, No.</th>
<th>Log-Rank</th>
<th>Cox Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 26)</td>
<td>(n = 14)</td>
<td>χ²</td>
<td>P Value</td>
</tr>
<tr>
<td>T class, T1-T2/T3-T4</td>
<td>15/11</td>
<td>5/9</td>
<td>3.52</td>
<td>NS</td>
</tr>
<tr>
<td>N class, N0/N</td>
<td>19/7</td>
<td>6/8</td>
<td>6.32</td>
<td>.01</td>
</tr>
<tr>
<td>Stage, I-II/III-IV</td>
<td>13/13</td>
<td>2/12</td>
<td>6.77</td>
<td>.009</td>
</tr>
<tr>
<td>Treatment, surgery or RT/combined</td>
<td>10/16</td>
<td>10/4</td>
<td>4.47</td>
<td>.03</td>
</tr>
<tr>
<td>Chr X, &lt;46%/≥46%</td>
<td>8/18</td>
<td>10/4</td>
<td>6.99</td>
<td>.008</td>
</tr>
<tr>
<td>Chr 11, &lt;38%/≥38%</td>
<td>4/22</td>
<td>10/4</td>
<td>14.30</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Trisomy: Chr 11, &lt;35%/≥35%</td>
<td>26/0</td>
<td>7/7</td>
<td>25.50</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Polysomy: Chr 11, &lt;8%/≥8%</td>
<td>24/2</td>
<td>5/9</td>
<td>5.08</td>
<td>.02</td>
</tr>
</tbody>
</table>

Abbreviations: Chr, chromosome; NA, not applicable; NS, not significant; RT, radiotherapy.
In the present study, we demonstrate for the first time, to our knowledge, a correlation between numerical aberrations of chromosome X and prognosis in HNSCC. Patients having tumors with high frequencies of numerical aberrations of chromosome X (≥46% of nuclei) had a poor prognosis compared with those with low values (<46% of nuclei). Although our results give no information about specific genes located on chromosome X that may be involved, accumulated evidence indicates that genes located on chromosome X play an important role in tumor biological behavior in some types of tumors, such as the estrogen receptor gene (ER) in breast cancer and the androgen receptor gene (AR) in prostate carcinomas.22,23 Recently a group of genes known as testis cancer-specific antigen gene family (MAGE, BAGE, and GAGE) has been identified. Several genes in this family such as MAGE genes and GAGE genes are located on chromosome X.28,29 Expression of MAGE genes located in the q28 region of chromosome X has been observed in several tumor types including HNSCC but not in normal tissues.25,26 Expressions of MAGE gene products have also been reported to correlate with prognosis in ovarian cancer, non–small cell lung cancer, and esophageal SCC.30-32 Our findings are consistent with the hypothesis that these genes may be of importance also for tumor development and thus prognosis of HNSCC.

In the present study, we show in HNSCC that numerical aberrations of chromosome 11 was associated with the T and N classification and consequently with clinical stage. Our results are in accordance with the findings shown in previous genetic studies of other tumor types such as breast cancer and ovarian cancer.33,34 The results may reflect abnormality of a single gene or of a group of cancer-related genes located on chromosome 11. Previous studies on chromosome 11 in HNSCC have
mainly focused on the region 11q13,18,35 where several important cancer-related genes such as CCND1, hst-1, int2, EMS1, and Taos1 are located.12-14 It has been reported that chromosomal abnormalities at 11q13 and amplification of the cyclin D1 gene were associated with poor prognosis in HNSCC.18-21 In the present study, we show that amplification of chromosome 11 correlates with poor outcome, and interestingly, all 7 patients (50% of nonsurvivors) with a trisomy value of chromosome 11 of 35% or higher died from disease within 3 years. This is consistent with the 11q13 locus and cyclin D1 region being of importance and suggests a gene dosage effect implicated in tumor progression.

Our investigation suggests that numerical aberrations of chromosomes X and 11 are positively associated with more aggressive tumor growth and thus are of prognostic value in HNSCC. This finding should be confirmed by further study with a large number of patients with HNSCC.

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