Distinct Nonrandom Patterns of Chromosomal Aberrations in the Progression of Squamous Cell Carcinomas of the Head and Neck

Anja I. Soder, Anton H. N. Hopman, Frans C. S. Ramaekers, Christian Conradt, and Franz X. Bosch

Molecular Biology Laboratory, Ear, Nose, and Throat University Hospital [A. I. S., F. X. B.] and Department of Medical Biometry, University of Heidelberg [C. C.], 69120 Heidelberg, Germany, and Department of Molecular Cell Biology and Genetics, University of Limburg, P.O. Box 616, 6200 MD Maastricht, the Netherlands [A. H. N. H., F. C. S. R.]

ABSTRACT

Fifty-one randomly selected primary squamous cell carcinomas of the head and neck, derived from the larynx (n = 18) and pharynx (oropharynx, n = 18, and hypopharynx, n = 15) were analyzed with centromeric probes for chromosomes 1, 7, 9, 11, 17, and 18 to study numerical aberrations, chromosomal imbalances, and aneuploidy by fluorescence in situ hybridization. The tumors were grouped into nonmetastasizing (N0) tumors (from patients clinically free of lymph node metastases for at least 18 months after surgery, n = 25) and metastasizing (N1) tumors (n = 26). We found a significant association between the tumor ploidy and the nodal status; in the N0 group, diploidy prevailed, and the most common aberration was loss to monosomy for chromosome 9 (44%), whereas in the N1 group, aneuploidy predominated (P = 0.002). Specifically, these genomic changes associated with progression to metastasis were: (a) tetrasomy or polyploidyic chromosomes were detected in 17 of 26 N1 tumors but in none of the 25 N0 tumors (P < 0.0001); (b) heterogeneous chromosomal copy numbers (i.e., extensive chromosomal imbalances) were also much more frequent in the N1 tumors (69.2% versus 24.0% in the N0 group; P = 0.018); and (c) loss of chromosome 9 (73%) and gains of chromosomes 7 (35%) and 17 (31%) persisted, but in addition, loss of chromosome 18 occurred in 31%. Overexpression of the p53 protein correlated with an increased incidence of chromosomal imbalances and aneuploidy (P < 0.001) and, hence, constituted an additional risk factor. The lower metastatic potential of larynx tumors as compared with tumors from the pharynx was reflected by a lower incidence of these genomic changes. These specific patterns of chromosomal aberrations can characterize and distinguish different stages of tumor progression of squamous cell carcinomas of the head and neck and should be valuable diagnostic and prognostic markers.

INTRODUCTION

HNSCC represent 2–3% of all human malignancies, with increasing incidence predominantly among people who have a high tobacco and alcohol consumption (1, 2). Due to technical difficulties encountered in SCC cytogenetics, our knowledge on clonal chromosomal abnormalities in HNSCC, derived from karyotyping of tumor cell lines or short-term culturing of primary tumors, is very limited (3–7). These studies showed extensive chromosomal changes, predominantly losses of genetic material of chromosomes 3p, 5q, 8p, 9p, 18q, and the sex chromosomes, while gains were observed at lesser frequency for chromosomes 3q, 5p, 7p, 8q, and 11q. A significant fraction of mixed karyotypic populations was also observed (7). However, it is not clear as to what extent variations were attributable to the tissue culture conditions used (8).

Allelotypes of HNSCC have been established recently using a large panel of microsatellite markers that were analyzed after PCR amplification. Allelic losses were found to be extensive, involving chromosomes 3, 4, 5, 6, 8, 9, 11, 13, 17, and 19 (9, 10). In a study focusing on chromosome 9, 72% of 29 HNSCC examined showed allelic loss of heterozygosity on 9p, and this loss was found to occur already in dysplastic preneoplastic lesions (11). Comprehensive information about structural chromosomal alterations, i.e., deletions and amplifications, can be obtained by CGH (12, 13). Using CGH on HNSCC, Speicher et al. (14) detected a high incidence of both losses and gains (including high-level amplifications) on the same chromosomes, in particular chromosomes 3 and 5 (in up to 10 of 13 cases). On the other hand, they found loss of 9p in only 2 of 13 cases; in one of which, loss also involved the 9q arm, whereas in another case, they found gain of 9q accompanied by high-level amplification of 9p. Without targeted in situ hybridization, no information on quantitative numerical changes at the chromosomal/centromeric level leading to chromosomal imbalances and aneuploidy can be obtained. Such information is provided by interphase cytogenetics, i.e., by applying FISH to interphase nuclei using chromosome-specific DNA probes. Like with allelotyping or CGH, the genetic alterations observed by FISH are authentic alterations and not induced upon culturing. However, FISH has distinct advantages. It allows the assessment of the topographical distribution of the most prominent changes that may yield valuable information on alterations correlating with the development of tumor cell heterogeneities and with tumor progression, and these changes can be detected at the level of the individual cell. The FISH technique is fast, requires only small biopsies, and is, therefore, suitable for routine screening of clinical material. The FISH technology has been applied to solid tumors in both single-cell suspensions (15–19) and in frozen or formalin-fixed, paraffin-embedded sections (20, 21). Recently, in a study on paraffin-embedded tissues of head and neck tumors, an increase in polysomies (three or more signals/tumor cell nucleus) of chromosomes 7 and 17 has been reported during head and neck multistage tumorigenesis (22).

To gain more insight into the chromosomal aberrations occurring in the carcinogenesis of HNSCC, we have studied 51 HNSCC by means of FISH, using six different biotinylated centromere-specific DNA probes. We paid particular attention to the question of whether certain patterns of chromosomal compositions could distinguish nonmetastasizing from metastasizing tumors. Besides probes to the chromosomes 7, 9, 11, and 17 mentioned thus far, we have also included probes to chromosomes 1 and 18 because in a recent detailed karyotypic analysis of HNSCC, frequent changes were observed on these chromosomes and the changes in chromosome 18 were found to be associated significantly with tumor progression (7).

Another question which we wanted to tackle using FISH related to a possible correlation between overexpression of the tumor suppressor protein p53 and the degree of genomic instability and aneuploidy in HNSCC. p53 has been shown to control the cell cycle and to prevent accumulation of genetic damage (reviewed in Ref. 23). In the genesis of HNSCC, p53 mutations occur in most cases very early in tumor development and p53 overexpression, due to mutation, is detectable also in tumor-distant, histologically normal-appearing mucosal biop-
sies (24). If such a correlation existed, this would strengthen the diagnostic usefulness of immunohistochemical p53 staining of tumor biopsies. Furthermore, the incidence of overexpression of wild-type and functionally active p53 might be indicated by those cases not showing an increased aneuploidy. To test these possibilities, we have compared the chromosomal status of the HNSCC with the immunohistochemical status of the p53 protein.

MATERIALS AND METHODS
Sample Preparation

Fresh tissue of HNSCC from 51 patients were collected immediately after resection, snap-frozen in isopentane/liquid nitrogen, and stored at −80°C. Normal tissues, to be used for control hybridizations, were obtained from five nontumor patients. Five-μm-thick frozen sections were mounted onto silanecoated slides, air dried, and stored at −80°C. For preparation of the cell suspensions, 50-μm-thick sections were collected on uncoated slides and stored at −80°C.

DNA Probes

The centromeric-specific DNA probes used were: pUC17.7 (sat.III type, 1.77-kbp insert, for chromosome 1; Ref. 25); p71 (alphoid type, 0.68 kbp, for chromosome 7; Ref. 26); pHUR98 (sat.III, 0.16 kbp, for chromosome 9; Ref. 27); pLC11A (alphoid, 0.85 kbp, for chromosome 11; Ref. 28); p71H8 (alphoid, 2.70 kbp, for chromosome 17; Ref. 29); and L1.84 (alphoid, 0.68 kbp, for chromosome 18; Ref. 30). These probes were propagated as described in the cited references. We used a commercially available kit (GIBCO-BRL, Eggenstein, Germany) to label the probes by nick translation with biotinylated dUTP. In a number of experiments, the same probes were obtained from the manufacturer (Vysis, Framingham, MA) labeled by direct conjugation with the fluorochrome and used in parallel.

FISH

Tumor Cell Processing for in Situ Hybridization. From each specimen, suspensions of single-cell nuclei from a 50-μm-thick frozen section were first prepared by proteolytic digestion with pepsin (from porcine stomach mucosa, 2500–3500 units per mg protein; Sigma Chemical Co., Deisenhofen, Germany) at a concentration of 100 μg/ml in 0.01 M HCl for 15 min. They were then spun onto silane-coated microscopic slides in a cytospin centrifuge (5 min at 600 rpm). The cells were “acid dehydrated” for 2 min by incubation in 70% ethanol + 0.01 M HCl, followed by further ethanol dehydration and air drying. The cells were then fixed in PBS-buffered 1% formaldehyde (made from paraformaldehyde) for 5 min. After 5 “dip washes” each in PBS and bidistilled water, the slides were dehydrated in ascending ethanol and air dried. Although such cytopsins are always “contaminated” with normal cells from the connective tissue or lymphoid cells, the strongly improved accessibility of these nuclei to various probes allows a sensitive screening of these suspensions for chromosomal aberrations. Since all tumor specimens contained fibroblasts that could serve as internal controls for hybridization efficiency and truncation of chromosomal aberrations. Since all tumor specimens contained fibroblasts that could serve as internal controls for hybridization efficiency and truncation of chromosomal aberrations. Since all tumor specimens contained fibroblasts that could serve as internal controls for hybridization efficiency and truncation of chromosomal aberrations. Since all tumor specimens contained fibroblasts that could serve as internal controls for hybridization efficiency and truncation of chromosomal aberrations. Since all tumor specimens contained fibroblasts that could serve as internal controls for hybridization efficiency and truncation of chromosomal aberrations. Since all tumor specimens contained fibroblasts that could serve as internal controls for hybridization efficiency and truncation of chromosomal aberrations.
### Table 1 Summary of FISH results for the 25 N0 and the 26 N1–3 HNSCC using the centromere-specific DNA probes for chromosomes 1, 7, 9, 11, 17, and 18

<table>
<thead>
<tr>
<th>Case</th>
<th>TNM/G/Lo/p53</th>
<th>Mean copy number</th>
<th>Mean ploidy</th>
<th>Status of the individual chromosomes</th>
<th>Losses/gains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T2N2MxG2/I0+/</td>
<td>2.03</td>
<td>di</td>
<td>I</td>
<td>+1, +7</td>
</tr>
<tr>
<td>2</td>
<td>T3N2MxG3/I0+</td>
<td>2.17</td>
<td>tri</td>
<td>Tr</td>
<td>Tr D D D D</td>
</tr>
<tr>
<td>3</td>
<td>T1N2MxG2/L+</td>
<td>1.96</td>
<td>di</td>
<td>D</td>
<td>D D D D Tr</td>
</tr>
<tr>
<td>4</td>
<td>T1N2MxG/Ox+</td>
<td>2.22</td>
<td>het</td>
<td>D</td>
<td>D Tr D Tr D</td>
</tr>
<tr>
<td>5</td>
<td>T1N2MxG/L+</td>
<td>2.18</td>
<td>tri</td>
<td>Tr</td>
<td>Tr D Tr D</td>
</tr>
<tr>
<td>6</td>
<td>T2N2MxOx+</td>
<td>1.93</td>
<td>di</td>
<td>D</td>
<td>D Tr D D D</td>
</tr>
<tr>
<td>7</td>
<td>T2N2Mx/O+</td>
<td>2.16</td>
<td>tri</td>
<td>Tr</td>
<td>Tr D Tr Tr</td>
</tr>
<tr>
<td>8</td>
<td>T2N2Mx2H+</td>
<td>1.97</td>
<td>di</td>
<td>D</td>
<td>D D D D Tr</td>
</tr>
<tr>
<td>9</td>
<td>T2N2MxG/L+</td>
<td>1.99</td>
<td>het</td>
<td>Tr</td>
<td>Tr M nd D D</td>
</tr>
<tr>
<td>10</td>
<td>T2N2MxG/L+</td>
<td>2.04</td>
<td>het</td>
<td>Tr</td>
<td>Tr M D D Tr</td>
</tr>
<tr>
<td>11</td>
<td>T2N2MxG/L+</td>
<td>1.97</td>
<td>di</td>
<td>D</td>
<td>D D D Tr</td>
</tr>
<tr>
<td>12</td>
<td>T2N2MxG/L+</td>
<td>2.18</td>
<td>tri</td>
<td>Tr</td>
<td>Tr M Tr Tr</td>
</tr>
<tr>
<td>13</td>
<td>T2N2MxOx+</td>
<td>1.99</td>
<td>het</td>
<td>Tr</td>
<td>Tr D D D D</td>
</tr>
<tr>
<td>14</td>
<td>T2N2Mx/O+</td>
<td>1.93</td>
<td>di</td>
<td>D</td>
<td>D D D D Tr</td>
</tr>
<tr>
<td>15</td>
<td>T2N2Mx/O+</td>
<td>1.80</td>
<td>di</td>
<td>D</td>
<td>M Md M D +1, +18</td>
</tr>
<tr>
<td>16</td>
<td>T2N2MxG/I+</td>
<td>1.88</td>
<td>di</td>
<td>D</td>
<td>M Md M +9, +18</td>
</tr>
<tr>
<td>17</td>
<td>T2N2MxG/L+</td>
<td>2.07</td>
<td>di</td>
<td>Tr</td>
<td>nd D D</td>
</tr>
<tr>
<td>18</td>
<td>T2N2Mx/Ox+</td>
<td>1.99</td>
<td>di</td>
<td>D</td>
<td>D D Tr D</td>
</tr>
<tr>
<td>19</td>
<td>T2N2MxG/I+</td>
<td>1.91</td>
<td>het</td>
<td>Tr</td>
<td>M D M D Tr</td>
</tr>
<tr>
<td>20</td>
<td>T2N2Mx/G2/I+</td>
<td>2.05</td>
<td>het</td>
<td>Tr</td>
<td>Tr M D Tr D</td>
</tr>
<tr>
<td>21</td>
<td>T2N2Mx/O+</td>
<td>2.11</td>
<td>tri</td>
<td>Tr</td>
<td>nd Tr D Tr</td>
</tr>
<tr>
<td>22</td>
<td>T2N2MxG/L+</td>
<td>1.92</td>
<td>di</td>
<td>D</td>
<td>D D</td>
</tr>
<tr>
<td>23</td>
<td>T2N2MxG/O+</td>
<td>2.60</td>
<td>het</td>
<td>M</td>
<td>Te D Te P D</td>
</tr>
<tr>
<td>24</td>
<td>T2N2MxG/I+</td>
<td>2.29</td>
<td>het</td>
<td>Te</td>
<td>M nd Tr D</td>
</tr>
<tr>
<td>25</td>
<td>T2N2MxG/L+</td>
<td>2.70</td>
<td>het</td>
<td>Te</td>
<td>Te Tr P nd</td>
</tr>
<tr>
<td>26</td>
<td>T2N2MxG/Ox+</td>
<td>2.05</td>
<td>het</td>
<td>Tr</td>
<td>Tr D D Tr</td>
</tr>
<tr>
<td>27</td>
<td>T2N2MxG/Ox+</td>
<td>2.22</td>
<td>het</td>
<td>M</td>
<td>D nd P Tr</td>
</tr>
<tr>
<td>28</td>
<td>T2N2MxG/Ox+</td>
<td>2.50</td>
<td>tri</td>
<td>Tr</td>
<td>Tr Te Tr T</td>
</tr>
<tr>
<td>29</td>
<td>T2N2Mx/G2/I+</td>
<td>2.57</td>
<td>pol</td>
<td>P</td>
<td>P P Tr</td>
</tr>
<tr>
<td>30</td>
<td>T2N2MxG2/I+</td>
<td>2.28</td>
<td>tri</td>
<td>Tr</td>
<td>Tr D nd Tr</td>
</tr>
<tr>
<td>31</td>
<td>T2N2MxG2/I+</td>
<td>2.30</td>
<td>het</td>
<td>Te</td>
<td>P D P Tr</td>
</tr>
<tr>
<td>32</td>
<td>T2N2MxG2/I+</td>
<td>2.23</td>
<td>tri</td>
<td>Tr</td>
<td>Tr D Tr D</td>
</tr>
<tr>
<td>33</td>
<td>T2N2MxG2/I+</td>
<td>2.27</td>
<td>het</td>
<td>Tr</td>
<td>Tr D nd P</td>
</tr>
<tr>
<td>34</td>
<td>T2N2MxG2/I+</td>
<td>2.11</td>
<td>het</td>
<td>Tr</td>
<td>Tr nd D D</td>
</tr>
<tr>
<td>35</td>
<td>T2N2MxG2/I+</td>
<td>2.13</td>
<td>het</td>
<td>D</td>
<td>D nd D Tr</td>
</tr>
<tr>
<td>36</td>
<td>T2N2MxG2/I+</td>
<td>2.06</td>
<td>di</td>
<td>Tr</td>
<td>D D D Tr D</td>
</tr>
<tr>
<td>37</td>
<td>T2N2MxG2/I+</td>
<td>2.72</td>
<td>het</td>
<td>Te</td>
<td>D Te Te Tr</td>
</tr>
<tr>
<td>38</td>
<td>T2N2MxG2/I+</td>
<td>2.54</td>
<td>het</td>
<td>P</td>
<td>P D Te Tr</td>
</tr>
<tr>
<td>39</td>
<td>T2N2MxG2/I+</td>
<td>2.16</td>
<td>het</td>
<td>Tr</td>
<td>Tr D nd Te</td>
</tr>
<tr>
<td>40</td>
<td>T2N2MxG2/I+</td>
<td>2.50</td>
<td>het</td>
<td>P</td>
<td>P D Tr D Tr</td>
</tr>
<tr>
<td>41</td>
<td>T2N2MxG2/I+</td>
<td>2.16</td>
<td>het</td>
<td>D</td>
<td>Te D Te Tr</td>
</tr>
<tr>
<td>42</td>
<td>T2N2MxG2/I+</td>
<td>2.11</td>
<td>het</td>
<td>Te</td>
<td>Te D Te Tr</td>
</tr>
<tr>
<td>43</td>
<td>T2N2MxG2/I+</td>
<td>2.21</td>
<td>het</td>
<td>Tr</td>
<td>P D nd D P</td>
</tr>
<tr>
<td>44</td>
<td>T2N2MxG2/I+</td>
<td>2.12</td>
<td>tr</td>
<td>Tr</td>
<td>Tr D Tr Tr</td>
</tr>
<tr>
<td>45</td>
<td>T2N2MxG3/O+</td>
<td>3.77</td>
<td>het</td>
<td>P</td>
<td>P Te P Tr</td>
</tr>
<tr>
<td>46</td>
<td>T2N2MxG4/H+</td>
<td>2.05</td>
<td>het</td>
<td>Tr</td>
<td>Tr D D Tr</td>
</tr>
<tr>
<td>47</td>
<td>T2N2MxG/O+</td>
<td>2.19</td>
<td>tri</td>
<td>D</td>
<td>D Tr M/Tr Tr</td>
</tr>
<tr>
<td>48</td>
<td>T2N2MxG/O+</td>
<td>2.49</td>
<td>het</td>
<td>nd</td>
<td>P nd Te P</td>
</tr>
</tbody>
</table>

* TNM classification was according to the guidelines from the International Union Against Cancer, except for cases 35, 42, and 48, which were initially diagnosed as nonmetastasizing. \( G \), histological grading. For evaluation of the FISH signals and p53 status, see "Materials and Methods."

* nd, not done; Lo, localization; L, larynx; O, oropharynx; H, hypopharynx; M, monosomy; D, disomy; Tr, trisomy; Te, tetrasomy; P, polysomy; di, diploid; tri, triploid; tet, tetraploid; pol, polyploid; het, heterogeneous. For the calculation of the mean copy number and of deviations of individual chromosomes (gains and losses) from the mean ploidy, see "Results." Gains and losses were also indicated by bold letters.

Number of the other chromosomes was indicated as gain or loss by bold letters. However, many tumors showed extensive chromosomal imbalances, resulting in the loss of a distinct ploidy, i.e., when less than four chromosomes yielded the same copy number. These were called tumors with heterogeneous chromosomal copy numbers (abbreviated "het" in Table 1). In addition, a mean copy number was listed for each tumor, calculated by dividing the sum of the number of signals by the number of cells counted (200). For example, in case 10 of Table 1, 18 cells showed one signal, 116 cells showed two signals, and 66 cells showed three signals with the chromosome 1 probe, yielding \((18 \times 1) + (116 \times 2) + (66 \times 3)/200 = 2.24\) chromosome 1 signals/cell. Correspondingly, there were 2.06 chromosome 7 signals/cell, only 1.69 chromosome 9 signals/cell, 1.98 chromosome 11 signals/cell, 2.17 chromosome 17 signals/cell, and 2.07 chromosome 18 signals/cell. The resulting mean copy number of 2.04, which is close to diploidy, severely obscured the marked heterogeneity of copy numbers (het) observed in this tumor. Therefore, for the assessment of the distinct deviations of individual chromosomes that were still evident in the tumors with heterogeneous chromosomal copy numbers (Table 1), the mean copy number could not be used. Instead, we used the mean ploidy of the chromosomes examined (not shown in the Table) to see whether individual chromosomes deviated in excess of 0.5 ploidy units from the mean ploidy. In case 10 of Table 1, trisomy \((3 + 3 + 3 + 2 + 2 + 3 + 3) = 14:6 = 2.3\), this being the mean ploidy. From this figure, chromosome 9 distinctly deviated as being underrepresented (−9), whereas trisomy of chromosomes 1, 17, and 18 was scored as gain of these chromosomes (+1, +17, and +18). It is to be noted that the term "heterogeneous" in Table 1 refers only to the chromosomal copy number, circumscribing extensive chromosomal imbalances. It does not infer a differential topographical distribution of aneuploid cells within tumors (intratumor heterogeneity). This phenomenon was not
followed up in detail in this study, although it could be observed in several instances. A single example (case 50 of Table 1) is presented, in which an admixture of cells with monosomy and trisomy of chromosome 9 was seen.

Some representative results of FISH analyses on cytospins of tumor cell nuclei and on tissue sections are shown in Fig. 1. Positive chromosome signals appear as yellow spots in the nuclei. As illustrated in Fig. 1A, single nuclei from a tonsillar carcinoma specimen (case 46 of Table 1) showed four or five signals for chromosome 7. The tumor cell nuclei of a 5-μm frozen section from the same biopsy showed more than four spots for chromosome 7 in many nuclei (Fig. 1B). Fig. 1C demonstrates the case of a monosomy for chromosome 9 (case 19) with 37% of the nuclei containing only one FISH signal for chromosome 9. Fig. 1D shows a control hybridization on a cytospin prepared from a buccal mucosa of a nontumor patient with a chromosome 7 probe. Fig. 1E shows the FISH signals for chromosome 7 in a tumor-distant buccal mucosa. Both hybridizations yielded a diploid set of signals.

Evaluation of the FISH Results

Uneven Distribution of HNSCC from the Anatomical Localizations Larynx and Pharynx in the N₀ (Nonmetastasizing) and N₁₋₃ (Metastasizing) Groups of Tumors. It is evident from Table 1 that the randomly selected tumors from the different anatomical localizations showed an uneven distribution between the groups of N₀ and

Fig. 1. Examples of FISH analyses. A. FISH with the chromosome 7 probe on a single-cell suspension from a tonsillar carcinoma specimen (case 46 in Table 1); four or five signals/per nucleus are visible. B, FISH with the chromosome 7 probe on a 5-μm frozen section from the same biopsy. Again, more than four spots/nucleus are visible. C, FISH with the chromosome 9 probe on a single-cell suspension from a hypopharynx SCC (case 19 in Table 1). One or two signals/nucleus, indicating a monosomy for chromosome 9. D, control hybridization with a chromosome 7 probe on a single-cell suspension prepared from a mucosal biopsy from a nontumor patient. Two signals/nucleus. E, FISH with the chromosome 7 probe on a frozen section of a tumor-distant buccal mucosa. Most epithelial cells show two signals.
CHROMOSOMAL ABERRATIONS IN HNSCC CARCINOGENESIS

Table 2 Distribution of tumor ploidy and copy numbers of individual chromosomes in N0 and N1,3 tumors in relation to tumor localization

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>N0</th>
<th>N1,3</th>
<th>N0</th>
<th>N1,3</th>
<th>N0</th>
<th>N1,3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Larynx</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>9</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneuploid</td>
<td>5</td>
<td>3</td>
<td>6</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OR1 = 5.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OR2 = undefined</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pharynx</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneuploid</td>
<td>80</td>
<td>14</td>
<td>58</td>
<td>84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P &lt; 0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Fisher’s exact test)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OR1 = 18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OR2 = 3.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**A. Distribution of diploid and aneuploid tumors**

- **Diploid:** 9/1, 5/0, OR1 = 5.4, OR2 = undefined
- **Aneuploid:** 5/3, 6/22, P < 0.0001 (Fisher’s exact test)

**B. Distribution of tetrasomic/polysomic chromosomes**

- **Tetra, poly:**
  - Diploid: 0/8, 0/38, P < 0.0001 (Fisher’s exact test)
  - Aneuploid: 80/14, 58/84, OR1 = 18, OR2 = 3.8

**C. Distribution of tumors with heterogeneous chromosomal copy numbers**

- **Heterogeneous:** 2/3, 4/15
- **Other:** 12/1, 7/7
- **Total:** 14/4, 11/22

**Correlation between tumor ploidy and N (lymph node) status:** OR1 = 17.4 (Mantel-Haenszel Weighted Odds Ratio); P = 0.002 (Mantel-Haenszel Summary χ²).

**Correlation between tumor ploidy and the lymph node status of the HNSCC Analyzed.** Table 2A demonstrates that there was a significant association between the tumor ploidy and the lymph node status; diploid tumors were more frequent in the N0 group, and aneuploid tumors were more frequent in the N1,3 group of HNSCC (P = 0.002, Mantel-Haenszel Summary χ², stratified for tumor localization larynx versus pharynx).

**Losses and Gains of Individual Chromosomes.** The evaluation of individual chromosomal losses and gains is summarized in Table 3. The most frequently observed chromosomal change was the loss of chromosome 9. It was observed in 44% of the N0 tumors and in 73% of the N1,3 tumors (overall 59%). Loss of chromosome 9 was apparently independent of the p53 status, since it occurred with similar frequency in the p53-negative (15 of 27, 56%) and p53-positive tumors (14 of 22, 64%). The other chromosomal changes showed an increasing incidence from the N0 to the N1,3 group, except gain of chromosome 1, which was found more often in N0 tumors (Table 3).

**Triploid Tumors.** Of the 10 triploid primary HNSCC, five each were N0 tumors and N1,3 tumors. All five triploid N0 tumors displayed an underrepresentation of a single chromosome, whereas four of the five trisomic N1,3 tumors contained two deviations from trisomy (Table 1).

**Losses and Gains of Individual Chromosomes.** The evaluation of individual chromosomal losses and gains is summarized in Table 3. The most frequently observed chromosomal change was the loss of chromosome 9. It was observed in 44% of the N0 tumors and in 73% of the N1,3 tumors (overall 59%). Loss of chromosome 9 was apparently independent of the p53 status, since it occurred with similar frequency in the p53-negative (15 of 27, 56%) and p53-positive tumors (14 of 22, 64%). The other chromosomal changes showed an increasing incidence from the N0 to the N1,3 group, except gain of chromosome 1, which was found more often in N0 tumors (Table 3).

**Gain of chromosome 11 was observed in only two cases.**

**Relationship between p53 Overexpression and Chromosomal Imbalances and Aneuploidy.** In 49 primary HNSCC the immunohistochemical status of the p53 tumor suppressor protein was determined using the panreactive mAb Bp53-11 (see Table 1). Of the total number of cases analyzed, 45% were p53 positive. Among the p53-positive cases, only 23% were diploid tumors, whereas among the p53-negative cases, 37% were diploid (Table 1). We next asked whether p53 overexpression had an influence on the incidence of the aneusomic individual chromosomes in the N0 and N1,3 tumors, separated into larynx and pharynx. As shown in Table 4, the number of aneusomic chromosomes was significantly higher in the p53-positive tumors in both the N0 and N1,3 groups, independent from the anatomical localization (P < 0.001, Mantel-Haenszel Summary χ² test, stratified for lymph node status and anatomical localization).

**Chromosomal Analysis of Lymph Node Metastases.** It was of interest to examine whether the chromosomal aberration frequency enhanced further in lymph node metastases, in comparison with primary HNSCC. Therefore, the lymph node metastases of five cases (corresponding to cases nos. 30, 33, 34, 36, and 38 in Table 1) were also included single cases with tetraploidy and polyploidy. These contained, as additional deviations, loss of chromosomes 9 and 18 (cases 40 and 32 in Table 1, respectively).

**Tumors with Heterogeneous Chromosomal Copy Numbers: Increased Incidence in N1,3 Tumors.** As shown in Table 2C, the proportion of tumors with extensive chromosomal imbalances, resulting in heterogeneous chromosomal copy numbers, was distinctly higher in the N1,3 group (18 of 26 cases, 69.2%) than in the N0 group (6 of 25 cases, 24%). This difference narrowly failed statistical significance after α adjustment (P = 0.018, Mantel-Haenszel Summary χ², stratified for tumor localization larynx versus pharynx). Interestingly, chromosome 9 remained to be clearly underrepresented when compared with the other chromosomes in the great majority of cases (Table 1).

**Table 3 Distribution of chromosomal losses and gains in N0 and N1,3 HNSCC**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>N0 (% of 25 total)</th>
<th>N1,3 (% of 26 total)</th>
<th>% of 51 HNSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chr.*</td>
<td>Loss</td>
<td>Gain</td>
<td>Loss</td>
</tr>
<tr>
<td>1</td>
<td>1 (4)</td>
<td>8 (32)</td>
<td>4 (15)</td>
</tr>
<tr>
<td>7</td>
<td>0 (0)</td>
<td>4 (16)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>9</td>
<td>11 (44)</td>
<td>0 (0)</td>
<td>19 (73)</td>
</tr>
<tr>
<td>11</td>
<td>1 (4)</td>
<td>0 (0)</td>
<td>4 (15)</td>
</tr>
<tr>
<td>17</td>
<td>1 (4)</td>
<td>6 (24)</td>
<td>5 (19)</td>
</tr>
<tr>
<td>18</td>
<td>1 (4)</td>
<td>4 (16)</td>
<td>8 (31)</td>
</tr>
</tbody>
</table>

*Chr., chromosome.
The single apparently diploid metastasizing tumor (case 39 of Table evaluated the observed chromosomal ploidy patterns in the individual chromosome-specific DNA probes, the results presented provide a to a lower level of ploidy. Underrepresentation of chromosome 9 was similar to the primary tumors (cases 30 and 38, respectively). Cases 2 and 4 showed a switch to higher ploidy, but case 3 showed a change to a lower level of ploidy. Underrepresentation of chromosome 9 was still a distinct feature.

**DISCUSSION**

In this study, we have used the FISH technique to directly examine biopsy material from 51 primary HNSCC. Using six centromeric, chromosome-specific DNA probes, the results presented provide a detailed view of the numerical chromosomal changes occurring in the progression of HNSCC from low-stage nonmetastasizing tumors to high-stage metastasizing tumors and lymph node metastases. We have evaluated the observed chromosomal ploidy patterns in the individual cases (Table 1) in the context of the clinical state of the disease, i.e., lymph node involvement at a time point at least 18 months after resection. In addition, a comparison was made with the immunohistochemical status of the p53 protein.

The evaluation of the ploidy of the different tumor stages in this study revealed that, altogether, the metastasizing primary HNSCC displayed a markedly increased frequency of chromosomal aberrations, while the majority of the HNSCC characterized clinically as nonmetastasizing had retained a diploid set of chromosomes. We found a high correlation ($P < 0.0001$) between metastasizing potential and the aneuploidy of the tumors; thus far, all tumors in which a single chromosome or more than one chromosome showed four (tetrasomy) or more signals (polysomy) were metastasizing. Most notable, three $N_{1-3}$ tumors (cases 35, 42, and 48) were initially diagnosed as nonmetastasizing ($N_0$). Case 42 was tetrasomic for chromosome 17; case 48 even contained two tetrasomic and three polysomic chromosomes. This represents the most important finding of this study and provides a basis for future diagnostic patient management and assessment of prognostic significance.

Another important observation was the development of heterogeneous chromosomal copy numbers, reflecting extensive chromosomal imbalances. This feature was also associated with an increased metastasizing potential (six of 25 $N_0$ tumors versus 18 of 26 $N_{1-3}$ tumors; $P = 0.018$, not significant after $a$-adjustment). Certainly, the analysis of a larger cohort of HNSCC will reveal a significant association between heterogeneous chromosomal copy numbers and metastatic potential. Thus, $N_0$ tumors with heterogeneous chromosomal copy numbers have a high risk of conversion to metastatic spread. This is in line with a recent karyotypic study on HNSCC in which a higher frequency of chromosomal imbalances has been noted in metastasizing tumors (36). Similarly, triploid $N_0$ tumors might also be regarded as potentially becoming metastatic, in particular those with two deviations from trisomy, as exemplified by case 35 of Table 1 (triploid with two deviations), which was also initially diagnosed as $N_0$ tumor. The single apparently diploid metastasizing tumor (case 39 of Table 1) attracted our suspicion, and we wondered whether in this case the analysis of chromosomes other than those of this study would yield different information. Indeed, while chromosome 15 appeared to be trisomic (resulting in a heterogeneous copy number), a centromeric chromosome 12 probe gave four hybridization signals in over 25% of tumor cell nuclei, “explaining” the metastatic character of this tumor (data not shown).

It is a longstanding clinical observation that laryngeal SCC (mostly supraglottic and glottic carcinomas) are less metastasizing than HNSCC from the oropharynx/oral cavity and hypopharynx. We have confirmed this observation; 14 of the randomly selected 25 $N_0$ tumors but only 4 of the randomly selected 26 $N_{1-3}$ tumors were from the larynx. Since the extent of carcinogenic exposure (to tobacco-associated carcinogens and to alcohol) of the larynx is generally regarded as being equivalent to that of the pharynx, the lower metastatic potential of the laryngeal SCC as compared to the other HNSCC can be only partly explained. One reason is that due to symptoms of hoarseness, glottic tumors are often recognized earlier than other HNSCC. As another reason, it is widely assumed that this phenomenon is due to an impaired access to the cervical lymph node system of laryngeal tumors as compared to other HNSCC. Based on this assumption, we should have expected the aneuploidy rate of some of the nonmetastasizing laryngeal HNSCC to be as high as that of the metastasizing tumors, irrespective of the anatomical localization. However, our FISH data clearly show that this is not the case. Instead, the nonmetastasizing laryngeal HNSCC appear to have fewer genomic aberrations than those from the pharynx and, hence, require more additional changes (and more time) in order to metastasize to the regional lymph nodes. Similar observations have been reported recently (36). This suggests that either the laryngeal squamous epithelia are less susceptible to genetic damage, or the laryngeal tumors are less susceptible to genetic instability, or both. It should be an interesting subject of future studies as to which factor(s) contribute to this phenomenon.

The most consistent and characteristic numerical aberration detected by FISH, in comparison to the mean tumor ploidy changes, was a loss of chromosome 9. In total, loss of chromosome 9 to monosomy or its underrepresentation compared to the other chromosomes examined was found in 59% of the HNSCC. Since this observation was made with significant frequency (44%) in the low-stage tumors without lymph node involvement ($N_0$ tumors), this suggests that loss of genetic information contained in chromosome 9 may be a general and relatively early mechanism in the progression of these neoplasias. Loss of heterozygosity of chromosome 9p has been described in many different tumor types, including leukemia (37), cancer of the lung (38), bladder (39), brain (40, 41), in familial melanoma (42), as well as in HNSCC and their preneoplastic precursor lesions (7, 9, 11). The question remained open, however, whether these losses represented specific deletions on 9p (9, 11) or whether deletions also involved the 9q arm (10, 43). The latter was supported by recent CGH analyses (14, 44). Due to the nature of the centromeric probe (which also recognizes a heterochromatin block on 9q adjacent to the centromeric region), our

**Table 4 Relationship between chromosomal ploidy and p53 status (by IHC)**

<table>
<thead>
<tr>
<th>Larynx</th>
<th>Pharynx</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_0$</td>
<td>$N_{1-3}$</td>
</tr>
<tr>
<td>p53-</td>
<td>p53+</td>
</tr>
<tr>
<td>Disomic</td>
<td>33</td>
</tr>
<tr>
<td>Aneuploidy</td>
<td>12</td>
</tr>
</tbody>
</table>

$OR_1 = 3.3$, $OR_2 = 8.3$, $OR_3 = 2.4$, $OR_4 = 2.6$.

$\text{OR} = 2.9$ (Mantel-Haenszel Weighted Odds Ratio; $P < 0.001$; Mantel-Haenszel Summary $X^2$).

**Table 5 FISH results on lymph node metastases using the centromere-specific DNA probes for chromosomes 1, 7, 9, 11, 17, and 18: comparison with primary tumors**

<table>
<thead>
<tr>
<th>Case*</th>
<th>Mean copy number of m/p</th>
<th>Mean ploidy of m/p</th>
<th>Status of the individual chromosomes in m</th>
<th>Losses/gains in m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (30)</td>
<td>2.28/2.22</td>
<td>het/het</td>
<td>D M D D P Tr -7, +17</td>
<td></td>
</tr>
<tr>
<td>2 (33)</td>
<td>2.61/2.28</td>
<td>tet/bi</td>
<td>Tr Te D Te Tr Te -1, -9</td>
<td></td>
</tr>
<tr>
<td>3 (34)</td>
<td>2.01/2.30</td>
<td>het/het</td>
<td>Tr Tr D Tr Tr Tr Tr nd -9, +1, +7, +17</td>
<td></td>
</tr>
<tr>
<td>4 (36)</td>
<td>2.98/2.57</td>
<td>het/het</td>
<td>Tr P nd Tr P nd -1, -11, +7, +17</td>
<td></td>
</tr>
<tr>
<td>5 (38)</td>
<td>2.05/2.13</td>
<td>het/het</td>
<td>Tr D M D Tr Tr -9, +1, +17</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, refer the case of primary HNSCC (see Table 1 for details). Gains and losses are indicated by bold letters, m, metastasis; p, primary tumor.
results argue that allelic loss of the entire chromosome 9 may occur early in tumor progression of HNSCC, rather than only loss of genetic material around 9p21. This observation might be of relevance since a group of affected candidate genes is located around 9p21 and includes MTS1 and MTSII (multiple tumor suppressors; Ref. 45), encoding p16INK4 (CDKN2) and p15INK4B. Both proteins are inhibitors of the cdk4 and cdk6 and, hence, appear to be key regulators of cell cycle progression (46–48). It is viewed presently that a threshold level of the cdk inhibitors (including also p21WAF/CIP and p27KIP; for a review, see Ref. 49) has to be overridden by the cyclin/cdk complexes in order for the cell cycle to proceed from G1 into S-phase and from G2 into mitosis (reviewed in Refs. 50 and 51). Accordingly, the frequent loss of chromosome 9 could generate an imbalance and disadvantage of the cdk inhibitors compared to their counteracting cyclins/cdks, giving rise to cell clones with a growth advantage. This could obviate mutations within the cdk-inhibitor genes in the HNSCC affected by loss of chromosome 9 (for an overview on the p15/p16 literature, see Refs. 52 and 53).

Recently, Voravud et al. (22) have shown that the copy numbers of chromosomes 7 and 17 increased as early events during head and neck tumorigenesis. In accordance, we have also found gains for chromosome 7 (25%) and 17 (27%), which were already detectable in the group of low-stage (N0) tumors (16 and 24%, respectively). Aneuploidy of chromosome 7 has also been found to be significantly associated with aggressiveness and poor prognosis in prostate cancer (54). In addition to gains in chromosomes 7 and 17, however, we have observed a higher frequency (32%) of chromosome 8 gains in the N1, N2 tumors. Due to losses of chromosome 1 at later stages, this value decreased to a total of 27%. Thus, common numerical chromosomal changes occurring in N1, N2 tumors of early stages are loss of chromosome 9 and gain of chromosome 1, followed by gains of chromosomes 17, 7, and 18. In contrast, there was a conspicuous increase in the number of cases in the N1–3 group showing a loss of chromosome 18 (31%; Table 3). This finding appears to support recent studies (7) in which a significant association between loss of 18q and a shortened survival of HNSCC patients was reported.

p53 mutations occur very frequently in HNSCC and constitute, in most cases, a very early event in the genesis of these tumors (24). A crucial function of the wild-type p53 protein is the control of the cell cycle to prevent accumulation of genetic damage. It was, therefore, of interest to ask whether p53 overexpression, which can be easily monitored by immunohistochemical staining, reliably indicates functional inactivation, either due to mutations in the DNA-binding domain (55) or due to binding by cellular or viral oncoproteins. If so, p53 overexpression should correlate with enhanced genomic instability and tumor progression. We have, therefore, included in this study a comparison of the ploidy and aneuploidy levels to the status of the p53 protein (in a few cases, the mutations have been identified by PCR-based sequencing of exons 5 to 8; data not shown). When the ploidy of p53-positive and p53-negative tumors was examined, it was found that the p53-negative (assumed to be wild-type in most cases) tumors were more often diploid than the p53-positive (assumed to be mutant) tumors. A profound difference between the p53-positive and -negative tumors became apparent when the copy number of the individual chromosomes was compared, thus incorporating any chromosomal imbalances into the analysis (Table 4). The number of aneuploidy chromosomes was significantly higher in the p53-positive tumors, in both the N1 and N1–3 groups, and these changes occurred similarly in larynx and pharynx tumors (P < 0.001, Mantel-Haenszel Summary χ² test). A similar association of p53 overexpression to polyploidy for chromosomes 7 and 17 was recently found in a large cohort of bladder cancer cases (56). Our data indicate that p53 overexpression is, in most cases, a good indicator for its functional inactivation, and this constitutes an additional risk factor for an increased genomic instability/aneuploidy and an accelerated tumor progression in HNSCC. Overexpression of the functional wild-type protein should not lead to genomic instability and aneuploidy.

Since chromosomal aberrations have a strong impact on tumor aggressiveness and on patient prognosis, the task following the study presented here will be to define the association between the aneuploidy rate of HNSCC with patient survival times, by analyzing a larger cohort of cases and after a longer period of follow-up time.

It was notable that in the few cases of lymph node metastases analyzed in comparison to the primary HNSCC, a further increase in chromosomal aberration rate was not evident. Whether even more extensive aberrations as seen here in the primary tumors can occur awaits the examination of a larger number of cases.

In conclusion, we have identified specific features of numerical chromosomal aberrations associated with different phases of HNSCC progression. In early phases, a distinct loss of chromosome 9 and gain of chromosomes 1, 7, and 17 was observed. As tumors progressed, chromosomal imbalances increased, leading to heterogeneous chromosomal copy numbers. Particularly, the development of tetrasomy/polysomy in individual chromosomes was strongly associated with the acquisition of metastatic potential of HNSCC. These findings should help to improve the diagnosis of the tumor stage at the time of resection and patient management.

ACKNOWLEDGMENTS

We are greatly indebted to our medical colleagues for their help in collecting and processing the tissue specimens. We specifically thank Prof. H. Weidauer and Drs. H. Maier and C. Reißer for continuous support, encouragement, and helpful discussions on clinical aspects. We very much appreciate the technical assistance of A. Schuhmann and W. Klein and the discussions with T. Andl, R. Erber, and A. Pfuhl, as well as the helpful practical suggestions by M. Vallinga, M. Jansen, J. Herbergs, and C. Voorter. We are indebted to Dr. I. A. Born, Institute of Pathology, University of Heidelberg, for kindly performing the histopathology.

REFERENCES


Distinct Nonrandom Patterns of Chromosomal Aberrations in the Progression of Squamous Cell Carcinomas of the Head and Neck


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/55/21/5030

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.