Genomic heterogeneity has been observed in several solid tumor types. To investigate this phenomenon in head and neck squamous cell carcinoma (HNSCC), we analyzed macroscopically distinct tissue samples of 12 resected tumors by a combination of fluorescence in situ hybridization (FISH) and DNA flow cytometry. Using a panel of centromeric DNA probes, numerical chromosomal aberrations were detected in 10 tumors, 9 of which showed a single DNA aneuploid peak. Imbalances in chromosomal copy numbers resulted in unique patterns of chromosomal aberrations for each tumor case. Two types of tumors could be distinguished, i.e., tumors \( n = 5 \) containing a single aneuploid clone and tumors \( n = 5 \) with multiple aneuploid clones. The center of this latter group of tumors was shown to be genetically more heterogeneous than the tumor margin.

In conclusion, this study showed that 1) the pattern of chromosomal aberrations varies greatly between different HNSCC, 2) a major clone with a specific pattern of chromosomal aberrations has spread throughout most HNSCC, and 3) a subgroup of HNSCCs contains additional clones with a different pattern of chromosomal aberrations. Based on these results, HNSCC can be divided into a genetically more homogeneous and a genetically more heterogeneous group. Cytometry (Comm. Clin. Cytometry) 34:113–120, 1998.

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Key terms: head and neck squamous cell carcinoma; heterogeneity; fluorescence in situ hybridization; DNA flow cytometry
tissue material was analyzed from two macroscopically distinct parts of each tumor, i.e., one biopsy taken from the central part of the tumor and one from the tumor margin. All specimens were analyzed by DNA flow cytometry and by FISH using a panel of centromere-specific probes.

MATERIALS AND METHODS
Patient Material

Tissue samples from 12 patients with HNSCC were included in this study (Table 1). Specimens were collected from both the center and the border of the resected tumors, snap-frozen in liquid nitrogen, and stored at −80°C. A 5 µm thick frozen section of each specimen was stained with hematoxylin-eosin and the presence of tumor cells was verified by an experienced pathologist.

DNA Flow Cytometry

A nuclei suspension was prepared from a 50 µm thick frozen section of each specimen by a proteolytic digestion step with pepsin from porcine stomach mucosa (2,500–3,500 U/mg protein; Sigma Chemical Co., St. Louis, MO) at a concentration of 100 µg/ml in 0.01 N HCl for 20 min at 37°C (12). The nuclei were thereafter treated with 500 µg/ml RNase (Serva, Heidelberg, Germany) and stained with 50 µg/ml propidium iodide (Calbiochem, La Jolla, CA). The fluorescence intensity was analyzed in a FACSort with 50 µg/ml propidium iodide (Calbiochem, La Jolla, CA). The fluorescence intensity was analyzed in a FACSort (Becton Dickinson, Sunnyvale, CA) and displayed as a histogram of DNA content versus the number of nuclei. The DNA index was calculated by dividing the aneuploid histogram of DNA content versus the number of nuclei. The coefficient of variation did not exceed 10% in any diploid peak.

FISH

A nuclei suspension was prepared from each frozen tumor specimen, as described above. This suspension was cytocentrifuged onto poly-L-lysine-coated slides, post-fixed in 1% paraformaldehyde/0.1 M phosphate buffer, and air-dried. Individual slides were hybridized with centro-

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Tumor localization</th>
<th>TNM classification (UICC 1987)</th>
<th>Histological differentiation grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>54</td>
<td>M</td>
<td>Larynx</td>
<td>T4N2bM0</td>
<td>Poor</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>M</td>
<td>Larynx</td>
<td>T4N0M0</td>
<td>Well</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>M</td>
<td>Larynx</td>
<td>T2N0M0</td>
<td>Moderate</td>
</tr>
<tr>
<td>4</td>
<td>59</td>
<td>M</td>
<td>Oral cavity</td>
<td>T2N2bM0</td>
<td>Moderate</td>
</tr>
<tr>
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<td>25</td>
<td>M</td>
<td>Tongue</td>
<td>T2N0M0</td>
<td>Well</td>
</tr>
<tr>
<td>6</td>
<td>83</td>
<td>M</td>
<td>Larynx</td>
<td>T4N0M0</td>
<td>Moderate</td>
</tr>
<tr>
<td>7</td>
<td>60</td>
<td>F</td>
<td>Larynx</td>
<td>T4N2cM0</td>
<td>Moderate</td>
</tr>
<tr>
<td>8</td>
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<td>M</td>
<td>Larynx</td>
<td>T4N0M0</td>
<td>Moderate</td>
</tr>
<tr>
<td>9</td>
<td>63</td>
<td>M</td>
<td>Pharynx</td>
<td>T4N1M0</td>
<td>Moderate</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>M</td>
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<td>T4N0M0</td>
<td>Poor</td>
</tr>
<tr>
<td>11</td>
<td>65</td>
<td>M</td>
<td>Larynx</td>
<td>T3N2bM0</td>
<td>Poor</td>
</tr>
<tr>
<td>12</td>
<td>81</td>
<td>M</td>
<td>Larynx</td>
<td>T3N0M0</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

Clinicopathological Characteristics of the HNSCC Cases Examined in This Study

No. | Patient Age (years) | Sex | Tumor localization | TNM classification (UICC 1987) | Histological evaluation classification |
--- | --------------------- |-----|-------------------|--------------------------------|-------------------------------------|
1   | 54 M Larynx          | T4N2bM0 | Poor                |
2   | 58 M Larynx          | T4N0M0 | Well                |
3   | 58 M Larynx          | T2N0M0 | Moderate            |
4   | 59 M Oral cavity     | T2N2bM0 | Moderate            |
5   | 25 M Tongue          | T2N0M0 | Well                |
6   | 83 M Larynx          | T4N0M0 | Moderate            |
7   | 60 F Larynx          | T4N2cM0 | Moderate            |
8   | 55 M Larynx          | T4N0M0 | Moderate            |
9   | 63 M Pharynx         | T4N1M0 | Moderate            |
10  | 60 M Larynx          | T4N0M0 | Poor                |
11  | 65 M Larynx          | T3N2bM0 | Poor                |
12  | 81 M Larynx          | T3N0M0 | Moderate            |

The patients included 1 woman and 11 men (Table 1), ranging in age from 25 to 83 years (mean 60 years). Histologically all tumors were squamous cell carcinomas (2 well, 7 moderately, and 3 poorly differentiated). Most tumors were localized in the larynx (n = 9), while other sites were the oral cavity (n = 1), the tongue (n = 1), and the pharynx (n = 1). No or only minor variation was observed in the degree of differentiation of the two...
different tissue samples (center versus border) analyzed for each neoplasm.

### DNA Flow Cytometry

Three of the tumors displayed a diploid DNA content, and nine tumors were aneuploid (Table 2). In none of the tumors was a discrepancy detected among the two different samples (border versus center) regarding the overall ploidy pattern. The DNA indices of the aneuploid cell populations ranged from 1.3 to 2.5, with none of the 9 aneuploid tumors exhibiting a major difference between the DNA indices of the aneuploid stemline in the different samples. The percentage of cells in the aberrant DNA peak was in general higher in the center biopsy sample (mean 24%, ranging from 10 to 38%) than in the border biopsy sample (mean 36%, ranging from 11 to 47%) than in the border biopsy sample (mean 24%, ranging from 10 to 38%). All DNA aneuploid tumors showed a single aneuploid stemline.

### FISH

Single- and double-target FISH on nuclei suspensions, using DNA probes for chromosomes 1, 7, 9, 17, and 18, revealed numerical aberrations for one or more chromosomes in 10 of the 12 analyzed tumors. Aneuploidy numbers detected in this series included mainly trisomies and trisomies, but also hexasomies and octasomies. Table 2 summarizes these chromosomal copy number aberrations of the major clone(s) present in the tumors. From Table 2 it is clear that chromosomal copy number imbalances are present in almost all tumor cases. Only the two disomic tumors (nos. 1 and 2) and one tetrasomic tumor (no. 10) did not show these imbalances. Imbalances were not restricted to specific chromosomes, but seemed to occur more or less randomly, although chromosome 7 showed a relative gain in four cases and was never underrepresented. Chromosomal imbalances were in all cases verified by double-target FISH. Repeated single- and double-target FISH experiments revealed identical results in this study, with only a minor variation in the percentage of aneuploid cells present in each specimen (results not shown). As a result of these imbalances, a unique pattern of chromosomal aberrations was identified for each of the tumors in this series. A clone containing this unique pattern of chromosomal aberrations was present in both biopsies taken from different sites of the tumor. However, in some cases, multiple aneuploidy clones were detected. Based on the number of aneuploid clones detected by FISH, the tumors could be distinguished as follows.

**Tumors containing a single tumor cell population.**

These tumors (n = 5) showed a specific pattern of numerical chromosomal aberrations, however, several general features were observed:

1. The majority of nuclei (40–90%) contain two FISH signals for most chromosomes and are classified as disomic. These cells represent normal stromal and epithelial cells and/or diploid tumor cells.
2. A major aberrant fraction (exceeding 10% of the total fraction) is detected for one or more chromosomes. Chromosomal copy number imbalances are present in most cases.
3. Next to this major aneuploidy fraction, low percentages of nuclei are detected with other copy numbers than the copy number of the major fraction. These nuclei comprised 0.5–10% of the total number of nuclei.
4. Since no major differences are detected in the FISH pattern of the two samples from the same tumor, it appears that the same clone is present in both macroscopically distinct sites of the tumor. The percentage of aneuploidy cells is in general lower in the border sample compared to the center sample.

As a typical example of these tumors, Figures 1A,B, 2A,B, and 3A,B show the FISH pattern obtained for the two samples of tumor no. 8.

**Tumors containing multiple aneuploid clones.**

In these tumor cases (n = 5), multiple genetically aberrant clones were detected in at least one of the two samples analyzed for each case.

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Table 2: Results of DNA Flow Cytometry and Single-Target FISH on Nuclei Suspensions From 12 HNSCC*

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>DNA Index</th>
<th>Aneuploidy (%)</th>
<th>Status of the individual chromosomes</th>
<th>Gains (+) or losses (−)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Center</td>
<td>Border</td>
<td>Center</td>
<td>Border</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>1.3</td>
<td>1.4</td>
<td>47</td>
<td>16</td>
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<td>5</td>
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<td>30</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>1.9</td>
<td>n.e.</td>
<td>11</td>
<td>n.e.</td>
</tr>
<tr>
<td>12</td>
<td>2.4</td>
<td>2.5</td>
<td>38</td>
<td>17</td>
</tr>
</tbody>
</table>

*For FISH the data of border and center biopsies are combined. In case of differences between these regions, this is indicated as follows: a only detected in the center biopsy and b only detected in the border biopsy. n.e., not evaluable; Di, disomy; Tr, trisomy; Te, tetrasomy; He, hexasomy; Oc, octasomy.
In three cases, copy number heterogeneity was restricted to a single chromosome. An example of such a case (tumor no. 3) is shown in Figures 1C,D, 2C,D, and 3C,D. In the center biopsy of this tumor, trisomy for chromosome 1 was present in 20% of all nuclei, but no aberrations were present for this chromosome in the border biopsy. Trisomy for chromosome 7, however, was detected in both the center (67%) and the border biopsy (54%). Double-target FISH with probes for these two chromosomes revealed the presence of two aneusomic subpopulations in the center biopsy (chromosomal status of the two clones: disomy 1/trisomy 7 and trisomy 1/trisomy 7), whereas only one of these populations was detected in the border biopsy (disomy 1/trisomy 7). In tumor no. 4, two populations, one with trisomy for chromosome 1 and one with tetrasomy for chromo-

![Fig. 1. Representative patterns of chromosomal aberrations identified by FISH in two macroscopically distinct biopsies of three HNSCC. A,B: Pattern of the center biopsy (A) and the border biopsy (B) of case no. 8 is depicted. C,D: Patterns identified in the same biopsies of case no. 3. E,F: Patterns identified in these biopsies of case no. 12.](image-url)
some 1, were detected. Again, also a "normal" disomic population and a small fraction of nuclei with different amounts of FISH signals were detected with the chromosome 1 probe. The populations with trisomy 1 and tetrasomy 1 were present in both samples of this tumor. For the other chromosomes one aberrant copy number was detected in both samples of this tumor (trisomy 7, tetrasomy 9, trisomy 17, and disomy 18). The center biopsy of tumor no. 5 did not contain numerical aberrations above the cutoff percentage for chromosome 17, whereas in the border biopsy trisomy for chromosome 17 was found in 16% of all nuclei. The status of the other

Fig. 2. Frequency histograms of double-target FISH results in different parts of three HNSCC. A,B: Histogram of the center biopsy (A) and the border biopsy (B) of patient no. 8 is depicted. C,D: Histograms obtained in the same biopsies of patient no. 3. E,F: Histograms identified in these biopsies of patient no. 12.
chromosomes was identical in both biopsy sites of this tumor.

In two cases, copy number heterogeneity was detected for four of the five analyzed chromosomes. As an example, the results of tumor no. 12 are depicted in Figures 1E,F, 2E,F, and 3E,F. In this case, multiple aneusomic cell fractions were identified in the center biopsy, whereas the border biopsy contained only one of the aneusomic
clones. Extensive chromosomal copy number imbalances were also detected in this tumor; the chromosome copy numbers varied from disomy for chromosome 18 to octasomy for chromosome 7. In case no. 9, the center biopsy also contained multiple clones with different copy numbers for most chromosomes, whereas only one of these populations was present in the border biopsy (except with the probe for chromosome 17 with which a trisomic and a tetrasomic population were detected in both biopsies).

**DISCUSSION**

ITH has been reported in several malignancies, and many investigators have suggested that the occurrence of this phenomenon has a clinical impact. Different clonal subpopulations of a tumor may, e.g., differ in their capacity to metastasize (3,16) and/or their response to therapeutic modalities (17,18). HNSCC comprises a clinically and histopathologically heterogeneous group of tumors, but as yet little is known about the genetic basis underlying this heterogeneity.

For this study, the chromosomal content of tumor cells isolated from two macroscopically distinct parts was determined in 12 resected HNSCC. One sample was taken from the center of the tumor and one part was taken close to the border of the same tumor. The genetic constitution of these tumor areas was studied by means of DNA flow cytometry (FCM) and FISH. We defined ITH as the presence of more than one DNA aneuploid (FCM) or chromosomally aberrant (FISH) population within the same tumor.

FCM analysis of these samples showed that 9 of the 12 tumors contained a single DNA aneuploid stemline with a nearly identical DNA index in the two different samples from the same tumor. Although similar results have been obtained in some studies (19,20), they seem to be in sharp contrast with others (21,22). We feel that the ITH reported in the latter two studies can be explained by the variable admixture of normal diploid cells with aneuploid tumor cells, and hence does not reflect the presence of tumor cells with different DNA contents. This is also reflected in our results showing a difference in the percentage of aneuploid cells between the different samples of the same tumor. The border biopsy in general contained less aneuploid cells, a finding that was verified by FISH and might well be explained by the fact that more non-tumor cells were present in these samples.

We conclude that DNA FCM is not sensitive enough for the detection of the genetically distinct clones present within HNSCC because this technique provides integrated data on the DNA content. In contrast, the FISH technique, enabling the detection of single chromosomes in individual cells, did show ITH in a considerable portion of the HNSCC included in this study.

FISH was used to study numerical aberrations of chromosomes 1, 7, 9, 17, and 18 in isolated nuclei from the same samples. The FISH results showed that 10 of the 12 tumors contained numerical chromosomal aberrations, 9 of which were DNA aneuploid as shown by FCM. Two cases displayed a concordant diploid DNA content and disomy for all chromosomes in both samples. These results once again show that most HNSCC exhibit numerical chromosomal aberrations. The patterns of chromosomal aberrations obtained showed great differences between the tumors included in this study. These were due to the presence of chromosomal copy number imbalances in most tumors. In fact, only one DNA aneuploid tumor case (no. 10) did not show imbalances for the chromosomes studied. In contrast to the results of Soder et al. (2), the presence of these chromosomal copy number imbalances was not associated with lymph node metastasis in our study; i.e., five of the seven N\textsubscript{0} tumors contained such imbalances. Comparison of the different samples from the same tumor showed that a tumor clone with an identical pattern of chromosomal aberrations was present throughout most tumors. The cytogenetic similarities observed between macroscopically distinct tumor lesions point to the presence of a genetically homogeneous cell population in these tumors. It might be hypothesized from these results that the complete pattern of chromosomal aberrations, including the extensive chromosomal copy number imbalances found in some tumors, is more or less stable during clonal expansion of the aneuploid cell population.

The present study, however, also provides evidence for ITH in HNSCC. Using single- and double-target FISH and by applying strict counting criteria, multiple cell populations with different numerical chromosomal aberrations were detected in five tumors.

Three tumors (nos. 3, 4, and 5) showed copy number heterogeneity for a single chromosome in one or both of the analyzed samples, whereas an identical copy number was detected for the other chromosomes in both samples. In two of these cases (nos. 4 and 5) DNA FCM showed the presence of a single aneuploid peak, while the tumor from case no. 3 was DNA diploid. FISH revealed trisomy for chromosomes 1 and 7 in the center biopsy of this tumor, while the border biopsy only contained trisomy 7. From these results, it might be deduced that trisomy 7 was present in the expanding clone before trisomy 1. Interestingly, chromosome 7 was frequently overrepresented (tumor nos. 3, 6, 8, and 12), a finding also reported by others (2).

In two tumors, the center biopsy contained at least two aneuploidic clones with differences in the copy number of four of the five analyzed chromosomes. In contrast, the border biopsy of these tumors contained only one of the clones detected in the center biopsy. These two tumors contained extensive chromosomal copy number imbalances and a polysomy (more than four copies) for at least one of the chromosomes. It has been suggested (6) that cells with excessive chromosome numbers may be cytogenetically unstable, resulting in a random loss of chromosomes during subsequent cell divisions. The fact that biopsies taken from the tumor border contained a lower percentage of tumor cells than the center biopsies, thereby reducing the chance that one or more aneuploid cell fractions are present above the cutoff percentage of 10%, explains why less subpopulations were detected in these biopsies. Another explanation might be that several clones develop in the center of the tumor, while only the most
expansive clone (selected on the basis of growth advantage and/or protection against apoptosis) will spread and will be found in the tumor border.

Finally, we want to comment on our evaluation criteria. Strict evaluation is essential to circumvent false classification due to technical artifacts or poor nuclear morphology. These evaluation criteria are in our opinion and the opinion of others (11) essential for the detection of genuine ITH, but have not always been applied in studies reported in the literature. A disadvantage of using these criteria is that we were unable to classify small cell populations, a problem also described by Schapers et al. (7). In most cases, such small subpopulations were identified next to the major tumor cell fraction. At this moment, we are reluctant to classify these findings as proof for ITH since they might partially result from technical shortcomings. We feel that the genetic content of small subsets can best be analyzed on tissue sections allowing a direct correlation of the genetic data with the histological appearance of each tumor area (see refs. 23, 24).

In conclusion, single- and double-target FISH on multiple biopsies of HNSCC revealed that 1) great intertumor heterogeneity exists in the pattern of chromosomal aberrations, 2) a major clone containing a specific pattern of chromosomal aberrations has expanded relatively stable throughout most of these tumors, and 3) a subgroup of HNSCC contains additional clones with a different pattern of chromosomal aberrations, thereby providing evidence for ITH in these tumors. These results show that the FISH technique can be used to divide HNSCC into a genetically more homogeneous and a genetically more heterogeneous group. The clinical relevance of these findings will have to be investigated in a larger series of HNSCC with known follow-up.

LITERATURE CITED