Patterns of Chromosomal Alterations in Metastasizing and Nonmetastasizing Primary Head and Neck Carcinomas

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Abstract

In an attempt to define chromosomal alterations that are associated with the metastatic phenotype, we investigated a total of 29 metastasizing (pN+) and 19 non-metastasizing (pN0) head and neck squamous cell carcinomas by comparative genomic hybridization (CGH). The analysis indicated that the pN0 tumors carried preferentially overrepresentations of chromosomes 5p, 6p, and 7p and that the pN+ tumors were frequently characterized by deletions on chromosomes 7q, 10q, 11p, 11q, 15q, and 20p and overrepresentations of the chromosomes 19q and 20q. In particular, the use of difference histograms and statistical analysis indicated that the deletions on chromosomes 10q25–q26 and 11p13–p14 were highly significant for metastasizing carcinomas. The findings on chromosome 10q were supported by loss of heterozygosity analysis in the primary tumors and eight synchronous lymph node metastases using four microsatellite polymorphisms. The data suggest that distinct patterns of genetic lesions are responsible for the metastatic phenotype of head and neck squamous cell carcinomas.

Introduction

Cancer of the respiratory tract has the highest incidence of all solid tumors, is the leading cause of cancer death, and is associated with a distinct epidemiological background. The vast majority of lung neoplasms is related to smoking alone, whereas squamous cell carcinomas of the head and neck are caused by the combined effects of the carcinogens alcohol and tobacco. Despite refined diagnostic tools and treatment modalities, the survival rates have not improved during the last decade (1). The pathological staging, and in particular the nodal stage, is known to be the most important prognostic factor in HNSCC (2). The metastatic phenotype, however, is highly variable. For instance, there are lymph node metastases occurring from small carcinomas that escape clinical diagnosis. On the other hand, there are primary tumors that grow to enormous size without forming metastases.

Cytogenetics, allelotyping, and CGH have supplied insight into the prevailing genetic alterations of HNSCC (3–5). Recently, molecular genetic studies have led to a tumor progression model (6). In accordance with this model, we identified a distinct pattern of genetic alterations in highly differentiated HNSCCs by CGH consisting of deletions on chromosomes 3p and 9p, along with the overrepresentation of 3q. The study revealed that poorly differentiated tumors are characterized by additional changes, i.e., deletions on chromosomes 4q, 8p, 11q, 13q, 18q, and 21q and overrepresentations on 1p, 11q13, 19, and 22q (7).

Materials and Methods

Tumor Samples. The characteristics of the study cohort are shown in Table 1. All tumor samples were obtained from surgical resections at the Department of Otorhinolaryngology of the University Hospital Charité. Tumor and normal tissue from the salivary gland were frozen in liquid nitrogen within 1 h after surgery and stored at −80°C. Tumors were staged and classified according to the Union International Contre Cancer and WHO guidelines. DNA was extracted from several 30-µm cryostat tissue sections by proteinase K digestion and phenol-chloroform extraction. The tumor tissue was verified to consist of a minimum of 70% tumor cells in each case. In addition to the primary tumors, metastatic lesions were investigated in eight cases by LOH analysis. In the pN0 group, we selected carcinomas of advanced size to enrich this group for tumors that obviously do not have the capability to metastasize.

CGH. The protocols for the CGH preparation steps have been published previously (8). Briefly, 5 µg of tumor and normal DNA were labeled by nick translation with biotin-dUTP and digoxigenin-dUTP, respectively. One µg of each, together with human Cot1 DNA, was hybridized to normal metaphase chromosomes prepared from peripheral blood lymphocytes. After 3 days of hybridization at 37°C, the tumor and the normal DNA were specifically detected by fluorescein-avidin (Vector Laboratories, Burlingame, CA) and anti-digoxigenin-rodhamine (Boehringer Mannheim, Mannheim, Germany), respectively, whereas the chromosomes were counterstained with DAPI. All hybridizations were performed with sexual neutrality, i.e., tumor DNA, reference DNA, and metaphase chromosomes were either derived from male or female donors.

Digital Image Analysis. Image acquisition and digital image analysis were performed as described (8, 9). Briefly, three images per metaphase (DAPI, FITC, and TRITC) were acquired using a epifluorescence microscope with appropriate filter sets mounted by a cooled CCD camera. A ratio (FITC/TRITC) image was calculated representing the over- and underrepresented DNA segments of a single metaphase. The chromosomes were arranged to a CGH karyogram by DAPI banding patterns. Up to 15 metaphases/karyogram were analyzed and CGH sum-karyograms as well as mean ratio profiles with confidence intervals (Fig. 2A) were calculated for each case. Alterations were determined by the deviations of these mean FITC/TRITC profiles from the normal ratio of 1.0, which were tested for significance by a Student’s t test. Deviations of the ratio profile with at least 99% significance in the Student’s t test were scored as DNA gains or losses, i.e., only those imbalances in which the ratio profile with its 99% confidence interval exceeded the line of the normal ratio 1.0 to the same side (Fig. 2A) were included in the evaluation.

By this way, CGH histograms of the metastasizing and nonmetastasizing carcinomas were calculated. They represent the incidence of DNA gains and losses of a tumor group along each chromosome, e.g., the maximum value of 100% is reached if all tumors of the same tumor group carry a change at a specific chromosomal region. The difference histogram of Fig. 1 indicates the genetic imbalances of both tumor groups. The percentage of changes occurring
only in the pN+ tumors is represented by the green color, whereas the excess of changes in the pN+ tumor group is shown in red. The white areas beneath the colored part of the histogram represent the percentage of changes that are present in both entities. A wide colored area thus indicates a pronounced difference between the tumor groups. The differences were tested for significance by a \( \chi^2 \) test. Areas with 95% significance (0.01 < \( P \) < 0.05) are depicted in bright gray, and areas with 99% significance (\( P < 0.01 \)) are depicted in dark gray.

**LOH Analysis.** Paired samples of the primary tumor and normal DNA from all 48 HNSCC patients as well as 8 metastatic lesions were assessed for allelic loss by microsatellite polymorphism analysis. Four different markers that were located between 10q22–q24 (D10S569, D10S541, and D10S185) and at 10q25–q26 (D10S169) were selected from the Généthon genetic linkage map of chromosome 10 (10). Primer sequences were obtained from the genome database and commercially synthesized (MWG Biotech, Ebersberg, Germany). One primer of each primer pair was labeled with an IR dye. PCR was performed with approximately 200 ng of DNA, 20 pmol of each primer, and 2.5 mM deoxynucleotide triphosphates. The PCR conditions were assessed as described previously (11). The DNA fragments were checked on a 2% agarose gel and then separated on a Licor 4000 automatic DNA sequence (MWG). The intensities of the alleles were evaluated visually as well as by a custom-made digital image analysis software. We scored a LOH under the condition that the relative intensity difference between the allele of the tumor and CGH data of the metastatic lesion was at least less than 60% of the corresponding allele of the normal sample.

**Statistical Analysis.** The LOH data were tested for significance by \( \chi^2 \) test from the statistical software package NCSS supplied by Unisoft (Augsburg, Germany).

**Results**

**CGH.** In general, the HNSCCs were characterized by a high incidence of complete or partial deletions of chromosomes 3p, 4, 5q, 6q, 8p, 9p, 11, 13q, 18q, and 21q. DNA overrepresentations were more pronounced for chromosomes 3q, 11q13, 8q, 9q, 19, 22q, 16p, 17q, and 1pter. Differences between HNSCCs with and without lymph node metastases were derived from the histogram representation of Fig. 1. Nonmetastasizing tumors revealed higher percentages of overrepresentations than pN+ tumors. In particular, we observed more frequently overrepresentations of the entire chromosomal arms 5p, 6p, and 7p. The statistical analysis suggested that the overrepresentations of the chromosomal bands 5p15, 6p22, and 7p15 were of 99% significance for the pN0 tumors. There was no deletion detectable that was significantly associated with the nonmetastatic phenotype.

The metastasizing HNSCCs carried more deletions on chromo-
process (14). Although the significance of these regions has to be confirmed by additional studies, e.g., by fluorescence in situ hybridization with specific probes, they might harbor genes that stimulate the growth of nonmetastatic tumors.

The metastasizing HNSCCs carried DNA losses more frequently, supporting the previous notion that the accumulation of deletions is equally important for tumor progression as amplifications (8, 15). The statistical analysis revealed two regions of high significance, i.e., chromosomal bands 10q25—q26 and 11p13—p14. Interestingly, the CD44 gene is located at chromosome 11p13, which has been implicated in metastases formation (16) and has recently been defined as a metastasis suppressor gene in prostate cancer by microcell fusion-mediated chromosomal transfer (17).

The chromosome 10q loss has been described to be important in tumor progression, particularly of brain and prostate cancer (18, 19). We observed that allelic loss is a typical finding in advanced squamous cell carcinomas of the lung and is significantly associated with the metastatic phenotype (11). Two candidate tumor suppressor genes have been identified on this chromosomal arm, the MXII gene at 10q24—q25 and the MMAC/PTEN gene at 10q23 (20—22). Meanwhile, the MXII gene has been evaluated in prostate cancer and carcinomas of the lung (11, 23). Because both studies did not find mutations within the MXII region, this gene is an unlikely candidate for HNSCC. Our CGH analysis showed DNA losses particularly at the chromosomal bands 10q21 and 10q24—q25, suggesting that MMAC at 10q23 is not the important gene for metastases formation. Although the distinct indication of a chromosomal band by the statistical analysis has to be carefully interpreted, the LOH data support the notion that a gene located more to the telomere might be of importance for the metastatic phenotype in HNSCC.

The incidence of DNA loss on 10q as indicated by the CGH histogram amounted to 45%, which is less than for other chromosomes, e.g., 3p and 9p. These two chromosomal changes are important for early tumor stages (6, 7). We think that the lower incidence for chromosome 10q deletions is related to the fact that only primary tumors were investigated by CGH. The primary tumors are composed...
of cells with and without the capability to metastasize. Thus, the genetic lesions mediating metastases formation might be masked by the admixture of tumor cell clones that do not disseminate. This is supported by the fact that we observed additional allelic losses in the cases in which primary tumors and lymph nodes were investigated, yielding LOH in six of eight cases.

Allelic loss on chromosome 7q has been frequently identified in breast cancer (24). The region affected involves the locus of the MET proto-oncogene, which encodes the receptor of the scatter factor/hepatocyte growth factor. Because the scatter factor is involved in tumor dissemination (25), it is tempting to speculate that alterations of the MET gene might also be implicated in metastases formation. However, MET has not yet been shown to carry properties of a tumor suppressor gene.

The result that deletions on chromosome 15q are associated with the metastatic phenotype correlates well with the finding that allelic loss on chromosome 15q has been implicated in tumor progression and dissemination (26). Similarly, the amplification of chromosome 19q is a typical finding of small cell lung cancer, which is characterized by early and widespread metastases formation (8).

In summary, the study points to specific chromosomal alterations associated with progression to a metastatic stage in breast cancer. Oncogene, 12: 2488–2492, 1996.

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References


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