Frequent Novel DNA Copy Number Increase in Squamous Cell Head and Neck Tumors

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ABSTRACT

We have undertaken a study of DNA copy number changes in head and neck squamous cell carcinomas to identify novel DNA copy number changes and to determine the significance of previous findings of cytogenetic alterations in cultured cells. Comparative genomic hybridization was performed on genomic DNA extracted from ten tumors. A novel copy number gain on chromosome 3q26–27 and a loss of chromosome 3p were found at high frequency (≥50% of tumors). Many other novel chromosomal copy number changes were identified but occurred at a lower frequency. In addition, our data confirm that DNA copy number changes that frequently occur in cultured cells, such as loss of chromosome 3p, also occur in tumors. Frequently altered loci may encode oncogenes or tumor suppressor genes involved in head and neck squamous cell carcinoma tumorigenesis.

INTRODUCTION

Forty-two thousand new cases of head and neck cancer occurred in the United States in 1992 and there were 11,600 deaths (1). Tobacco and alcohol are the most important etiological agents (Ref. 1 and literature cited therein). A variety of genetic alterations and complex aberrant karyotypes have been described in cancer cells derived from HNSCC tumors, including copy number decreases of chromosome arms 3p (2–4), 11q (5, 6), and 18q (7–9) and loss of chromosomes 9, 12, 13, 8, and Y (10). Karyotyping and loss of heterozygosity analysis revealed rearrangements and translocations on chromosomes 1p, 1q, 3q, 8, 11q, 13p, 14p, and 15p (4, 9–11). Point mutations in the p53 tumor suppressor gene (12–16) have also been found in cultured cells. However, culture conditions are known to influence the karyotype of cells derived from head and neck tumors (10). Therefore, to corroborate the occurrence of the chromosomal aberrations reported in cell cultures and, thus, confirm their significance in head and neck tumorigenesis, and to identify novel alterations, molecular cytogenetic analysis of fresh tumor cells was performed.

We report here the analysis of 10 tumors with the use of CGH (17, 18), a novel cytogenetic method that allows the simultaneous determination of DNA copy number changes in a tumor specimen in a single experiment. CGH is very powerful because anonymous DNA copy number changes can be identified rapidly (19–22) and because tumors can be analyzed directly without culturing or karyotyping. We have found that a novel DNA copy number increase at 3q26–27 is the most frequent cytogenetic alteration in HNSCC. Common DNA copy number decrease at 3p, which was observed previously in cultured cells, is also found in tumors by CGH. We compare our data to a similar CGH analysis of head and neck tumors that was published recently (23).

MATERIALS AND METHODS

Patients. Ten tumors from patients with squamous cell carcinomas of the head and neck were analyzed by CGH. Specimens were obtained from surgical resections of the primary tumors or of recurrent tumors as indicated in Table 1. The tumor site and level of differentiation are also listed in Table 1. For each tumor, a pathologist removed a small specimen from the tumor mass while attempting to avoid the inclusion of contaminating normal cells. The “TNM” classification is based on a system described previously (24).

DNA Preparation. The DNA was prepared as described (19). Tumor tissue (0.3–0.5 g) was cut into small pieces with a razor blade and incubated in a solution of 10 mM Tris-HCl (pH 8)-100 mM NaCl-1 mM EDTA-0.5 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) at 42°C for 12 h. The following day the dissolved tissue was extracted with phenol-chloroform (1:1) four to five times and precipitated with ethanol. The DNA was resuspended in 50 μl of 10 mM Tris-HCl-1 mM EDTA (pH 8), and the concentration was determined by absorbance at 260 nm.

Comparative Genomic Hybridization. Hybridizations were performed essentially as described (17). Normal human male lymphocyte metaphase preparations were denatured in 70% formamide and 2 x SSC [1 x SSC is 0.15 M NaCl and 0.015 sodium citrate (pH 7)] at 78°C for 2.5–10 min depending on the age of the slides. Slides that had been stored at room temperature for more than 2 weeks or that had been stored frozen generally required longer denaturation (10 min) at higher temperatures (78°C) for optimal hybridization. The slides were then dehydrated in a sequence of 70, 85, and 100% ethanol washes. The slides were air dried and incubated at 37°C until the DNA probes were applied (<5 min). One hundred and twenty ng of biotinylated tumor DNA, 120 ng of digoxigenin-labeled normal reference DNA, and 5 μg of Cot-1-blocking DNA (GIBCO-BRL) were precipitated with ethanol. The pellet was resuspended in 3 μl of distilled H2O and mixed with 7 μl of hybridization buffer to give a final concentration of 50% formamide, 10% dextran sulfate, and 2 x SSC (pH 7). This probe mixture was denatured for 5 min at 77°C and incubated at 37°C for 10 min before being applied to the metaphase spreads. Coverslips were applied and sealed to the slides with rubber cement, and the slides were incubated in a humified chamber for 3 days at 37°C.

Slides were washed at 45°C in three changes of 50% formamide-2 x SSC (pH 7) followed by two washes with 2 x SSC and one wash with 0.1 x SSC (10 min in each wash). All subsequent manipulations were done at room temperature. The slides were washed for 5 min in 2 x SSC and blocked for 5 min in 2 x SSC-1% BSA (Pentax Fraction V; Sigma). The slides were then immunohistochemically stained with 5 μg/ml FITC-avidin (a green fluorochrome; Vector Laboratories, Burlingame, CA) and 2 μg/ml anti-digoxigenin rhodamine (a red fluorochrome; Boehringer Mannheim) in 2 x SSC-1% BSA for 30 min in the dark. The slides were then washed in the dark for 10 min successively in each of the following: 4 x SSC, 4 x SSC containing 0.1% Triton X-100 (Fluka Chemika), 4 x SSC and 0.01% Na2HPO4 and 0.1% NP-40. The slides were dried on a paper towel, and 7.5 μl of a solution of 0.1 μM DAPI in glycerol containing antifade was applied. Coverslips were applied and sealed with nail polish (Sally Hansen’s Hard as Nails).

Image Acquisition and Processing. The slides were examined with a Zeiss Axioplan fluorescence microscope. Metaphase images were acquired and stored as three color images (DAPI, FITC, and rhodamine) with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) interfaced with a
Sun 4/330 work station equipped with the quantitative image processing system. Chromosomes were identified based on the DAPI-banding patterns (17). The green:red fluorescence ratio profiles were determined by integration of the fluorescence intensity along the axis of each chromosome by use of the XWOOOLZ program (17). At least three metaphase spreads were analyzed to determine the ratio of green:red fluorescence for each chromosome. Five of the ten tumor samples were also analyzed with the normal and tumor fluorochromes reversed (digoxigenin dUTP for the tumor DNA and biotin dUTP for the normal DNA). If the green:red ratio was >1.2 or <0.8 the alteration was scored as a gain or a loss because, in our experience, control CGH experiments using normal-normal DNAs do not show this much variation in the green:red fluorescence ratio. The extent and location of each copy number change (based on the mean fluorescence ratio profiles) were plotted on an ideogram showing a schematic representation of the G-banding pattern of the chromosome. Heterochromatic chromosome regions (Ceni, Cen9, Cen10, 13p, 14p, 15p, 21p, 22p, and Yq12) were not interpretable because of suppression of hybridization with Cot-1 DNA in these regions.

RESULTS

CGH was performed on DNA from 10 HNSCC tumor specimens. Table 1 shows the clinical stage for each of the patients. Seven of the patients had received radiation therapy before and three had not at the time of surgery. The tumors varied somewhat in size and node status, and three showed metastases. Fig. 1A shows a representative CGH metaphase for case 5. Fig. 1B shows the corresponding green:red fluorescence ratio profiles. A green:red fluorescence ratio of >1.2 indicates a copy number increase of a particular chromosomal region, whereas a green:red fluorescence ratio of <0.8 indicates a copy number decrease. The tumor shown in Fig. 1 has copy number increases on chromosomes 3q (Fig. 1A, red arrows) and 9p. Because the HNSCC tumor is from a female patient and the normal DNA is from a male, chromosome X from the patient DNA shows a 2-fold increase in copy number resulting in a green appearance of chromosome X in the metaphase, and chromosome Y, which is absent from the female tumor, appears to be underrepresented, resulting in a red Y chromosome in the metaphase (green:red ratio is much smaller than 1).

A schematic representation of all DNA copy number changes from the 10 HNSCC cancers analyzed is shown in Fig. 2. Copy number changes on chromosome 3 occurred in 70% of the cases. Fifty % of the tumors showed a copy number decrease on chromosome 3p (the “p” arm is the top arm of each chromosome in Fig. 2) and 50% showed a copy number increase of chromosome 3q (Fig. 2, bottom arm), whereas 30% did not show an alteration on chromosome 3. Many tumors showed copy number changes on chromosomes other than 3 but only at frequencies of 40% or less. Copy number decrease of all of chromosomes 19, 22, and Y and copy number decreases on chromosome arms 11p and 16p occurred in 30% of the tumors. The telomeric regions of chromosomes (1p, 5q, and 19q) were decreased in copy number in 40% of the tumors. Copy number increases of chromosome bands 2q22–24.1, 2q32, 3q13.3, 4q11–27, 7q31.3, 8q23–24, and 13q21–31 occurred in 30% of the tumors analyzed.

A more detailed representation of the copy number changes on chromosome 3 for each individual case is shown in Fig. 3. Closer inspection reveals that the region of copy number increase on 3q in the individual tumors is not identical but that each spans the region at band 3q26–27. Three tumors (nos. 6, 7, and 8) showed a simultaneous copy number decrease of 3p and a copy number increase of 3q26–27, two showed only the 3p copy number decrease (nos. 5 and 9), and two tumors (nos. 2 and 10) showed the 3q26–27 copy number increase alone.

DISCUSSION

The cytogenetic alterations that arise in HNSCCs have been characterized extensively (1, 5, 9). They include frequent deletions at 3p, 5q, 8p, 9p, and 18q and frequent gains at 3q, 5p, 7p, 8q, and 11q (7). To corroborate the occurrence of the chromosomal aberrations reported in cell cultures and, thus, confirm their significance in head and neck tumorigenesis, and to identify novel alterations, molecular cytogenetic analysis of fresh tumor cells was performed. CGH is very powerful because it allows one to determine relative copy number of nearly the entire genome without growing or karyotyping tumor cells. CGH also allows previously unrecognized alterations to be identified (19, 21, 22, 25). Comparative genomic hybridization was performed on genomic DNA extracted from ten tumors. A novel copy number gain on chromosome 3q26–27 and a loss of chromosome 3p were found at high frequency (≥50% of tumors).

The most frequent DNA copy number changes detected were on chromosome 3. Several studies have described copy number decreases of chromosome 3p in cultured cells and primary tumors (2, 4, 26) and we confirm here that these copy number decreases occur frequently in primary tumors analyzed by CGH (50% of the cases). Copy number increases of the entire 3q chromosome arm have been described in cultured cells at a frequency of 50% (26). Our data confirm the high incidence of copy number increase on chromosome 3q demonstrating that classical and molecular cytogenetic methods can yield congruent findings. Moreover, our data indicate that the minimal common region of copy number increase on 3q occurs in a small region at 3q26–27. 3q26 is frequently involved in translocations with chromosome 21 in myelogenous leukemia (27, 28). LAZ3, a Zn finger protein, maps at 3q27. (3;14) and (3;4) translocations have been shown to lead to a disruption of LAZ3 in non-Hodgkin’s lymphoma (29, 30). BCL-6 is another Zn finger protein that maps to 3q27 (31, 32). Patients with diffuse, large cell lymphomas often show rearrangements within the 5’ non-coding region of the gene, suggesting that BCL-6 is a proto-oncogene. Because we find that 3q26–27 is frequently amplified, these two genes are candidates for involvement in tumor progression.
Fig. 1. A, a representative CGH metaphase with DNA from tumor no. 5. Tumor DNA was labeled with biotin dUTP and normal DNA with digoxigenin dUTP. Tumor DNA was detected with FITC-avidin (green), and normal DNA was detected with rhodamine anti-digoxigenin antibody (red). Green areas are due to DNA copy number increases in the tumor, and red areas are due to copy number decreases. Chromosomes are labeled at the p arm. Red arrow, a common amplification at 3q26-27. B, green:red fluorescence ratio profiles of the HNSCC tumor DNA shown in A. The green:red ratio is shown as the solid line in the diagram. Ratio profiles are presented with p arms to the left and q arms to the right of the vertical mark, which indicates the position of the centromeres. Dashed line, green:red fluorescence ratio of one; dotted lines, ratios 0.5 (lower) and 1.5 (upper). Numbers to the left of each trace indicate chromosome number; n, number of independent chromosomes analyzed. A green:red ratio >1.2 indicates a copy number increase, and a ratio <0.8 indicates a copy number decrease in the tumor DNA. The thin solid lines near the green:red ratio line are SDs.
in squamous cell head and neck cancer. Studies of the expression level of the respective proteins or the mRNAs might shed light on their possible involvement in HNSCC tumorigenesis.

Our data are quite consistent with a recently published CGH analysis of 13 head and neck tumors (23). In that study the most frequent copy number gain was found to be 3q26-qter, and the most frequent loss was 3p. These are also the most common alterations found in our study, although our data suggest a somewhat narrower region for the common copy number gain (3q26–27). A copy number gain on 5p was found in 8 of 13 tumors by Speicher et al. (23) whereas we found 5p increase in only 2 of 10 tumors. The difference may simply reflect the small number of tumors analyzed. Both studies suggest that copy number increase in the 3q26–27 region may be an important event in head and neck tumorigenesis. Amplification of 3q26 has also been observed recently in several other tumor types, including brain, bladder, ovarian, and cervical cancer. Whether a common oncogene is involved in tumorigenesis in all the tumor types could be determined through identification of the relevant cDNA(s).

In HNSCC tumors, copy number increase of BCL-1 and INT-2 on 11q13 and of the epidermal growth factor receptor on 7p have been reported (5, 6, 33). Only one of our patients [and two in the Speicher et al. (23) study] showed a copy number increase in this region. Most likely copy number increases of those loci are below the detection level of CGH. The amplicon size of the INT-2 regions has been reported to range from <1 to 4.5 MB (34). The minimal size of copy number changes that can be detected by CGH is about 2 MB (for 50-fold copy number increase) to 10 MB (for 5-fold copy number increase) for cultured cells. In CGH analysis of tumors, these values may be higher if the tumor is contaminated by normal cells, although in our analysis we analyzed regions of the tumors judged to contain few contaminating normal cells based on pathology. CGH on cultured HNSCC cells with known amplicon size could be used to further delineate the sensitivity of the technique.

We also found copy number increases and decreases on other chromosomes but at lower frequencies. All of these copy number changes occurred at a frequency of <40%. Other studies have found higher frequency copy number decreases and increases on other chromosomes in cultured cells derived from head and neck tumors (4, 10, 11, 35). The reason for the discrepancy could lie in the difference between primary tumors and cultured cells. However, because about 50% of HNSCC tumors have p53 mutations, one might expect that the resulting genomic instability would generate a large number of alterations in at least this fraction of tumors. Thus, an alternative explanation is that the resulting alterations are relevant to tumorigenesis but are below the limits of detection of CGH. Additional experiments are required to resolve this issue. Our data show that the other chromosomal copy number changes are qualitatively the same as described in the literature.

The high frequency of loss of chromosome 3p and gain of chromosome 3q26–27 indicates the likely involvement of these loci in HNSCC tumor formation and/or tumor progression.

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4 J. Gray, unpublished observation.
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REFERENCES

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