Detection of Chromosomal Aberrations in Cytologic Brush Specimens from Head and Neck Squamous Cell Carcinoma

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BACKGROUND. Detection of genetic changes in the mucosa of the upper aerodigestive tract may provide a target for the screening of cytologic specimens to identify premalignant transformation in this region. In this pilot study, the feasibility of the fluorescence in situ hybridization (FISH) technique to detect genetically aberrant cells in brush specimens was evaluated.

METHODS. Brush specimens taken from the tumors of 20 patients with head and neck squamous cell carcinoma (HNSCC) and from the normal mucosa of 8 control patients were analyzed by FISH using DNA probes for the chromosomes 1 and 7. The FISH results were compared with DNA flow cytometry and FISH results of the solid tumor specimens.

RESULTS. The results of this study showed that 15 of the 20 tumor brush specimens contained numeric chromosomal aberrations in at least 5% of the cells collected. Chromosomal aberrations were detected in all brush specimens taken from tumors that were DNA aneuploid and showed aneusomy. The presence of these aberrations correlated well with the classification “suspicious for malignancy,” which was based on Papanicolaou stained slides of the same specimens. In the control group the percentage of chromosomally aberrant cells did not exceed 2%; in addition, no suspiciously malignant cells were observed in this group.

CONCLUSIONS. This study reveals that the FISH technique can be applied diagnostically to brush specimens of HNSCC. The presence of chromosomal aberrations in > 5% of the cells in these specimens can be considered as a marker for malignancy.


KEYWORDS: fluorescence in situ hybridization, head and neck squamous cell carcinoma, cytology, DNA flow cytometry.

The detection of genetic changes in the cytologic specimens of persons at high risk for the development of head and neck carcinoma may provide a target for the screening of premalignant transformation in these persons. Epithelial cells can be obtained in a simple, noninvasive manner by brushing the mucosa. Although exfoliate cytology using standard Papanicolaou staining is a valuable tool for the diagnosis of several types of premalignancies and can be used in the head and neck region for tumor diagnosis, the validity of the method depends strongly on the representativity of the specimen and the experience of the investigator.1,2 Therefore, additional techniques have to be applied to obtain more objective diagnostic criteria. Fluorescence in situ hybridization (FISH) can be used to study the chromosomal constitution of cytologic specimens3 and recently has been reported to be of use in the cytopathologic diagnosis of cervical neoplasia.4,5

By using chromosome specific DNA probes, this technique allows a
targeted detection of numeric chromosomal aberrations in interphase nuclei on a single cell basis. Recently the FISH technique was applied to biopsy specimens obtained from head and neck squamous cell carcinoma (HNSCC). Specific chromosomal changes were reported to be associated with different stages of HNSCC progression. In the early stages (N0 tumors) a distinct loss of chromosome 9 and gain of chromosomes 1, 7, and 17 was observed. The development of tetrasomy or polysomy and the presence of chromosomal copy number imbalances were associated strongly with tumor progression. Hittelman et al. recently reported the presence of numeric aberrations for chromosomes 7 and 17 in morphologically normal epithelium adjacent to head and neck tumors, using in situ hybridization on paraffin tissue sections. It was suggested that these aberrations are markers of a generalized genetic change and also that the presence of these aberrations might be useful as a genetic biomarker for malignant transformation. To the authors' knowledge, until now studies on chromosomal aberrations in cytologic specimens of HNSCC have not been described. In the current study the feasibility of the FISH technique was tested for the identification of aberrant cells in cytologic brush specimens from patients with HNSCC.

MATERIAL AND METHODS

Patient Material

Brush specimens were obtained from 20 patients presenting with HNSCC (17 men and 3 women; age range, 42–79 years). Most tumors were localized in the larynx (n = 10); other sites were the palate (n = 2), the pharynx (n = 3), the tongue (n = 2), and the tonsils (n = 3). The tumors varied in UICC 87 stage from T1N0 to T4N2; in seven patients lymph node metastasis was identified. Under general anesthesia, prior to diagnostic evaluation, cells were brushed from the tumor area using an interdental cleaner (Enta-Lactona bv., Bergen op Zoom, The Netherlands). A control group was comprised of 8 patients who were admitted for nonmalignant disease. Brush specimens from the control group also were obtained under general anesthesia prior to surgery, by brushing healthy-appearing mucosa from the vocal cords, the posterior pharyngeal wall, and the floor of the mouth. Brushes were fixed immediately in Carbowax (2% polyethylene-glycol molecular weight 1450 [Sigma Chemical Co., St. Louis, MO] in 50% ethanol) and stored at 4 °C in this fixative. Fresh tumor tissue was collected from 12 patients after resection or biopsy, snap-frozen, and stored at −80 °C.

Histologic and Cytologic Examination

From 14 tumor and 24 control brush samples (taken from 3 regions of the 8 patients) a cell suspension was cytocentrifuged onto glass slides. The slides were coded, followed by the Papanicolaou staining procedure. The samples were graded as normal, atypia, and suspiciously malignant by an experienced pathologist. Tumor brush samples from six patients could not be evaluated with the Papanicolaou procedure. A 5-μm frozen tissue section from each of the 12 tumor biopsies was stained with hematoxylin and eosin and tumor diagnosis and grading were confirmed by the same pathologist.

Fluorescence In Situ Hybridization

From each specimen (both brush and frozen tissue) a nuclei suspension was prepared by a proteolytic digestion step with pepsin from porcine stomach mucosa (2500–3500 U/mg protein; Sigma) at a concentration of 100 μg/mL in 0.01 N HCl for 20 minutes at 37 °C. This suspension was cytocentrifuged onto poly-L-lysine coated slides, postfixed with 1% paraformaldehyde in 0.1 M phosphate buffer, and air-dried. Separate slides were hybridized with the biotin-11-dUTP (Boehringer, Mannheim, Germany) labeled chromosomes 1 (pUC1.77) and 7 (p7t1) centromere specific probes. When the single-target FISH procedure indicated an imbalance between the copy number of both chromosomes, double-target FISH was performed. This was done by hybridizing the same specimen simultaneously with a biotin-11-dUTP labeled probe for one of the chromosomes and a digoxigenin-11-dUTP probe for the other chromosome. Cytochemical detection of the biotin-11-dUTP probe was performed as previously described. Detection of the digoxigenin labeled probe was performed by subsequent incubation with mouse antidigoxigenin (MA-Dig, 1:2000; Sigma), tetramethylrhodamin isothiocyanate (TRITC)-conjugated rabbit antirabbit immunoglobulin (IG) G (RAMTRITC, 1:1000; Dako, Glostrup, Denmark), and TRITC-conjugated swine antirabbit IgG (SWARTRITC, 1:100; Dako). Nuclei were counterstained with 4,6-diamino-2-phenyl indole (DAPI; Sigma; 1.25 ng/mL) diluted in 0.2 M Tris-HCl, pH 8.0/glycerol (1:9; volume to volume) containing 2.3% of the antifading reagent 1,4-di-azobicyclo-(2,2,2)-octane (DABCO; Sigma).

Evaluation of the single-target FISH results was performed by counting the chromosome copy number of 200 nuclei per slide, according to the criteria described previously. In the control group, the mean plus 3 times the standard deviation was 1% for numeric chromosomal aberrations. To obtain reliable results, the cutoff percentage for the different aberrant chromosome copy numbers was set at 5%. A tumor sample was classified as disomic when the frequency of aberrant cells did not exceed this cutoff value. When the frequency of aberrant cells exceeded this percent-
age, the tumor was classified as trisomic when the cells contained three FISH signals per nucleus, tetrasomic with four signals per nucleus, and polysomic with more than four signals per nucleus. When cell populations with different aberrant copy numbers were present, a combined classification was given. Microphotographs were recorded with the In Situ Image System (Metasystems GmbH, Altlussheim, Germany) using a black and white CCD camera, with TRITC in red, fluorescein isothiocyanate in green, and DAPI in blue.

DNA Flow Cytometry
From each frozen tumor specimen, a nuclei suspension was prepared from a 50-μm section as described earlier. The nuclei were treated with 500 μg/mL RNase (Serva, Heidelberg, Germany) and stained with 50 μg/mL propidium iodide (Calbiochem, La Jolla, CA). The fluorescent intensity was analyzed in a FACSort (Becton Dickinson, Sunnyvale, CA) and displayed as a histogram of DNA content versus number of nuclei. The DNA index was calculated by dividing the aneuploid mean channel number by the intrinsic diploid mean channel number.

RESULTS
Detection of Chromosomal Aberrations in Cytologic Brush Specimens
In the control group of eight patients, the mean plus three times the standard deviation was 1% for numeric chromosomal aberrations. To obtain reliable results, the cutoff percentage for the different aberrant chromosome copy numbers was set at 5%. Figure 1A shows the disomic pattern that was obtained in this control group with double-target FISH. In the tumor group, numeric aberrations for the chromosomes 1 and 7 were detected above the cutoff percentage in 15 of the 20 tumor brushes for either 1 or both chromosomes (Table 1). Numeric chromosomal aberrations detected in the cytologic specimens included trisomies, tetrasomies, polysomies, or combinations thereof. Imbalances between the copy numbers of chromosomes 1 and 7 were observed frequently. Chromosome 7 showed a relative gain in seven cytologic specimens, whereas a relative gain for chromosome 1 was detected in two specimens. The presence of these imbalances was in all cases confirmed by double-target FISH. Figure 1 shows some examples of double-target FISH patterns in brush specimens of HNSCC.

Cytologic Grading and Comparison with the FISH Results
From the control group, 23 brush specimens were classified as normal and in 1 specimen a group of atypical cells were observed. In the tumor group, two specimens were classified as normal, atypical cells were observed in two specimens, and ten specimens contained suspiciously malignant cells. No numeric chromosomal aberrations were detected above the cutoff percentage in the two samples with atypia and in one normal sample, but a trisomy for chromosome 7 was detected in one sample without atypical cells. Chromosomal aberrations were detected in nine of the ten brush specimens that were classified as suspicious for malignancy (Table 1).

DNA Flow Cytometry and FISH Analysis of Solid Tumor Tissue
A frozen tissue specimen taken from the tumor was available for analysis from 12 of the 20 patients in this series. Flow cytometric analysis showed that 3 of these specimens contained diploid tumor cells (DNA-index = 1.0), whereas 9 tissue specimens contained DNA aneuploid tumor cells (DNA-index > 1.0) (Table 1). In addition, the FISH analysis of these solid tissue specimens showed that all DNA aneuploid tumors contained numeric aberrations for chromosomes 1 and/or 7. No chromosomal aberrations were detected in the DNA diploid tumors. Chromosomal aberrations were detected in all brush specimens taken from DNA aneuploid tumors with aneusomy. In addition, trisomy 7 was detected in one brush specimen whereas the matching solid tissue specimen did not show an aberrant copy number for this chromosome or an aberrant DNA content. Differences also were observed when the FISH results of the brush specimens and the matching solid tissue specimen were compared. In some cases a chromosome was classified as trisomic in one specimen but as tetrasomic in the other specimen taken from the same tumor. In addition a minor polysomic population was detected occasionally in the solid tissue specimen that could not be detected in the brush specimen. To exclude that these differences were the result of technical problems involving specimen pretreatment and classification on basis of 200 nuclei, the authors repeatedly analyzed these samples and obtained identical results (results not shown). The average percentage of cells harboring numeric chromosomal aberrations was lower in the brush specimen (20%) than in the solid tissue specimen (43%).

DISCUSSION
The feasibility of the FISH technique to detect chromosomal aberrations in cytologic brush specimens from tumors of the upper aerodigestive tract was examined in this study. For this purpose the copy number of chromosomes 1 and 7 was determined by interphase cytogenetics in cells brushed from tumor sites and in cells obtained from normal mucosa. In all specimens studied a high detection efficiency could
FIGURE 1. Fluorescence in situ hybridization (FISH) results in tumor brush specimens from head and neck squamous cell carcinoma. Probes hybridized to the centromeric regions of chromosomes 1 and 7 were detected with tetramethylrhodamin isothiocyanate (red) or fluorescein isothiocyanate (green). Nuclei were counterstained with 4',6-diamino-2 phenyl indole (blue). (A) Control sample showing nuclei with a disomy for both chromosomes 1 (green) and 7 (red). (B) Sample from Patient 15, showing nuclei with numeric aberrations for both chromosomes 1 (red) and 7 (green). (C) A nucleus from the sample from patient 2, with disomy for chromosome 1 (green) and trisomy for chromosome 7 (red). (D) Nucleus from the sample from Patient 7 showing trisomy for chromosome 1 (red). Nucleus from the sample from Patient 7 showing trisomy for chromosome 1 (red) and tetrasomy for chromosome 7 (green). (E) Nucleus from the sample from Patient 15 showing polysomy for chromosome 7 (Magnifications: A + B, ×1000; C – E, ×2000).

be achieved. Numeric aberrations for chromosome 1 and/or 7 were detected in all brush specimens taken from DNA aneuploid tumors. In total 75% of all brush specimens, taken randomly from tumors of different clinical stages, contained chromosomal aberrations, whereas in the control group the percentage of cells with abnormal chromosomal copy numbers did not exceed the cutoff percentage. The presence of chromosomal aberrations in the tumor brushes did not correlate with tumor localization, clinical stage, and/or stage of differentiation. In the current study, the cytologic specimens from 11 of the 13 NO tumors contained numeric aberrations for chromosomes 1 and 7. This points to an early accumulation of generalized genetic changes in head and neck tumorigenesis, and indicates that the detection of chromosomal aberrations in cytologic specimens is a relevant marker for risk assessment in (pre)clinical stages of this disease. Studies using flow cytometry to measure the DNA content of HNSCC showed that 60–70% of these tumors
were DNA aneuploid. In the current study 9 of 12 analyzed solid tumor tissue specimens showed DNA aneuploidy (75%) and chromosomal aberrations were detected in all these tissue specimens. This is in agreement with the study of El-Naggar et al., which reported the presence of chromosomal aberrations in all examined DNA aneuploid head and neck tumors. The results of the current study showed that chromosomal aberrations were detected in brush specimens from all of the DNA aneuploid tumors. Therefore this result validates the use of FISH in head and neck cytopathology. It is important to note that the percentage of cells with chromosomal aberrations in the brush specimens was in general lower than in the tissue specimens. This can be explained by contamination of tumor cells with normal mucosal cells in a brush specimen and by difficulties encountered in brushing (e.g., as for a glottic tumor, which often presents as a small lesion). In addition, aberrant cells present in deeper parts of the tumor will not be collected by the brush procedure.

In five cases the FISH technique did not reveal chromosomal aberrations in the brush specimen. Two cases had matching solid tumor specimens with a normal DNA index and disomy for the chromosomes 1 and 7. The remaining three cases had no suspiciously malignant cells in the brush specimen with Papanicolaou staining.

In six solid tissue specimens and in four brush specimens a mixed classification of the chromosomal copy numbers was given. The presence of such genetically different cell populations within one tumor is a strong indication of intratumoral heterogeneity. This also might explain the frequent differences that were detected between copy numbers in the brush specimens and solid tissue specimens and in particular the trisomy for chromosome 7 that was detected in the brush specimen of Patient 2, which could not be detected in the solid tissue sample from this patient.

In conclusion, this study showed that application of the FISH technique in cytologic specimens of tumors present in the upper aerodigestive tract provides valuable information regarding the genetic constitution of the tumor cells. Detection of chromosomal aberrations in >5% of the cells is a strong indication of malignancy.

REFERENCES


