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SPECIFIC STEPS IN ANEUPLOIDIZATION CORRELATE WITH LOSS OF HETEROZYGOSITY OF 9p21, 17p13 AND 18q21 IN THE PROGRESSION OF PRE-MALIGNANT LARYNGEAL LESIONS

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Laryngeal squamous-cell carcinoma is often preceded by pre-malignant lesions. In this study, pre-malignant as well as malignant laryngeal lesions were analyzed using p53 immunohistochemistry and *in situ* hybridization for chromosomes 1, 7, 9, 17 and 18. Microsatellite analysis was performed on laser-microdissected tissue fragments with the aim of studying loss of heterozygosity (LOH) of 9p21, 17p13 and 18q21. Sequential biopsies were analyzed from a few cases to study genetic progression in more detail. The following genetic progression patterns were observed: *(i)* histologically normal mucosa and hyperplastic lesions without malignant progression were typically disomic for all chromosomes tested and showed no or only basal cell layer positivity for p53 and no allelic loss; *(ii)* hyperplastic lesions preceding dysplastic/invasive growth frequently showed trisomy for chromosome 7 and LOH of 9p21 and 17p13, and small foci within these lesions sometimes showed tetraploidization and p53 positivity; *(iii)* dysplastic lesions were characterized by a tetraploid chromosome content, LOH of 9p21 and 17p13 and p53 positivity; *(iv)* carcinoma *in situ* lesions and invasive laryngeal carcinomas showed a more unbalanced chromosome pattern and an additional 18q21 LOH. These results show that different steps in aneuploidization correlate with LOH of 9p21, 17p13 and 18q21 in early laryngeal carcinogenesis. These genomic changes could be of potential use in the diagnosis and prognosis of pre-malignant laryngeal lesions.

Key words: *aneuploidization; heterozygosity; laryngeal lesions*

Head-and-neck squamous-cell carcinomas (HNSCC) often exhibit an aneuploid DNA content; loss of chromosomal regions 3p14-21, 9p21, 17p13 and 18q21; mutations in the *p53* gene; and amplifications of the *CCND1* proto-oncogene.[1–4] Many of these genetic alterations take place in pre-malignant stages of the disease, and some appear to be indicators of malignant transformation.[5–7] Although the timing of the different genetic changes in malignant transformation is gradually being resolved, little is known about the correlation between the different genetic changes. At present, *e.g.*, it is unclear whether loss of heterozygosity (LOH) of 9p21 and/or 17p13 occurs before, simultaneously with or after the aneuploidization process in early stages of head-and-neck carcinogenesis.

In this study, a series of pre-malignant laryngeal lesions were screened by *in situ* hybridization (ISH) using centromeric probes for chromosomes 1, 7, 9, 17 and 18 to investigate the presence and the pattern of chromosomal abnormalities in the different parts of these lesions. Selection of DNA probes was based on previous ISH studies on HNSCC.[8,9] In addition, p53 immunohistochemistry was performed on these lesions since there are strong indications that alterations in this tumor-suppressor gene may lead to overall genetic instability.[10–12] Based on ISH data, laser-facilitated microdissection was used to isolate small pre-malignant regions with a specific chromosomal content, followed by a fluorescent microsatellite analysis using markers for 9p21, 17p13 and 18q21. The choice of these loci was based on a detailed allelotype of HNSCC published by Field *et al.*[2] and on an LOH study by Califano *et al.*,[5] using pre-malignant lesions of the head and neck. This approach allowed us to draw conclusions *(i)* on the type and extent of genetic alterations present in different stages of laryngeal carcinogenesis, *(ii)* on the correlation between the process of aneuploidization and the loss of specific chromosomal regions and *(iii)* on the role of p53 in the progression of the disease.

MATERIAL AND METHODS

Patient material

Formalin-fixed, paraffin-embedded biopsy specimens were available from 27 (pre-)malignant laryngeal lesions of 18 patients. From all specimens, a series of 4-μm-thick sections was used for detailed histological examination, ISH, laser-facilitated microdissection and p53 immunohistochemistry. One section was stained with hematoxylin and eosin and reviewed by an experienced pathologist (FJB). Normal mucosa, hyperplasia, dysplasia, carcinoma *in situ* (CIS) or carcinoma areas were identified and the specific areas marked on the slide for detailed comparisons with the genetic data.

ISH

ISH was performed and evaluated essentially as described previously.[7,13] Briefly, after deparaffinization, sections were pre-treated with 85% formic acid containing 0.3% H_2O_2, followed by incubation at 80°C in 1 M sodium thiocyanate. After digestion with pepsin from porcine stomach mucosa (800 to 1,200 U/mg protein; Sigma, St. Louis, MO) at a concentration of 4 mg/ml in 0.02 N HCl, individual sections were hybridized using centromere-specific probes for human chromosomes 1, 7, 9, 17 or 18,[14] all labeled with biotin-11-dUTP (Boehringer-Mannheim, Mannheim, Germany). Immunocytochemical detection was performed by a standard avidin-biotin complex procedure. Signals were developed using diaminobenzidine/H_2O_2; sections were counterstained with hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany).

Based on the ISH results, lesions were classified as either disomic, trisomic, tetrasomic or polysomic according to the maximum chromosome copy number present in the lesion (2, 3, 4 or >4, respectively).

Laser-facilitated microdissection

Laser-facilitated microdissection was performed essentially as described previously,[15] with minor adaptations. Briefly, 4-μm-thick sections were mounted onto 0.17 mm glass slides, deparaffinized and stained with hematoxylin and eosin. The UV laser microbeam used for microdissection (Palm, Wolfratshausen, Ger-

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many) consisted of a pulsed nitrogen laser coupled through the fluorescence illumination path of the microscope. Unwanted cells and areas of surrounding tissue were brought into the laser beam using a motorized, computer-controlled microscope stage and eliminated by the high photon density within the laser focus. In this way, a tissue-free gap around the area of interest was created, and this area (often consisting of 1,000 or more cells) was collected manually under a stereomicroscope using a sterile syringe needle. Cells were transferred to a 1.5 ml microcentrifuge tube and could be stored for months at room temperature. Areas were selected for microdissection based on the histological classification and ISH results of each specimen. These areas were microdissected from 3 to 5 serial sections. As a source for normal DNA, stromal cells and lymphocytes present in the same biopsy specimen were used. Histologically normal mucosa showing a disomic chromosome content (as detected by ISH) was also included in the analysis when present adjacent to a pre-malignant lesion.

Fluorescent microsatellite analysis

Genomic DNA was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) with a slightly modified protocol. Incubation of the tissue with proteinase K was prolonged to about 60 hr at 55°C. After column purification, the DNA was dissolved in 30 μl of bidistilled water. PCR analysis targeted sequences containing highly polymorphic microsatellite repeat motifs at loci on chromosomes 9p21 (D9S161), 17p13 (TP53) and 18q21 (D18S35). These microsatellite markers were specifically chosen because the PCR products obtained were of very small size (varying between 101 and 147 bp), which made them suitable for amplifications from the DNA isolated from formalin-fixed and paraffin-embedded cells. Oligonucleotides were labeled with either 6-FAM (D9S161), HEX (TP53) or TET (D18S35). PCRs were performed according to Erber *et al.*,[16] using 3 μl of DNA and adding a final extension step of 30 min at 72°C. After amplification, PCR samples were diluted 1:5 in formamide, heated to 95°C for 2 min, chilled on ice and analyzed on an ABI PRISM 310 Genetic Analyzer (ABI Perkin-Elmer, Nieuwekerk, the Netherlands) with Genescan software. To estimate the degree of LOH, *i.e.,* the allelic loss in the heterozygously paired normal and (pre-)malignant samples, an LOH value was calculated according to Cawkwell *et al.*[17] as (T1/T2)/(N1/N2). T1 and N1 are the peak height values of the shorter allele for the (pre-)malignant and the normal samples, respectively, and T2 and N2 are the peak height values of the longer allele for the (pre-)malignant and normal samples, respectively. When the allele ratio calculated by this equation was above 1.0, the ratio was converted, using 1/[(T1/T2)/(N1/N2)] to give a result in the range 0.0–1.0. A value at or below 0.50 was taken to indicate LOH. Microsatellite instability was indicated by the presence of novel allele peaks in the pre-malignant or malignant DNA sample analyzed for a particular microsatellite marker, which was not present in the corresponding normal DNA analyzed for this marker.

p53 immunohistochemistry

An immunohistochemical assessment of (mutated) p53 protein expression was performed on 4-μm-thick paraffin sections. After deparaffinization, sections were pre-treated with 0.3% H_2O_2 in methanol to quench endogenous peroxidase activity. Antigen retrieval was performed by microwave heating in 0.01 M citrate buffer (pH 6.0). Both normal and mutant p53 proteins were detected using the monoclonal antibody (MAb) clone DO-7 (Dako, Glostrup, Denmark). After incubation with the biotinylated secondary antibody, immunocytochemical detection was further performed as described for ISH. In each analysis, known p53-negative and p53-positive oropharynx tumors served as controls. The extent of the p53 immunostaining reaction was scored visually as (I) negative, (II) nuclear staining limited to the basal cell layer, (III) scattered positive nuclear staining in 5% to 10% of cells or (IV) positive nuclear staining in most cells in the lesion.

RESULTS

ISH

Histologically normal mucosa, present in 15 of the 28 biopsy specimens, was used as internal control. Nuclei within these normal epithelia showed 0, 1 or 2 ISH signals for all chromosome probes used, with no evidence of nuclei with more than 2 ISH signals. Table I shows the ISH results obtained in 34 (pre-)malignant lesions, which were histologically identified in the 28 specimens of the 18 patients. In Figure 1*a–c*, examples of the ISH results are presented. Four major chromosomal patterns were distinguished: *(i)* disomy for all chromosomes tested, *(ii)* trisomy for chromosome 7 as the sole numerical chromosomal change, *(iii)* tetrasomy for all chromosomes tested and *(iv)* chromosomal copy number imbalances and polysomy in 1 or more of the chromosomes tested. In general, patterns *(i)* and *(ii)* were observed in hyperplastic lesions and patterns *(iii)* and *(iv)* were observed in dysplastic lesions, CIS and SCC.

In most cases, a homogeneous chromosomal pattern was observed in a specific histological area. In 4 cases, however, small foci of 10 to 100 nuclei containing a chromosomal pattern that deviated from the pattern observed in the rest of the lesion were identified. Trisomy for chromosome 7 was observed in part of the hyperplastic lesion in case 5. Cases 6, 17 and 18 contained small foci of nuclei with tetrasomy (and polysomy) for all chromosomes, while the rest of the lesion showed a trisomy for chromosome 7.

Polysomy (*i.e.*, >4 ISH signals per nucleus) was observed in many dysplastic lesions and most of the CIS and SCC lesions, often as a small subpopulation within a tetrasomic lesion. Polysomy occurred more or less randomly for chromosomes 1, 7, 17 and 18. Polysomy for chromosome 9, however, was not detected. Imbalances in chromosomal copy numbers occurred predominantly in the group of CIS and SCC lesions and appeared to be present throughout the lesion.

Microsatellite analysis of 9p21, 17p13 and 18q21

Laser-facilitated microdissection was performed on tissue sections stained with hematoxylin and eosin from all specimens. Microdissection was guided by histology and ISH results, by which tissue compartments with and without chromosomal changes were precisely mapped (Fig. 1). DNA was isolated, and amplification for the microsatellites D9S161 (located on 9p21), TP53 (located on 17p13) and D18S35 (located on 18q21) was performed. Microsatellite data for each specimen are presented in Table I. Figure 1g–k illustrates typical LOH results. For these microsatellites, the presence or absence of allelic loss could easily be determined based on the LOH value; all lesions classified as LOH contained values between 0 and 0.49, whereas all lesions classified as presenting no LOH showed values between 0.60 and 0.98. Histopathologically normal mucosa, isolated from 15 specimens, did not show allelic loss when compared to control non-mucosal samples. Most hyperplastic lesions also showed retention of heterozygosity, though allelic loss for 9p21 was observed in all hyperplastic areas of case 17 and allelic loss for 17p13 was observed in a hyperplastic region adjacent to a dysplasia in case 18. Virtually all dysplastic lesions, CIS and SCC displayed LOH for at least 1 of the 3 loci. Allelic loss at 9p21 and 17p13 was shown at high frequencies in both dysplastic lesions as well as CIS and SCC. Only 1 of 6 dysplastic lesions displayed LOH of 18q21, whereas 2 of 3 CIS and 2 SCCs revealed allelic loss at this locus. In only 1 case (case 15) was microsatellite instability observed for the microsatellite TP53, both in the dysplastic lesion and in the invasive carcinoma diagnosed 4 years later (Fig. 1*k*).

p53 immunohistochemistry

Normal mucosa, present in 15 specimens adjacent to the (pre)-malignant lesions, showed either no p53 staining or only faintly positive nuclear staining in the basal cell layer. Identical results were obtained in most hyperplastic lesions, whereas a majority of the dysplastic lesions, CIS and SCC showed nuclear p53 staining in the majority of cells (Table I, Fig. 1*d*).

TABLE I – OVERVIEW OF THE HISTOLOGICAL AND GENETIC CHANGES OBSERVED IN THE LARYNGEAL BIOPSY SPECIMENS

Case	Biopsy	Histology	ISH CH.1	ISH CH.7	ISH CH.9	ISH CH.17	ISH CH.18	P53 IHC	9p21	17p13	18q21
1	A	Hyperplasia	2	2	2	2	2	—	—	—	NI
2	A	Hyperplasia	2	2	2	2	2	—	—	—	—
3	A	Hyperplasia	2	2	2	2	2	BL Pos	—	—	—
4	A	Hyperplasia	2	2	2	2	2	—	—	—	—
5	A	Hyperplasia 1	2	2	2	2	2	—	—	—	—
		Hyperplasia 2 (small focus)	2	3	2	2	2	S Pos	—	—	—
6	A	Dysplasia 1	2	3	2	2	2	—	LOH	—	NI
		Dysplasia 2 (small focus)	4	6–8	4	4	4	Pos	n.e.	n.e.	n.e.
7	A	Dysplasia	4	4	4	4	4/6–8	Pos	LOH	LOH	NI
8	A	Dysplasia	4	4	4	4/6–8	4	S Pos	LOH	LOH	—
9	A	Dysplasia	4/6–8	4/6–8	4	4/6–8	4	Pos	—	LOH	—
10	A	Dysplasia	4/6–8	4/6–8	4	4	4	S Pos	LOH	NI	—
11	A	CIS	2	3	4	4	4	Pos	LOH	LOH	LOH
12	A	Hyperplasia	2	2	2	2	3	—	—	—	—
		CIS	4	4	4	4	3/6–8	—	LOH	n.e.	—
13	A	CIS	2	4	2	4/6–8	4	S Pos	—	n.e.	n.e.
14	A	CIS	4/6–8	4/6–8	4	4	4	Pos	LOH	—	LOH
15	A	Dysplasia	2	3	n.e.	n.e.	n.e.	Pos	—	MSI	n.e.
	B	SCC	2	3	2	2	2	—	LOH	MSI	n.e.
16	A	Dysplasia	4	4	4	4	4	Pos	LOH	LOH	—
	B	Dysplasia	4	4	4	4	4	Pos	LOH	LOH	—
	C	SCC	4/6–8	4/6–8	4	4/6–8	2/3	Pos	LOH	LOH	LOH
17	A	Hyperplasia	2	3	2	2	2	BL Pos	LOH	NI	NI
	B	Hyperplasia	2	3	2	2	n.e.	—	LOH	NI	NI
		Hyperplasia (small focus)	4	4/6–8	4	4	n.e.	—	LOH	NI	NI
	C	Hyperplasia (small focus)	4	4	4	4	4	Pos	LOH	NI	NI
		Dysplasia (small focus)	2	2	2	2	2	—	—	NI	NI
18	A	Hyperplasia	2	2	2	2	2	BL Pos	NI	—	—
	B	Hyperplasia (small focus)	3/6–8	4	4	4	4	—	n.e.	n.e.	n.e.
		Dysplasia (small focus)	2	2	2	2	2	—	n.e.	n.e.	n.e.
	C	Hyperplasia	2	3	2	2	2	—	NI	LOH	—
		Dysplasia (small focus)	3	4/6–8	4	4	4	—	NI	LOH	LOH
	D	SCC	4	4/6–8	2/3	4	2/3	—	NI	LOH	LOH
	E	Hyperplasia	2	2	2	2	2	BL Pos	NI	—	—

CH., chromosome; IHC, immunohistochemistry; A–E, subsequent biopsies; CIS, carcinoma *in situ;* 2, disomy; 3, trisomy; 4, tetrasomy; 6–8, polysomy; −, no expression/no LOH; BL Pos, basal cell layer-positive; S pos, scattered-positive; pos, positive; MSI, microsatellite instability, NI, not informative; n.e., not evaluable.

Correlation between histology, ISH analysis, p53 immunohistochemistry and microsatellite analysis

The approach we chose allowed detailed comparison between the genetic changes as detected by the different techniques in the different histological stages of laryngeal carcinogenesis. Table II illustrates the accumulation of genetic alterations with histological progression. Table III shows the increase in LOH and p53 positivity with subsequent steps of aneuploidization as detected in these lesions by ISH. Neither LOH nor p53 over-expression was detected in lesions with disomy for all chromosomes. In contrast, 4 of 7 lesions with sole trisomy for chromosome 7 displayed LOH of 9p21 and/or 17p13, and 2 of these lesions showed extensive p53 immunostaining. The percentage of lesions with LOH and p53 over-expression further increased in lesions that contained more extensive chromosomal alterations such as tetraploidization and chromosomal instability. LOH at 18q21 was detected only in lesions showing chromosomal imbalances or polyploidization. LOH at either 9p21 or 17p13 is strongly correlated with an increased copy number (aneusomy) for the corresponding chromosomes, as detected using a centromere-specific DNA probe in the ISH. In contrast, only part of the lesions with aneusomy for chromosome 18 showed 18q21 LOH. Immunostaining of the *p53* gene (located on 17p13) was present in lesions that typically contain multiple copies of chromosome 17 and show allelic loss of the p53 locus.

Genetic changes correlated with progression of individual cases

In Table IV, a detailed scheme is presented of 4 cases from which progressive lesions were available. Genetic alterations such as allelic loss at 9p21, p53 positivity and chromosomal duplication were shown to persist in many of the biopsy specimens from these cases, even when the time interval between different biopsies was 1 to 4 years. In case of histological progression, additional genetic changes, such as LOH of 18q21, arose (Fig. 1*g–j*). Furthermore, the occurrence of chromosomal copy number imbalances and polyploidization was typical in these later stages.

In 2 of the cases depicted in Table IV, genetic changes were observed in a hyperplastic area but not in the dysplastic area located in the same biopsy specimen. A small but clearly identified dysplastic region of case 17 (biopsy c) did not display numerical chromosomal alterations or p53 over-expression. Microdissection of this dysplastic region followed by microsatellite analysis did not reveal LOH for 9p21 (the other 2 markers were not informative). In contrast, a small hyperplastic region present in the same biopsy specimen contained tetrasomy for all chromosomes tested and was p53-positive. Microsatellite analysis of this region showed clear LOH at 9p21. In case 18 (biopsy b), a similar result was obtained by ISH, but unfortunately, the 2 regions were too small (both contained approx. 50 nuclei/tissue section) for PCR amplification.

DISCUSSION

In this study, histologically different stages of laryngeal carcinogenesis were analyzed by chromosome ISH, p53 immunohistochemistry and microsatellite analysis. The ISH and immunohistochemical techniques were applied directly onto tissue sections and allowed a detailed investigation of the chromosomal content and the p53 status in different areas present in a biopsy specimen. Based on the results of these analyses, specific tissue compartments were isolated using laser-facilitated microdissection, and a fluorescent microsatellite analysis was performed to detect LOH of chromosomal loci 9p21, 17p13 and 18q21. In our opinion, this approach has several advantages over the conventional approach, whereby regions are isolated for genetic analyses on the basis of

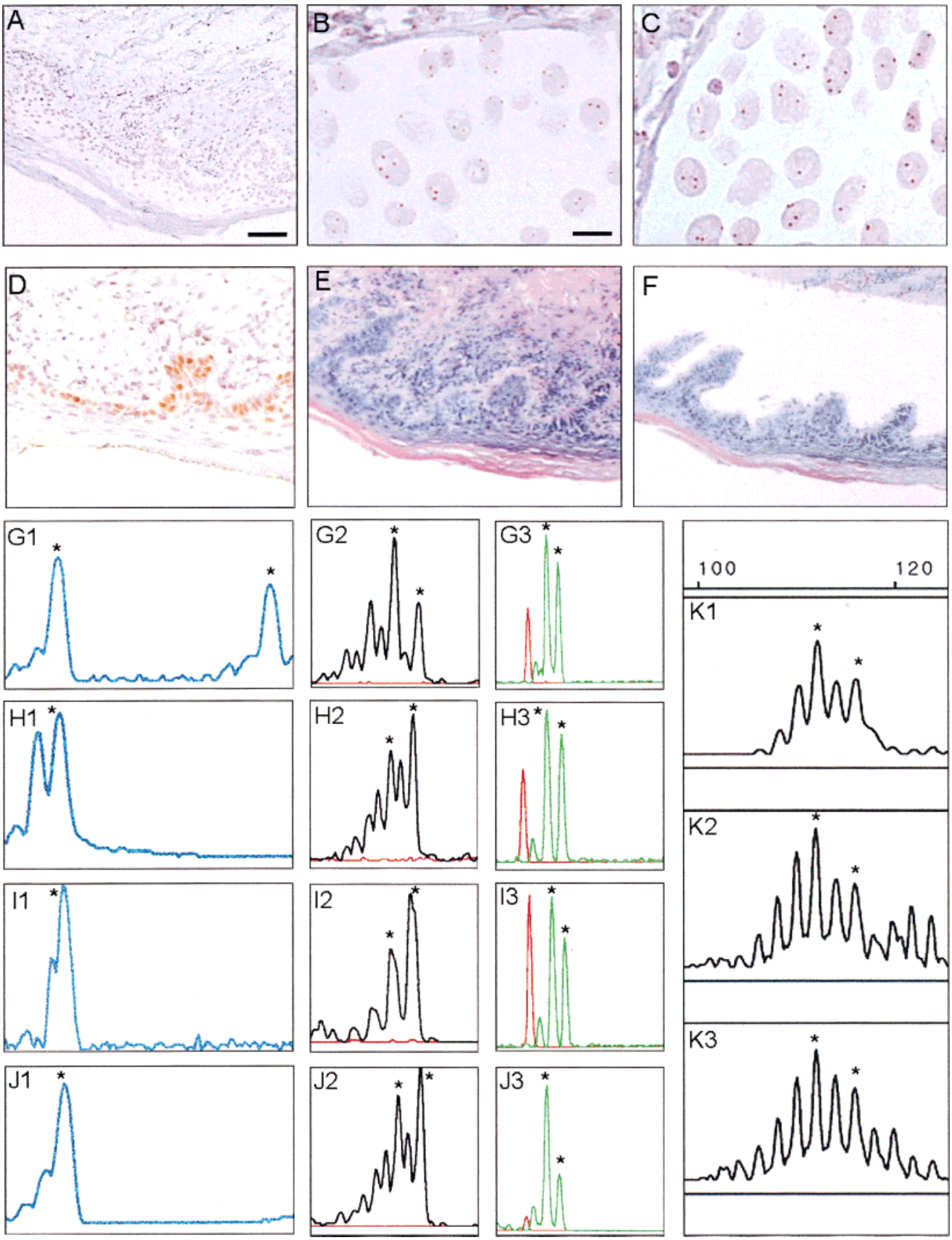

FIGURE 1.

TABLE II – CORRELATION BETWEEN HISTOLOGY, LOH DATA AND p53 STATUS

Histology	Chromosomal locus			p53 IHC positive staining[1]
	9p21	17p13	18q21	
Hyperplasia	4/11[2] (36)	1/10 (10)	0/8 (0)	2/15 (13)
Dysplasia	6/9 (67)	7/8 (88)[3]	1/6 (17)	8/12 (67)
CIS/LSCC	5/6 (83)	4/5 (80)[3]	4/5 (80)	4/7 (57)

[1]The positive group includes both lesions with scattered and those with complete positive nuclear staining.–[2]Values are given in loss/informative cases (% loss).–[3]Including microsatellite instability in 1 lesion.

TABLE III – CORRELATION BETWEEN ISH PATTERN, LOH DATA AND p53 STATUS

ISH chromosomal pattern[1]	Chromosomal locus			p53 IHC positive staining[2]
	9p21	17p13	18q21	
Overall disomy	0/6[3] (0)	0/7 (0)	0/5 (0)	0/9 (0)
Sole trisomy 7	4/6 (67)	3/5[4] (60)	0/2 (0)	2/7 (29)
Overall tetrasomy	7/7 (100)	4/4 (100)	0/4 (0)	6/9 (67)
Imbalances/polysomy[5]	4/6 (67)	5/6 (83)	5/7 (71)	6/8 (75)

[1]Chromosomal patterns based on ISH results of all 5 chromosomes tested.–[2]Positive group includes both lesions with scattered and those with complete positive nuclear staining.–[3]Values are given in loss/informative cases (% loss).–[4]Including microsatellite instability detected in 2 lesions.–[5]Chromosomal copy number imbalances and/or polysomy, detected for at least 2 of the 5 chromosomes studied.

histology. Firstly, as pointed out by many authors,[18] the histopathological features of pre-malignant lesions of the aerodigestive tract are subtle and overlap with non-neoplastic reactive processes. It does not appear logical to use the subjective morphological method as a starting point for a detailed molecular analysis that requires the use of a pure population with a minimal amount of non-aberrant "normal" cells. Screening the biopsy specimens by chromosome ISH and p53 immunohistochemistry easily identifies tissue compartments within these specimens harboring the most altered (genetically) cell population. It is this cell population that was our target for the microsatellite analysis and was, therefore, isolated by laser-facilitated microdissection. Secondly, this approach allows a detailed comparison of genetic alterations during the carcinogenic process, such as allelic loss of specific chromosomal regions, different steps in the aneuploidization process and alterations in expression of the *p53* tumor-suppressor gene.

Based on the results obtained in this study, a 3-step genetic progression model for early laryngeal carcinogenesis emerges (Tables II, III): *(i)* the earliest detectable genetic changes appear to be acquisition of trisomy for chromosome 7 and allelic loss at 9p21 and 17p13, and the tissue containing these genetic alterations is typically hyperplastic; *(ii)* this is followed by over-expression of the (presumably mutated) *p53* gene and tetraploidization, and the tissue containing these genetic alterations is typically mildly to moderately dysplastic; *(iii)* chromosomal copy number imbalances and polyploidization occur together with allelic loss at 18q21. The tissue containing these genetic alterations is typically CIS or SCC.

A small proportion of laryngeal lesions remain disomic during progression, *e.g.*, as illustrated by case 15. In this case, microsatellite instability was observed at the TP53 locus, indicating that another form of genetic instability is present. In addition, all results were obtained in a relatively small cohort and should be confirmed in a larger series.

In agreement with the genetic progression model proposed by Califano *et al.*,[5] we observed LOH of 9p21 and 17p13 to be among the earliest detectable genetic changes in head-and-neck carcinogenesis. These changes appear to occur before tetraploidization, in contrast to LOH of 18q21, which was found only in advanced precursor lesions containing chromosomal imbalances and polysomy. These data suggest that LOH of 9p21 and 17p13, containing the important cell-cycle regulators $p16^{INK4a}$ and p53, respectively, may contribute to chromosomal missegregation, resulting in aneuploidy. Our present results as well as of a previous study by our group on oral precursor lesions[19] indicate that p53 over-expression indeed precedes the tetraploidization process. These data support literature indicating that alterations in the *p53* tumor-suppressor gene lead to overall genetic instability.[10–12] These genetic changes persist within the pre-malignant epithelium for several years and, thus, may be used in the follow-up of a given pre-neoplastic lesion. LOH of 18q21, although not of importance in the early stages of laryngeal carcinogenesis, is very likely of relevance in tumor progression.[20,21] Candidate tumor suppressors on this locus are the *DCC* (deleted in colon cancer) and the *DPC4* (deleted in pancreatic cancer) genes.[22,23]

How can LOH be explained in light of the aneuploidization process? If a duplication of the whole chromosome 9 occurs before allelic loss at 9p21, this loss would have to take place on 2 duplicate chromosome copies, whereas only 1 chromosome copy has to be affected if LOH occurs before duplication. This has also been discussed by Varella-Garcia *et al.*[24] studying the relation between chromosomal duplication and allelic loss in non-small-cell lung carcinoma. Our data strongly suggest that the allelic loss observed at 9p21 and 17p13 occurred before tetraploidization, whereas allelic loss at 18q21 took place after tetraploidization. In support of this hypothesis, allelic loss of 9p21 was present in the entire dysplastic area of case 6, whereas disomy for chromosome 9 was observed in the majority of this lesion. Interestingly, in a small focus of about 10 nuclei located within the dysplasia, chromosomal duplication as well as nuclear p53 immunostaining were

FIGURE 1 – Typical examples of results obtained by ISH, p53 immunohistochemistry, laser-facilitated microdissection and fluorescent microsatellite analysis. *(a–f)* ISH and p53 immunohistochemistry on the biopsy specimen of case 7. Tetrasomy for chromosomes 7 (overview in *a*, detail in *b*) and 17 *(c)* is present in the dysplastic region, which also shows p53 positivity *(d)*. This region was microdissected from a section stained with hematoxylin and eosin *(e)* by laser-facilitated microdissection *(f)*. Stromal cells surrounding the region were eliminated, and the region was manually collected. *(g–j)* LOH results of biopsies A *(h)*, B *(i)* and C *(j)* of case 16 for the loci D9S161 (lane 1), TP53 (lane 2) and D18S35 (lane 3). *(g)* Results for normal mucosa from this case are depicted for comparison. For reasons of clarity, peaks representing specific alleles are marked with an asterisk; other alleles are so-called stutter bands caused by inefficient amplification. D9S161 shows complete loss of the second allele in both dysplastic lesions and SCC. TP53 shows a significant reduction of the first allele in the same lesions. Retention of heterozygosity is observed for D18S35 in the 2 dysplastic lesions, whereas SCC displays LOH. *(k)* Microsatellite instability observed for TP53 in dysplasia (lane 2) as well as SCC (lane 3) of case 15. Scale bar = 80 μm *(a,d,e,f)* and 10 μm *(b,c)*.

TABLE IV – HISTOLOGICAL AND GENETIC CHANGES OBSERVED IN 4 CASES WITH PROGRESSIVE DISEASE

Case	Biopsy 1	Biopsy 2		Biopsy 3		Biopsy 4	Biopsy 5
15							
Year	1992	1996					
Histology	Dysplasia	SCC					
ISH	Trisomy 7	Trisomy 7					
p53 IHC	p53-positive	p53-negative					
LOH/MSI	MSI 17p13	LOH 9p21/MSI 17p13					
16							
Year	1987	1991		1996			
Histology	Dysplasia	Dysplasia		SCC			
ISH	Tetrasomy	Tetrasomy		Polysomy/imbalances			
p53 IHC	p53-positive	p53-positive		p53-positive			
LOH/MSI	LOH 9p21/17p13	LOH 9p21/17p13		LOH 9p21/17p13/ 18q21			
17							
Year	1982	1985		1986			
Histology	Hyperplasia	Hyperplasia	Hyperplasia, small focus	Hyperplasia, small focus	Dysplasia, small focus		
ISH	Trisomy 7	Trisomy 7	Tetrasomy	Tetrasomy	Disomy		
p53 IHC	p53-negative	p53-negative	p53-negative	p53-positive	p53-negative		
LOH/MSI	LOH 9p21	LOH 9p21	LOH 9p21	LOH 9p21	No LOH		
18							
Year	1986	1988		1990		1991	1992
Histology	Hyperplasia	Hyperplasia, small focus	Dysplasia, small focus	Hyperplasia	Dysplasia, small focus	SCC	Hyperplasia
ISH	Disomy	Tetrasomy	Disomy	Trisomy 7	Tetrasomy/polysomy/imbalances	Polysomy/imbalances	Disomy
p53 IHC	p53-negative	p53-negative	p53-negative	p53-negative	p53-negative	p53-negative	p53-negative
LOH/MSI	No LOH	n.e.	n.e.	LOH 17p13	LOH 17p13/18q21	LOH 17p13/18q21	No LOH

In this scheme, the results of chromosome ISH, p53 immunohistochemistry and microsatellite analysis are depicted for each lesion of these 4 cases. IHC, immunohistochemistry; MSI, microsatellite instability; n.e., not evaluable.

observed, possibly representing a next step in the carcinogenic process. In the majority of cases, however, both chromosomal duplication and allelic loss were observed in the same region of a biopsy specimen, often together with over-expression of the tumor-suppressor gene *p53*, suggesting that although LOH of 9p21 and 17p13 occurs as a first change, this is followed by chromosomal duplication and alterations in the *p53* gene.

How can LOH at 18q21 take place after tetraploidization? The follow-up biopsies studied from case 16 illustrate that allelic loss at this locus occurs after tetraploidization. It can be hypothesized that the presence of chromosomal instability is essential for LOH to take place in the later stages of tumorigenesis, when most chromosomes are present in 4 copies. This instability appears to be enhanced after tetraploidization and results in chromosomal gains and losses.[7,25] It remains to be determined whether there is complete loss of 1 of the alleles on 18q21 or deletion of 1 of the 4 copies resulting in allelic imbalance. Chromosome 18 showed a relative copy number loss in 2 of 3 invasive carcinomas, both of which showed LOH at 18q21, suggesting that LOH at 18q21 is one of the direct results of chromosome instability and that this allelic loss may be important for malignant progression of laryngeal lesions.

Our ISH analysis frequently detected small groups of cells with chromosomal alterations, situated within lesions that showed an overall disomic chromosomal pattern. In addition, in 2 cases (cases 17 and 18), genetic changes were observed in a hyperplastic region but not in the dysplastic region located in the same biopsy. This kind of disparity between genotype and phenotype has also been witnessed by Mao *et al.*,[26] studying LOH in advanced lesions of the head and neck following chemoprevention therapy. These observations underline the importance of ISH as a screening method for the identification of the most altered (genetically) cell population in a biopsy specimen. Small groups of cells can be identified within a tissue section on the basis of a deviation in the chromosomal content, microdissected and studied by PCR amplification. In this study, a minimum of approximately 1,000 nuclei was required for microsatellite analysis. Use of a whole genome amplification step before microsatellite analysis will most probably reduce the acquired number of cells to an amount where even small groups of about 10 cells with a specific chromosomal content can be studied for the presence of LOH.[27,28] In addition, this will allow the investigation of allelic loss at multiple loci. These approaches may prove to be invaluable for the implementation of microarray-based techniques in these research fields.

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