Histol Histopathol (2003) 18: 1053-1057

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

3p21, 5q21, 9p21 and 17p13 allelic deletions accumulate in the dysplastic spectrum of laryngeal carcinogenesis and precede malignant transformation

J. Sanz-Ortega, C. Valor, M.C. Saez, L. Ortega, E. Sierra, J. Poch, S. Hernández and J. Sanz-Esponera

Department of Pathology, San Carlos Clinic Hospital, Madrid,Spain

Summary. A tissue field of somatic genetic alterations precede the histopathological phenotypic changes of carcinoma. Loss of Heterozygosity (LOH) at the sites of known or putative tumor suppressor genes is a common genetic abnormality detected in precancerous conditions. These genomic changes could be of potential use in the diagnosis and prognosis of pre-malignant laryngeal lesions. Recently the concept of laryngeal intraepithelial neoplasia (LIN) was introduced. To evaluate patients with an increased risk of developing invasive laryngeal carcinoma via a dysplasia-carcinoma progression we investigated 102 microdissected cell populations. Cell populations were procured from 15 laryngectomy specimens with different peritumoral histological changes adjacent to the squamous cell carcinoma cells and 15 laryngeal endoscopic biopsies with no evidence of malignant transformation in a 6-10-year follow-up period. Histological diagnoses were subdivided into keratosis without dysplasia (KWD), with mild dysplasia (LIN 1), with moderate dysplasia (LIN 2), and with severe dysplasia or carcinoma in situ (LIN 3). Microsatellite analysis was performed with the aim of studying LOH of 5q21 (APC), 9p21 (p16), 3p21 and 17p13 (p53) chromosomal regions. Frequent allelic losses were found in carcinoma cells at p53 (54%), p16 (66%), 3p21(87%) and 5q21(58%). Identical LOH patterns were determined in 100% of the LIN3 peritumoral cells, 60% of LIN2, 50% of LIN 1 and 25% of KWD. In contrast, histologically normal mucosae, KWD and LIN1 lesions without malignant progression showed no allelic loss. These results show that dysplasia correlates with LOH at 3p21, 5q21, 9p21 and 17p13 in early laryngeal carcinogenesis. These genomic changes in pre-malignant laryngeal lesions could be of potential use as markers for cancer risk assessment.

Key words: Carcinoma, Microdissection, Larynx, Loss of heterozygosity

Offprint requests to: Dr. J. Sanz-Ortega, Departamento de Anatomía Patológica, Hospital Clínico San Carlos, Martín Lagos s/n, 28040 Madrid, Spain

Introduction

Squamous cell laryngeal carcinoma (SCLC) accounts for 1% of all cancer deaths and 95% of all laryngeal malignancies. It is most frequently found in smokers over 40 years of age. Patients with early-stage disease are often cured with surgery or radiotherapy. However, the majority of patients present with advanced disease, for which the outcomes have not markedly improved despite advances in combined-modality therapy (Vokes, 1993). Pre-malignant lesions often precede SCLC. A wide spectrum of lesions ranging from dysplasia to in situ carcinoma have to be considered when dealing with laryngeal precancerous conditions. The evaluation of dysplastic features in laryngeal epithelium is the best indicator of an increased risk of malignant transformation. Recently the concept of laryngeal intraepithelial neoplasia (LIN) was introduced. The incidence of progression to infiltrative carcinoma in a series of 259 consecutive cases of laryngeal keratosis was 7.14% for LIN 1, 21.42% for LIN 2, and 9.37% for LIN 3 (Gallo et al., 2001)

Loss of Heterozygosity (LOH) and microsatellite instability (MSI) have been recognized as important events in the carcinogenesis of many cancers, including squamous cell carcinoma of head and neck (SCCHN) (Frachiolla et al., 1995; Ku et al., 1999). LOH has also been reported in dysplastic lesions (Van der Riet, 1994; Mao et al., 1996; Gallo et al., 1997; Veltman et al., 2001). Allelic deletions are somatic genetic alterations frequently found in tumor cells and precancerous conditions, representing a predominant mechanism for inactivating one of the two alleles of each tumor suppressor gene. Indeed, the determination of allelic deletions by LOH studies is a rapid method for detection of tumor suppressor genes implicated in the evolution of the carcinogenetic process (Vogelstein and Kinzler, 1993). Molecular analysis of genotypic alterations in laryngeal biopsies from mucosae with pre-malignant changes may improve the characterization of biological groups of patients at different risks of malignization.

Deletion of 9p21 and 17p13 is a common event in many human tumors, including HNSCC. The gene CDKN2, which encodes the protein p16, a cyclin-dependent kinase-4 inhibitor, maps to 9p21. LOH at 9p21 has been reported in HNSCC ranging from 15-89% (Van der Riet, 1994; Bouckmuhl et al., 1996; Bai et al., 1998; Rizos et al., 1998; Wu et al., 1999). The tumor suppressor gene p53 maps to 17p13. LOH at 17p13 was determined in HNSCC ranging from 30-75%, indicating the dominant role of the suppressor in comparison with the mutator pathway in HNSCC carcinogenesis (Golusinski et al., 1997; Jin et al., 2000; Veltman et al., 2001). Univariate analyses showed that LOH at 9p or 17p were significantly associated with drug resistance (Cabelguenne et al., 2000). Allelic losses of the 5q21 chromosomal region, have been detected in several human solid tumors, including gut, colon, stomach, lung and kidney (González et al., 1996; Sanz-Ortega et al., 1996, 1999). This chromosomal region harbors the adenomatous polyposis coli (APC) gene, an important tumor suppressor gene. The APC gene is frequently mutated in colon carcinoma. LOH at 5q21 has also been described as a frequent event in HNSCC and lung cancer (Sanz Ortega et al., 1999). Deletions of DNA sequences in chromosomal band 3p21 observed in a variety of human tumors suggests the presence of one or more tumor suppressor genes within this region. LOH studies show frequent allelic loss at chromosomal regions 3p21 in non-small cell lung cancer (NSCLC). Allelic loss at 3p21 has also been reported in premalignant epithelial lesions of the bronchus and in histologically normal bronchial cells (Thiberville et al., 1995; Mao et al., 1997; Wistuba et al., 1997;Kohno et al., 1999; Sanz-Ortega et al., 2001).

To clarify the role of allelic deletions as a potential marker for individuals with a high risk of developing carcinoma, we decided to study the incidence of LOH at 3p21, APC, p16 and p53 chromosomal regions.

Materials and methods

Cell samples

102 microdissected cell populations were obtained from formalin-fixed, paraffin-embedded tissues. 30 cases were randomly obtained from the files of the Hospital "San Carlos", Madrid, Spain: 15 laryngectomy cases with dysplastic changes adjacent to SCC and 15 endoscopic biopsies with laryngeal epithelium with KWD and LIN1. Patients with SCC had not received chemotherapy or radiation before surgery, and ranged in age from 35 to 76 years-old (average, 58.8). In the group of cases with endoscopic biopsies showing KWD and LIN1, patients ranged in age from 33 to 72 years-old (average, 58.2) and had no evidence of malignancy in a 6-10 year follow-up period. In two carcinoma cases several previous endoscopic biopsies with no evidence of malignancy were available.

Microdissection technique

Our microdissection technique allows a separation of the different cell populations with 80-100% purity. From carcinoma cases we obtained different cell populations: tumor cells; squamous epithelium adjacent to carcinoma with and without dysplasia; respiratory epithelium; and inflammatory-normal cells. Squamous epithelium with dysplasia was subdivided into LIN1, LIN2 and LIN3 following the Gallo et al. criteria. Microdissected inflammatory cells were considered to be representative of normal DNA from each patient. From endoscopic biopsies with no evidence of malignant transformation, epithelium and normal cells were microdissected from paraffin-embedded tissues.

DNA extraction

Microdissected procured cells were resuspended in a solution containing 0.1 mg/ml proteinase K, and incubated 48 hours at 55 ºC. The mixture was boiled for 10 minutes to inactivate proteinase K. 1.5-ul of this mixture was used as a template in each PCR-based microsatellite analysis.

PCR analysis

All microdissected cell populations were subjected to PCR analysis. Four-color fluorescent-labeled primers were obtained from Research Genetics (Huntsville, AL). Microsatellite markers at 5q21 (D5S346), 3p21 (D3S1300), 9p21 (D9S157) and 17p13 (TP53) were used in the study. All PCR reactions were performed on a 2400 Thermal Cycler from PE Applied Biosystems. Each 10-ul reaction consisted of 1.5 mM $MgCl_2$, 0.5 uM of each primer and 0.2 units of Taq polymerase from PE. Amplification was done at 94º for 5 min, followed by 35 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 1 min.Water blanks controls were included in each PCR.

LOH analysis

1 μl of labeled amplified DNA was mixed with 12.5 μl of formamide and 0.5 μl of Genescan 500 TAMRA. The samples were denatured for 5 min at 94 ºC and then analyzed by capillary electrophoresis on the PE Applied Biosystems 310 Genetic Analyser with Genescan2.1 software. The Genotyper labels the alleles of the normal lymphocytes or inflammatory cells and the corresponding peaks in lesional cells and tumor tissue. All DNA templates were coded such that investigators were unaware of the cytological and pathological data from patients until the analysis was complete. Only primers that demonstrated Heterozygosity in lymphocyte or inflammatory cell DNA were considered informative.Standard criteria for semiautomatic quantitative assessment of LOH and microsatellite instability were used (Canzian et al., 1996).

Microsatellite instability is also called replication error (RER) and when present it invalidates the assessment of LOH (Canzian et al., 1996). Therefore, cases showing RER were considered as not informative cases. Non-informative cases included those with homozygous alleles in normal tissue and cases in which allelic patterns could not be clearly distinguished by the capillary electrophoretic methods used.

Allelic loss (LOH) was calculated by comparison of the allele ratio of normal cells with the allele ratio in dysplastic and tumor cells. The criterion for LOH was an at least 50% reduction of the lesional allele with the subsequent modification of the allele ratio. To assess the reproducibility of the LOH patterns we microdissected different areas from the same tumor section. A consistent pattern was observed in all cases when studies were repeated (data not shown). None of the normal tissue samples showed inappropriate allelic dropout (artifactual allelic loss occurring in the PCR assay).

Results

Table 1 summarizes the results of our study for 15 laryngectomy cases. We compared the patterns for one 3p21 microsatellite (D3S1300), one 5q21 microsatellite at the site of APC gene (D5S346), one 9p21 microsatellite at the site of CDKN2/p16 (D9S171) and one p53 microsatellite (TP53) in microdissected cells populations of KWD, LIN1, LIN2 and/or LIN3 epithelium adjacent to SCC. We also microdissected respiratory epithelium, carcinoma cells and normal tissue counterparts in each case. Frequent allelic loss

Table 1. Microsatellite analysis of laryngectomy specimens (15 cases) at chromosomes 5q21, 3p21, 9p21 and 17p13.

CASE	SAMPLE	CELLS	5q21	3p21	9p21	17p13
1	1	Tumor	LOH	Ins	Het	Het
	2	LIN2	LOH	Ni	Het	Het
	3	Resp	Het	Ni	Het	Het
	4	Normal	Het	Ni	Het	Het
2	5	Tumor-a	Ni	LOH	LOH	Het
	6	Tumor-b	Ni	LOH	LOH	Het
	7	LIN2	Ni	Het	LOH	Het
	8	Resp	Ni	Het	Het	Het
	9	Normal	Ni	Het	Het	Het
3	10	Tumor	LOH	Ni	Ni	Ni
	11	LIN1	LOH	Ni	Ni	Ni
	12	LIN2	LOH	Ni	Ni	Ni
	13	Normal	Het	Ni	Ni	Ni
	14	Resp	Het	Ni	Ni	Ni
4	15	Tumor	LOH	LOH	LOH	LOH
	16	LIN2	LOH	Nw	LOH	Nw
	17	Normal	Het	Het	Het	Het
	18	Resp	Het	Het	Het	Het
5	19	Tumor	Het	Ni	Het	LOH
	20	LIN1	Het	Ni	Het	Het
	21	Normal	Het	Ni	Het	Het
	22	Resp	Het	Ni	Het	Het
	23	KWD*	Het	Ni	Het	Het
	24	Normal*	Het	Ni	Het	Het
6	25	Tumor	Het	LOH	Ni	LOH
	26	LIN1	Het	LOH	Ni	LOH
	27	Normal	Het	Het	Ni	Het
	28	Resp	Het	Het	Ni	Het
7	29	Tumor	Het	LOH	LOH	Het
	30	LIN1	Het	Het	Het	Het
	31	LIN2	Het	Het	Het	Het
	32	Normal	Het	Het	Het	Het
	33	Resp	Het	Het	Het	Het
8	34	Tumor	LOH	LOH	LOH	Ni
	35	LIN3	LOH	LOH	LOH	Ni
	36	Normal	Het	Het	Het	Ni
	37	Resp	Het	Het	Het	Ni
9	38	Tumor	Ni	LOH	LOH	Het
	39	KWD	Ni	Het	Het	Het
	40	Normal	Ni	Het	Het	Het
	41	Resp	Ni	Het	Het	Het
10	42	Tumor	Ni	Ni	LOH	LOH
	43	LIN2	Ni	Ni	Het	LOH
	44	Normal	Ni	Ni	Het	Het
	45	Resp	Ni	Ni	Het	Het
11	46	Tumor	Het	Ni	LOH	LOH
	47	LIN3	Het	Ni	LOH	LOH
	48	LIN1	Het	Ni	LOH	Nw
	49	Normal	Het	Ni	Het	Het
	50	Resp	Het	Ni	Het	Het
12	51	Tumor	LOH	Het	Ni	Het
	52	LIN1-a	Het	Het	Ni	Het
	53	LIN1-b	Het	Het	Ni	Het
	54	KWD	Het	Het	Ni	Het
	55	Normal	Het	Het	Ni	Het
	56	Resp	Het	Het	Ni	Het
13	57	Tumor	LOH	LOH	Het	Ni
	58	KWD	Het	LOH	Het	Ni
	59	Normal	Het	Het	Het	Ni
	60	Resp	Het	Het	Het	Ni
14	61	Tumor	LOH	Ins	LOH	LOH
	62	KWD	Het	Ins	Het	LOH
	63	Normal	Het	Het	Het	Het
	64	Resp	Het	Het	Het	Het
	65	KWD*	Het	Het	Het	LOH
	66	Normal*	Het	Het	Het	Het
	67	KWD**	Het	Het	Het	LOH
	68	Normal**	Het	Het	Het	Het
15	69	Tumor	Het	Ni	Het	Ni
	70	KWD	Het	Ni	Het	Ni
	71	Normal	Het	Ni	Het	Ni
	72	Resp	Het	Ni	Het	Ni

Tumor: tumor cells; LIN1: Laryngeal intraepithelial neoplasia grade 1; LIN2: Laryngeal intraepithelial neoplasia grade 2; LIN3: Laryngeal intraepithelial neoplasia grade3; LIN1a and LIN1b: two different LIN1 areas from the same patient; KWD: keratosis without dysplasia; Resp: normal respiratory epithelium; Normal: inflammatory cells representing the allele status of normal cells. *: cell populations obtained from endoscopic biopsies performed 3 months in advance of laringectomy. **: cell populations obtained 5 months in advance to laryngectomy. Symbols of the LOH study: LOH: loss of Heterozygosity, Het: Heterozygosity, Ni: not informative due to homozygosity or to not a clear Heterozygous normal pattern, NW: Not working, Ins: genomic instability and therefore not valid for the LOH study.

was determined in tumor cells on chromosome 17p13 (54%), 9p21 (66%), 3p21(87%) and 5q21(58%). Microsatellite instability (MI) was rarely found in tumor cells: only 2 cases showed MI at 3p21. Identical LOH patterns were determined in 100% of the LIN3 peritumoral cells, 60% of LIN2, 50% of LIN 1 and 25% of KWD. Histologically normal mucosae, peritumoral respiratory epithelium (Table 1) and KWD and LIN1 lesions without malignant progression showed no allelic loss.Microsatellite instability at 3p21 was already present in one case in the peritumoral-analyzed cells.

Laryngeal endoscopic biopsies were performed prior to the carcinoma diagnosis in two cases (Table 1). In case 14, two endoscopic biopsies with the diagnosis of KWD were performed in advance to the laryngectomy (3 and 5 months before surgery, respectively). Identical microsatellite patterns were detected in all the KWD cell populations obtained from the endoscopic biopsies and peritumoral KWD from the laryngectomy specimen: Heterozygous for 5q21, 3p21, and 9p21, and LOH at 17p13. In case 5, one endoscopic biopsy was performed 3 months in advance of the carcinoma diagnosis showing KWD. Case 5 showed LOH at 17p13 in tumor cells which was not determined in the peritumoral LIN1 cells from the laryngectomy specimen. LOH at 17p13 was neither determined in KWD from previous endoscopic biopsies.

LOH was not determined in laryngeal epithelium microdissected from endoscopic biopsies with no evidence of malignant transformation (data not shown). In each case (n=15), microdissected laryngeal epithelium cells were compared with normal-inflammatory cell counterparts.

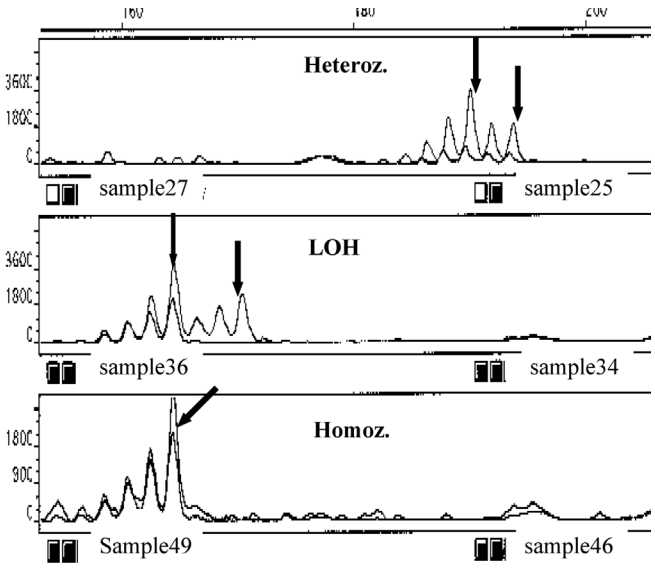

Fig. 1. The figure illustrates 3 examples of the semiautomated assessment of the allele status. The peak heights in fluorescent units are shown on the Y axis on the left. The DNA fragment size (base pairs) are shown on the X axis. Upper figure: Heterozygous pattern. Intermediate figure: complete deletion of the second ellele (LOH) on tumor cells. Lower figure: Homozygous pattern. Each figure shows two overlapping traces representing the fluorescent analysis oftumor cells (darker trace) and inflammatory cells(gray trace) for a given locus. The real alleles are labeled with arrows, while the decreasing peaks preceding each allele represent the characteristic “shadow bands” of microsatellite regions.

Discussion

Our analyses of carcinoma cell DNA match the incidence rates of LOH at 3p21, 9p21, 5q21 and TP53 previously reported (Frachiolla et al., 1995; Kiaris et al., 1995; Jares et al., 1997). We also determined a few expansions and contractions (MI) of these microsatellite regions, in agreement with other reports.Our results also show that the laryngeal epithelium surrounding the malignancy contained identical LOH at 3p21, 5q21 and 9p21 in association with dysplastic changes: 100% of the LIN3 peritumoral cells, 60% of LIN2, 50% of LIN 1 and 25% of KWD. LOH was absent in peritumoral respiratory epithelium or hyperplastic lesions without malignant progression. Therefore, our data may favor a sequential carcinogenetic model where LOH accumulates during the progress of laryngeal squamous cells through the dysplastic spectrum of phenotypic changes. Allelic loss at 3p21, 5q21, 9p21 and 17p13 play a role in the inactivation of tumor suppressor genes located at these loci: APC, p16 or p53. KWD and LIN1 lesions represent the laryngeal lesions with a lower potential of malignant transformation. In our series, striking differences in the incidence of LOH have been determined between peritumoral KWD -LIN1 lesions and KWD-LIN1 without progression in the follow-up study.Several reports also suggest the role of these genetic alterations in the malignant progression of precancerous conditions in HNSCC (Van der Riet et al., 1994; Mao et al., 1996; Gallo et al., 1997; Veltman et al., 2001). Indeed, similar genotypic changes have been described in the dysplastic spectrum of non-small-cell lung cancer precancerous conditions (Thiberville et al., 1995). In comparison with those studies, we analyzed a few specific chromosomal regions where a high concentration of LOH can be determined in small cell populations.

In conclusion our results indicate that a substantial proportion of cells in laryngeal mucosae adjacent to SCC carry identifiable genetic alterations that are unusual in laryngeal cells from patients with no evidence of malignancy. LOH analysis at 3p21, 5q21 and 9p21 may be useful to determine groups of KWD and LIN1 lesions with a higher risk of malignant transformation. We suggest that LOH studies can supplement the histopathological evaluation of cells to detect genotypic alterations in endoscopic biopsy specimens.

Acknowledgements. This work was supported by the grant FIS 98/794.

References

Bai S., Zhang X. and Wang W. (1997). Loss of heterozygosity on chromosome 13 in squamous cell carcinomas of the laruynx. Zhonghua Zhong Liu Za Zhi, 19, 35.37.

Bockmul U., Petersen I. and Dietel L. (1996). Allelverluste bei plattenepitel-karzinomen des larynx. Laryngorhinootologie 75, 48-62.

Calbelguenne A., blons H., de Waziers I., Carnot F., Hullier A.M., Soussi T., Brasnu D., Baune P., Laccourreye O. and Laurent-Puig P. (2000). p53 alterations predict tumor response to neoadjuvant chemotherapy in head and neck squamous cell carcinoma: a prospective series. J. Clin. Oncol. 18, 1465-1467.

Canzian F., Salovaara R., Hemminski A., Kristo P., Chadwick R.B., Altonen L.A. and de la Chapelle A. (1996). Semiutomated assessment of loss of heterozygosity and replication error in tumors. Cancer Res. 56, 3331-3337.

Frachiolla N.S., Pignataro L., Capaccio P., Trecca D., Boletini A., Ottaviani A., Polli E., Maiolo A.T. and Neri A. (1995). Multiple genetic lesions in laryngeal squamous cell carcinomas. Cancer 15, 1292-1301.

Gallo A., de Vicentiis M., Della Rocca C., Moi R., Simonelli M., Minni A. and Shaha A.R. (2001). Evolution of precancerous laryngeal lesions: a clinicopathologic study with long-term follow-up on 259 patients. Head Neck. 23, 42-47.

Gallo O., Santucci M. and Franchi A. (1997). Cumulative prognostic value of p16/CDKN2 and p53 oncoprotein expression in premalignant laryngeal lesions. J. Natl. Cancer Inst. 89, 1161-1163.

Golusinski W., Olofsson J., Szmeja Z., Szyfter K., Szyfter W., Biczysko W. and Hemminki K. (1997). Alteration of p53 gene structure and function in laryngeal squamous cell cancer. Eur. Arch. Otorhinolaryngol. Suppl. 1, S133-137.

González M.V., Pello M.F., López-Larrea C., Suarez C., Menendez M.J. and Coto E. (1995). Loss of heterozygosity and mutation analysis of the p16 (9p21) and p53 (17p13) genes in HNSCC. Clin.Cancer Refs. 1, 1043-1049.

Jares P., Fernandez P.L., Nadal A., Cazorla M., Hernandez L., Pinyol M. and Hernandez S. (1997). p16MTS1/CDK41 mutations and concomitant loss of Heterozygosity at 9p21-23 are frequent events in squamous cell carcinoma of the larynx. Oncogene 18, 1445-1453.

Jin X., Zhou L. and Zhao A. (2000). Mutants of p53 gene presence in laryngeal carcinoma and adjacent histopathologically normal tissue. ORL J. Otorhinolaryngol- Relat. Spec. 62, 140-142.

Kiaris X., Zhou L. and Zhao A. (2000). Mutants of p53 gene presence in laryngeal carcinoma and adjacent histopathologically normal tissue. ORL J. Otorhinolaryngol. Relat. Spec. 62, 140-142.

Kiaris H., Spanakis N., Ergazaki M., Sourvinos G. and Spandidos D.A. (1995). Loss of heterozygosity at 9p and 17q in human larngeal tumors. Cancer Lett. 20, 129-134.

Kohno H., Hiroshima K., Toyozaki T., Fujisawa T. and Ohwada H. (1999). p53 mutation and allelic loss of chromosome 3p, 9p of preneoplastic lesions in patients with non-small celllung carcinomas. Cancer 85, 341-347.

Ku J.L., Kim W.H., Lee J.H., Park H.S., Kim K.H., Sung M.W. and Park J.G. (1999). Establishment and characterization of human laryngeal squamous cell carcinoma cell lines. Laryngoscope 109, 976-982.

Mao L., Lee J.S., Kuri J.M., Fan Y.H., Lippman S.M., Lee J.J., Ro J.Y., Broxson A., Yu R., Morice R.C., Kemp B.L., Khuri F.R., Walsh G.L., Hittelman W.N. and Hong K.W. (1997). Clonal genetic alterations in the lungs of current and former smokers. J. Natl. Cancer Inst. 89, 857-862.

Mao L., Lee J.S., Fan Y.H. and Rojy J. (1996). Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer with risk assessment. Nat. Med. 2, 682-685.

Rizos E., Sourvios G. and Spandidos D.A. (1998). Loss of heterozygosity at 8p, 9p and 17q in laryngeal cytological specimens. Oral. Oncol. 34, 519-523.

Sanz-Ortega J., Sanz-Esponera J., Chuaqui R., Sobel M.E., Buck-Emmert M., Zuangping Z. and Merino M.J. (1996). LOH at the APC/MCC gene (5q21) in gastric cancer. Prognostic implications. Paholt. Res. Pract. 192, 1206-1210.

Sanz-Ortega J., Bryant B., Sanz-Esponera J., Balibrea J.L., Hernando F. and Merino M.J. (1999). LOH at the APC/MCC gene is frequent in early stages of NSCLC. Pathol. Res. Pract. 194, 677-680.

Sanz-Ortega J., Saez M.C., Sierra E., Torres A., Balibrea J.L., Hernando F., Sanz-Esponera J. and Merino M.J. (2001). 3p21, 5q21 and 9p21 allelic deletions are frequently found in normal bronchial cells adjacent to no-small-cell lung cancer while they are unusual in patients with no evidence of malignancy. J. Pathol. 194, 195-199.

Szyfter K., Szmeja Z., Szyfter W., Hemminki K., Banaszewski J. and Jaskula-Sztul R. (1999). Molecular and cellular alterations in tobacco smoke-associated larynx. Cancer Mutat Res. 30, 259-274.

Thiberville L. and Payne P. (1995). Evidence of cumulative gene losses with progression of premalignant epithelial lesions to carcinoma fo the bronchus. Cancer Res. 55, 5133-5139.

Van der Riet P., Nawroz H. and Hruban R.H. (1994). Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. Cancer Res. 54, 1156-1158.

Veltman J.A., Van Weert I., Aubele M., Bot F.J., Ramaekers F.C., Manni J.J., Hopman A.H. (2001). Speific steps in aneuploidization correlate with loss of Heterozygosity of 9p21, 17p13 and 18q21 in the progression of pre-malignant laryngeal lessions. Int. J. Cancer 91, 193-199.

Vogelstein B. and Kinzler K.W. (1993). The multisptep nature of cancer. Trends Genet. 9, 138-141.

Vokes E.E. (1993). Interactions of chemotherapy and radiation. Semin. Oncol. 20, 70-79.

Wistuba I.L., Lam S., Behrens C., Virmani A.K., Fong K.M., LeRiche J., Samet J.M., Srivastava S., Minna J.D. and Gazdar A.F. (1997). Molecular damage in the bronchial epithelium of current and former smokers. J. Natl. Cancer Inst. 89, 1366-1373.

Wu D., Fu W., Jiang L., Zhang X., Li F., Guo X. and Sun K. (1999). Loss of heterozygosity and microsatellite instability in laryngeal carcinoma near p16 gene. Zhonghua Yi, Xue Y. and Chuan Xue Za Zhi, 16, 99-102.

Xie M. (1997). Study of detecting p53 gene of laryngeal carcinoma of paraffin-embedded tissue sections by in situ polymerase chain reaction. Hunan Hi, Ke Da Xhue Xue Bao. 22, 459-461.

Accepted May 9, 2003