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17p13 (p53 locus), 5q21 (APC locus) and 9p21 (p16 locus) allelic deletions are frequently found in oral exfoliative cytology cells from smoker patients with non-small-cell lung cancer

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Summary. Molecular cytogenetic and LOH analyses of non-small cell lung cancer (NSCLC) have shown frequent allelic deletions in a variety of chromosomes where tumour suppressor genes are located. Allelic loss at 9p21 (p16 locus), 17p13 (p53) and 5q21(APC) has been frequently described in NSCLC and has also been described in premalignant epithelial lesions of the bronchus and normal bronchial cells. These findings suggest that a tissue field of somatic genetic alterations precedes the histopathological phenotypic changes of carcinoma. Similar changes have been described in oral and laryngeal epithelial tumours associated with smoke exposure. We previously reported frequent LOH at 5q21, 9p21 and TP53 in tumor cells and peritumoral normal bronchial cells from surgically resected NSCLC. We now analyze 96 cases of normal oral exfoliative cytology in which normal epithelial cells were obtained: 43 cases from smoker patients with NSCLC diagnosis, 33 smoker patients with no evidence of malignancy and 20 non-smoker patients with no evidence of tumour. All groups had a similar age and sex distribution. PCR amplification was performed utilising the specific markers D5S346, D9S157 and TP53. In normal oral mucosae cells from patients with NSCLC, we found that 21% of the informative cases showed LOH at any of the three analyzed loci distributed as follows: 14.3% of the informative cases showed LOH at 5q21, 7.7% at 9p21 and 22.2% at TP53. Within the smoker risk group only one case (4% of the informative cases) showed LOH at TP53, while no LOH was found at 5q21 or 9p21. No LOH was found in non-smokers. In conclusion, our results show that a significant number of patients with NSCLC have LOH at TP53, 5q21 and 9p21 in normal oral mucosae, while LOH at these loci is unusual in similar cells obtained from patients with no evidence of malignancy. Our study demonstrates that LOH studies can detect smoker patients with a mutated genotype in normal epithelial cells. Further prospective studies may confirm whether LOH studies can detect patients with a higher risk of NSCLC.

Key words: Carcinoma, Microdissection, Bronchogenic/genetics, Loss of heterozygosity

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Introduction

Non small cell lung cancer (NSCLC) is the leading cause of death in both women and men in the United States and many European countries (Ezzati and Lopez, 2003; Jemal et al., 2004). Molecular cytogenetic and LOH analyses of non-small cell lung cancer have shown somatic genetic alterations in a variety of chromosomes (Takahashi et al., 1989; Fong et al., 1995; Sanchez-Cespedes et al., 2001; Zochbauer-Muller et al., 2002). LOH of polymorphic DNA markers in tumors compared with normal tissue is a sign of somatic deletion. One major phenomenon underlying somatic deletion is the loss of tumor suppressor genes (TSG). Indeed, putative tumor suppressor genes such as p53, APC/MCC and p16 located in some of these loci have been found to be inactivated in a significant proportion of lung carcinomas (Endo et al., 1998; Thiagalingam et al., 2002; Zochbauer-Muller et al., 2002; Vineis et al., 2004). LOH studies show frequent allelic loss at 3p21, 5q21, 9p21 and 17p (TP53) chromosomal regions in NSCLC (Cooper et al., 1996; Mao et al., 1997; Zienolddiny et al., 2001).

Allelic loss at 9p21 and 5q21 has also been reported in premalignant epithelial lesions of the bronchus and in histologically normal bronchial cells (Thiberville et al., 1995; Cooper et al., 1996; Kohno et al., 1999). These findings suggest that a tissue field of somatic genetic alterations precedes the histopathological phenotypic changes of carcinoma. Multiple, sequentially occurring allele specific molecular changes commence in widely dispersed foci of histologically normal or mildly abnormal bronchial cells, early in the multistage pathogenesis of lung cancer. These somatic genetic changes continue to accumulate in histologically normal cells and appear to be more frequent in dysplasia and other precancerous conditions (Thiberville et al., 1995). Indeed, studies from the bronchial lavage of individuals who were referred with suspected lung cancer, found extensive and widespread allelic loss in confirmed lung cancer cases, as indicated by a high LOH score (Field et al., 1999; Kohno et al., 1999; Park et al., 1999; Powell et al., 2001). We also previously reported frequent LOH in normal bronchial cells from patients with NSCLC. The analysis of the peritumoral normal bronchial cells demonstrated that 52% of the smoker patients with NSCLC showed LOH at any of the 3 loci: 31.5% at 5q21, 23.5% at 9p21 and 23.5% at 17p13 (Sanz-Ortega et al., 2001). However, the detection of the LOH status in normal bronchial cells to detect patients with a higher risk of malignant transformation may be complicated from the practical and ethical points of view.

The aim of this study is to determine somatic genetic alterations in histologically normal, easy to obtain, oral mucosae cells. To clarify the role of LOH as a potential marker for individuals with a high risk of developing lung cancer we decided to compare the incidence of allelic deletions in smoker patients with NSCLC and smoker patients with no evidence of malignancy.

Materials and methods

Patients

We considered the WHO criteria to distinguish between smoker (more than 20 cigarettes per day during 10 years) and non-smoker individuals.

96 cytological smears (oral exfoliative cytology) from smoker patients with and without evidence of malignancy were selected. They were distributed in three groups: tumor group, risk group with no evidence of malignancy and an additional group with 20 non smoker patients. Patients ranged in age from 52 to73 years (median 63.7) in tumour group, from 53 to 78 (median 64.5) in the risk group and from 60-68 in the non-smokers group (median 63.2). Informed consent from the patient was obtained in every case for the research study. Superficial cell populations were separated with microdissection (Fig. 1) from benign cytological smears (oral exfoliative cytology). Constitutional DNA from each patient was obtained from blood samples with conventional protocols.

Cell populations from cytological smears were procured by microdissection with over 80% purity. Therefore, histological sections were selected in order to minimize (0-20%) the presence of stromal or inflammatory cells.

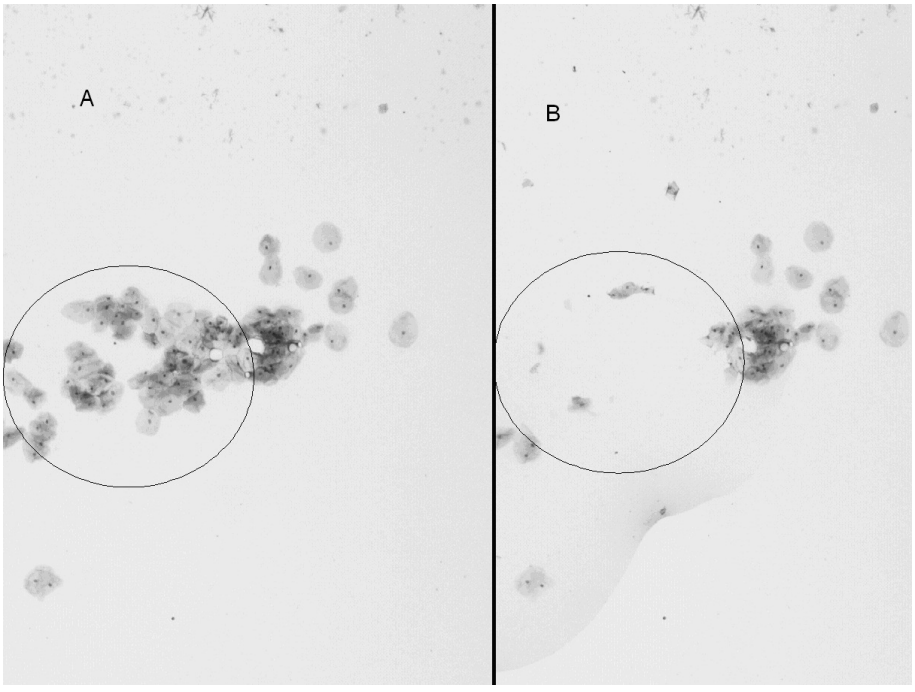

Fig. 1.A. shows a group of oral mucosae superficial cells selected for microdissection. **B.** shows the same area after microdissection.

DNA extraction from microdissected samples

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

PCR Analysis

Matched oral mucosae-normal cells cells were subjected to PCR analysis. Fluorescent labelled primers were obtained from Research Genetics (Huntsville, AL). Oligonucleotide primers flanking microsatellite polymorphisms at 5q21 (D5S346), 9p21 (D9S157) and 17p13(TP53) were used in the study. All PCR reactions were performed on the 2400 Thermal Cycler from PE Applied Biosystems. Each 10 μl 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 94° for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min. Water blanks were included in each PCR.

LOH analysis

1 μl of labelled 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°C 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 bronchial 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 semiautomated 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. 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 bronchial and tumor cells. The criterion for LOH was at least a 50% reduction of the lesional allele with the subsequent modification of the allele ratio (Fig. 2). 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 oral mucosae cells from smoker patients with NSCLC. Nine out of the 40 informative cases (21%) showed allelic deletions: 6 cases with LOH affecting only one locus and 3 cases with LOH on two chromosomal regions. LOH at 17p13 was found in 6 out of 27 informative cases (22.2%). LOH at 9p21 was found in 2 out of 26 informative cases (7.7%) and LOH at 5q21 was found in 4 out of 28 informative cases (9.3%).

Table 2 summarizes the results for oral mucosae

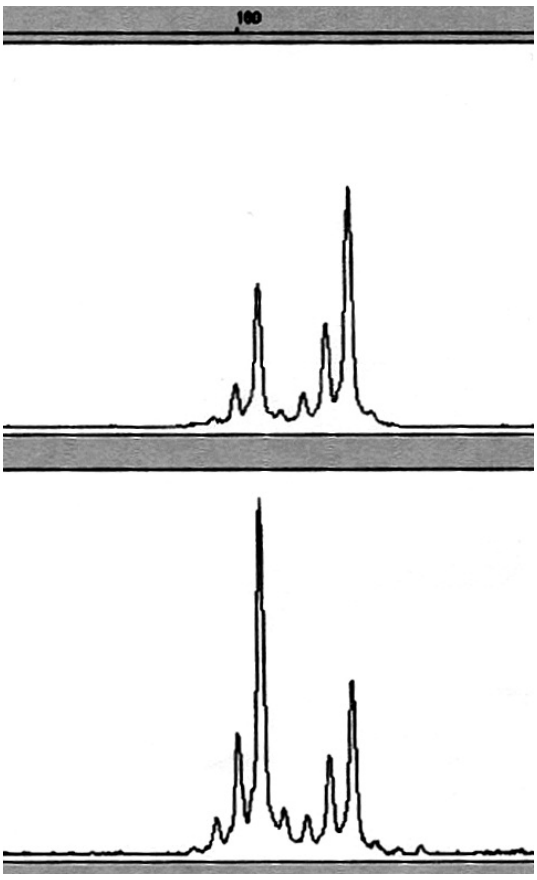

Fig. 2. The figure illustrates the semiautomated assessment of LOH. The peak heights in fluorescent units are shown on the Y axis on the left. Upper figure: amplification from oral mucosae cells. Lower figure: peripheral blood DNA from the same patient. Both samples exhibit the same heterozygous pattern with two alleles (162 and 170 bp). The decreasing peaks preceding each allele represent the characteristic "shadow bands" of microsatellite regions. The allele ratio was 0.6 for oral mucosae cell and 2.0 for normal DNA. There was a more than 50% decrease in the allele ratio of oral cells meaning that there was a loss of the 162 bp allele in that cell population.

Table 1. LOH in oral exfoliative cells from patients with lung cancer.

locus	Het	LOH	NI
17p13	21 (77.8%)	6 (22.2%)	16
9p21	24 (92.3%)	2 (7.7%)	17
5q21	24 (89.2%)	4 (10.8%)	15

Symbols of the LOH study: LOH, Het= heterozygosity, Ni= not informative due to homozygosity or not a clear heterozygous normal pattern.

Table 2. LOH in oral exfoliative cells from smokers with no evidence of lung cancer.

locus	Het	LOH	NI
17p13	24 (96%)	1 (4%)	8
9p21	22 (100%)	0	11
5q21	10 (100%)	0	15

Symbols of the LOH study: LOH, Het= heterozygosity, Ni= not informative due to homozygosity or not a clear heterozygous normal pattern.

from smoker patients with no evidences of malignancy. Only one case showed LOH at 17p13. LOH was not found at 9p21 or 5q21. LOH was not found at any loci in the group of non-smoker patients.

Discussion

Braakhuis et al. (2003) define the concept of field cancerization as areas of epithelial cells with somatic genetic changes and a clonal origin. These authors suggest that those areas may be considered as precancerous conditions similar to dysplastic lesions. Cytogenetic studies demonstrate that LOH is a frequent event in the multistep model of carcinogenesis (Waridel et al., 1997; Braakhuis et al., 2003). Pan et al. (2005), showed that LOH provides fingerprints for genetic heterogeneity in tobacco smoke-induced NSCLC.

In our series we analyse a few specific chromosomal regions where a high concentration of LOH can be determined. Three tumor suppressor genes (TSG) that play a mayor role in tumorigenesis are located within these regions: p16, p53 and APC genes. Allelic deletion is one of the most common mechanisms of TSG inactivation. The LOH status at these loci can be easily determined in a small population of cells. Determination of groups of cells that accumulate LOH at these loci indicate a mutated genotype and a progress through the multistep model of carcinogenesis .

Our data shows that LOH can be found both in oral mucosae (21% of the informative cases for any of the 3 analyzed markers) and bronchial (Sanz-Ortega et al, 2001) epithelium (52%) from patients with lung cancer. However, some slight differences between the genotype of oral epithelial cells and bronchial cells may take place. LOH at 5q21 appears to be more frequent in bronchial cells than in oral mucosae while LOH at 17p13 appears to be more frequent in oral epithelial cells (Tables 1,3). LOH at these loci is incidental in a group of smoker patients with a similar age and sex distribution but with no evidence of malignancy. These findings suggest that a mutated genotype is present throughout the respiratory tract with widespread allelic loss at chromosomal regions where important tumor suppressor genes are located .

In conclusion, our results indicate that a substantial proportion of cells in normal oral mucosae and histologically normal bronchial mucosae adjacent to lung cancer carry identifiable genetic alterations that are unusual in cells obtained from cytological smears from patients with no evidence of malignancy. Our data supports the field cancerization theory and we suggest that LOH studies can supplement the histopathological evaluation of oral or bronchial cells. Particularly, oral exfoliative cytology is easy to obtain and may be included in screening protocols to determine groups of patients with a higher risk of malignant transformation.

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