Oral mucosa and lung cancer: Are genetic changes in the oral epithelium associated with lung cancer?

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Abstract

Aim: To compare genetic aberrations in the oral epithelium of lung cancer patients with those without cancer.

Subjects and Methods: Buccal smears were performed to collect oral epithelium from each of the participants (smoker cancer patients n = 50, smoker control subjects n = 40, and nonsmoker control subjects n = 25). Cytogenetic changes in the samples were detected by micronuclei assay, whereas p53 and murine double minute 2 (MDM2) polymorphisms were genotyped using polymerase chain reaction-restriction fragment length polymorphism.

Results: p53 codon 72 polymorphism was seen in 44% of cancer patients versus 12.5% in smokers and 12% in nonsmokers of the control group. Similarly, MDM2 single nucleotide polymorphism 309 polymorphism was seen in 34% of patients with lung cancer as opposed to 12.5% of smokers (P = 0.038) and 8% of nonsmokers (P = 0.019) of the control group.

Conclusion: A higher proportion of individuals with lung cancer demonstrate genetic damage to oral mucosa compared to those without cancer.

Key words: Lung cancer, murine double minute 2, oral epithelium, p53, polymorphism

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Introduction

Long-term exposure to tobacco smoke that contains over 40 known carcinogens is by far the main contributor to lung cancer, although other risk factors (such as genetic tendency) have also been identified.¹,² In addition to its association with lung cancer, smoking is a primary risk factor for cancers of the oral, pharyngeal, and laryngeal mucosa.³ Inhaled smoke may cause similar changes in the lining epithelium through its journey from the oral cavity to the lungs. These changes produced in the lining epithelium may be associated with inhibition of tumor suppressor genes and activation of oncogenes.

Both p53 tumor suppressor gene and murine double minute 2 (MDM2) oncogene are crucial in carcinogenesis. The p53 is the most commonly mutated gene in most cancer types, including lung carcinomas. Studies have shown that p53 polymorphism at codon 72, which involves the substitution of an arginine (CGC) for proline (CCC) within the two alleles, is associated with increased lung cancer risk.⁴,⁵ The MDM2 inhibits p53 activity by promoting its degradation;⁶ hence, an overexpression of MDM2 increases the risk of tumor development.⁷ A single nucleotide
polymorphism (SNP) 309 in the promoter of MDM2 gene where T is substituted with G was found to be associated with enhanced MDM2 expression[9] and a higher risk of lung cancer.[9] As a result of such chromosome aberrations, the affected chromosome fragments are ejected from the nucleus.[9] Increased frequency of these chromosome fragments within cytoplasms (called micronuclei [MN]) has been shown to be associated with genetic defects and with a significant increase in cancer incidence.[10]

Collection of oral epithelial cells for cytogenetic and molecular biology studies as a pain-free, quick and noninvasive technique. As the oral mucosa is the most accessible region of the airway mucosa, its use as a surrogate for lung tissue to evaluate the mutagenic effects of inhaled cigarette smoke was proposed.[11] However, only a handful of studies were performed on the concept of analysis of oral mucosa identifying individuals predisposed to lung cancer.[12-14] The aim of this study was to compare genetic instability of oral epithelial cells collected from individuals who have lung cancer to those collected from individuals with no lung cancer assaying MN frequency and p53 and MDM2 gene polymorphisms. The null hypothesis of the study is that oral epithelial cells of smokers who have lung cancer are genotypically not different from those in smokers with no lung cancer.

Subjects and Methods

This is a cross-sectional, diagnostic case–control study conducted between January and October 2013. The study protocol was approved by the Ethics Committee of the Institutional Review Board of Suleyman Demirel University. The study was conducted in accordance with the Declaration of Helsinki guidelines and written informed consent for enrollment in the study was obtained from all participants.

Study population

A total of 52 consecutive lung cancer patients under the care of the Pulmonary Diseases Clinics of Suleyman Demirel University Research Hospital were screened for eligibility for recruitment to the study. Except for two subjects (one male and one female), all patients were (all male) current smokers at the time of diagnosis (n = 35) or former smokers (n = 15). All but two of the cancer patients gave up smoking after the diagnosis of lung cancer was established.

Age- and sex-matched volunteers were recruited from among those who sought treatment at the clinics of internal medicine and had not been diagnosed with lung cancer. Posteroanterior chest X-rays were obtained to exclude lung cancer. Individuals in the control group were divided into two subgroups as either smokers (n = 40) or nonsmokers (n = 25). Current (n = 25) and former (n = 15) smokers in the control group were matched with cancer patients in terms of cumulative cigarette smoking exposure.

Exclusion criteria were set as viral infection within the previous 2 weeks and exposure to any known carcinogens other than those related to tobacco.

Information on age, body mass index (BMI), history of cancer in the immediate family and in relatives, and exposure to passive smoking was recorded for each individual included in the study. In addition, the type of daily food consumption (intake of vegetables and fruits, alcohol, coffee, and tea) was also recorded using a questionnaire.

For current and former smokers, cumulative cigarette smoking exposure was determined in terms of pack-years by multiplying the number of years smoked with the average number of packs per day. For those with lung cancer, the histopathologic diagnosis, the stage of cancer, and the form of treatment were recorded.

Collection of samples

Epithelial cells were obtained from the buccal mucosa of both sides by swabbing, applying a firm manual pressure. Two sets of epithelial samples were collected. Epithelial cells for MN analysis were collected using a cotton swab, which was immediately placed into a tube containing 5 ml RPMI 1640 medium (Invitrogen-Life Technologies, CA, USA). Epithelial cells for analysis of nucleotide changes were collected using a cotton swab (Invitex, SalivaGene Buccal Swab Collection Set, Stratec Molecular, Berlin, Germany), which immediately after sample collection was placed into a collection tube (provided with the Buccal Swab Collection Set) containing DNA-preserving solution. The samples were then coded and sent to the molecular biology laboratory anonymously to enable analyses made in a blinded fashion.

Micronuclei analysis

Tubes containing cotton swabs were centrifuged (1000 rpm, 5 min) thereby creating a cell suspension. The cells were then washed 2 or 3 times by centrifugation at 1200 rpm for 10 min in the fixative solution (methanol/acetic acid, 3:1). Cell suspension was spread on a glass slide and allowed to air dry for 5–10 min. The slides were stained with 5% giemsa for 10 min and examined under a microscope. The frequency of cells that exhibit nuclear alterations (micronucleus, nucleus budding, and other nuclear changes) under microscope was recorded, and a total number of micronucleated cells per 1000 cells was calculated.

Genotype analysis

Genomic DNA was isolated from the epithelial cells collected from oral mucosa using a DNA isolation kit (Invitex, SalivaGene Buccal Swab Collection Set,
Stratec Molecular, Berlin, Germany). p53 codon 72 polymorphism (G→C variation) and MDM2 SNP309 polymorphism (T→G variation) were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism technique.

PCR analyses of the human p53 gene were performed with the isolated DNA samples. The primers correspond to the fourth exon of the gene were forward, 5'-GCCGTCCCAAAGGAATGGATGA-3' and reverse, 5'-CTGGGAAAGGACAGAAGTGAC-3'. Each PCR reaction mixture (50 µl) contained 200 ng of genomic DNA, 10 pmol of each primer, 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, 200 mM each dNTP, and 1 U Taq polymerase (Invitrogen-Life Technologies, CA, USA). The reaction involved 35 cycles of incubation at 94°C (30 s), 55°C (1 min), and 72°C (1 min). These gave rise to a 199 bp amplification product and followed by BstUI restriction enzyme (Thermo Scientific, MA, USA) digestion with 3 U of restriction enzyme at 37°C for 16 h. DNA fragments were submitted to electrophoresis through a 3% agarose gel and stained with ethidium bromide. The p53 Pro allele has a unique BstUI site that is absent in the Arg allele, resulting in bands of different sizes as follows: 113 bp, 86 bp for Arg/Arg, 199 bp, 113 bp, 86 bp for Arg/Pro, and 199 bp for Pro/Pro alleles.

Likewise, MDM2 promoter SNP309 was amplified as a 157 bp PCR product using the following primers: Forward, 5'-CGGGGATTCAGGGTAAAGG-3' and reverse, 5'-CTGAGTCAACCTGGCCACTG-3'. Each PCR reaction mixture (50 µl) was the same with p53. The reaction involved 30 cycles of incubation at 94°C (30 s), 60°C (1 min), and 72°C (1 min). Genotyping was then performed by digestion using 5 U restriction enzyme MspAI (New England Biolabs, Herts, UK) at 37°C for 4 h. The MDM2 promoter SNP309 polymorphic G allele has a unique MspAI site that is absent in the wild T allele resulting in bands of different sizes as follows: 110 bp, 47 bp for G/G; 157 bp for T/T; and 157 bp, 110 bp, and 47 bp for G/T alleles.

Subsequently, band pattern observed in agarose gel stained with ethidium bromide was controlled by DNA analysis. The frequency of GG, GC, and CC genotype; and allele for p53 protein and TT, TG, and GG genotype; and allele for MDM2 SNP309 was calculated.

Statistical analysis
All statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA). The mean occurrence of MN in every 1000 cells among the three groups was compared by Kruskal–Wallis test. Where there was a significant difference, pairwise comparisons were made using Mann–Whitney U-test. Independence of allele and genotype frequencies between cancer and control groups was tested using Chi-square test. Possible correlation of MN values with cumulative smoking exposure and age was analyzed with Spearman analysis. P < 0.05 was regarded as significant.

Results

Distribution of variables that belong to each group was provided in Table 1. The mean age of subjects in each group was comparable. In addition, cumulative cigarette smoking exposure was similar in cancer patients and smokers in the control group. Cancer patients had a lower BMI than those in the control group. Compared to the control group, a higher proportion of subjects in the cancer group were exposed to passive smoking, consumed alcohol, had a diet poor in fruits/vegetables, and had a positive family history of cancer. The distribution of variables related to cancer in lung cancer patients was provided in Table 2.

Micronuclei analysis
Figure 1 shows the mean (±standard deviation) number of multinucleated cells per 1000 epithelial cells in each group included in the study.
A representative image of a micronucleated cell is presented in Figure 2. The mean number of MN in 1000 cells collected from the oral mucosa of patients with lung cancer (15.1 ± 4.4) was greater than those collected from the oral mucosa of smokers (9.7 ± 4.4) and nonsmokers (7.3 ± 2.5) in the control group of individuals (P = 0.000 in both instance). Moreover, within the control group, the number of MN cells was greater in smokers than nonsmokers (P = 0.017).

While MN frequency was strongly correlated with pack-years (P = 0.000), it was not correlated with the age of the patients.

### Genotype analysis

#### p53 codon 72 aberration (G→C)

Table 3 shows genotype and allele distribution of p53 codon 72 in the three groups studied. The distribution of p53 codon 72 genotyping in individuals with lung cancer was significantly different compared to those in the control group (P = 0.005 for lung cancer vs. smoker control; P = 0.02 for lung cancer vs. nonsmoker control). Within the control group, however, the genotyping of p53 codon 72 was not statistically different between smokers and nonsmokers (P = 0.95). While 44% of lung cancer patients showed p53 codon 72 polymorphism (C/C) of the buccal cells, 12.5% of smokers, and 12% of nonsmokers in the control group exhibited cell polymorphism for p53 codon 72 (C/C).

#### Murine double minute 2 single nucleotide polymorphism 309 aberration (T→G)

The distribution of MDM2 SNP309 genotyping was significantly different in the cancer group compared to...
Previous studies demonstrated that the mean MN index of buccal mucosa collected from smokers was significantly higher than those collected from nonsmokers. In addition, an increased frequency of multinucleated oral epithelium was found in smokers compared to nonsmokers. This suggests a direct relationship between smoking status and cellular damage in the oral epithelium as assessed by MN assay.

In support of our results, Gabriel et al. reported that the degree of genomic methylation in the buccal cells of smokers and nonsmokers was not significantly different. Separate studies, on the other hand, demonstrated significant aberrations in gene expressions in smokers. Smith et al. reported that expression of 113 genes in the cells obtained from buccal mucosa significantly differed between smokers and nonsmokers. In addition, an increased frequency of polymorphism in smokers and nonsmokers was not significantly different.

The cytotoxic effects of inhaled smoke may exert comparable effects on the oral mucosa as well as bronchial mucosa. Bhutani et al. investigated whether molecular damage in the oral epithelium reflects molecular damage in the lungs. They compared promoter methylation status of the p16 and FHIT genes (two important tumor suppressor genes involved in early lung carcinogenesis) between cells collected from buccal epithelium and cells collected from various parts of bronchus of 127 chronic smokers. A strong association between genetic changes in the samples taken from oral and bronchial mucosa was found. For both genes, bronchial methylation index was higher in individuals who also showed oral mucosa methylation than those who had no methylation on the oral mucosa. To identify individuals predisposed to lung cancer, other means of molecular changes, i.e. antioxidant gene expressions in the oral epithelium were assessed. Spivack et al. examined epithelial cell samples taken from the buccal mucosa and from the lungs in subjects having been evaluated for lung cancer. They found a relationship between cells taken from the two sites with regard to expression of various carcinogen- or oxidant-metabolizing genes. However, the same group was unable to show that any gene expression pattern emerged as predictive of lung cancer. In another study, antioxidant enzyme transcript levels of buccal epithelium showed wide inter-individual expression and were unrelated to smoking status or cancer status.

In our study, we demonstrated that compared to those with no lung cancer, individuals with lung cancer had significantly higher frequency of multinucleated buccal cells and higher frequency of p53 and MDM2 polymorphism. Nevertheless, the sensitivity of these assays for detecting lung cancer was not significantly different.

Discussion

Tobacco smoking is a risk factor both for oral cancer and for lung cancer. An association between cancers in the oral cavity and in the lungs was reported. For instance, Keith et al. found that 3.5% of their patients with oral squamous cell carcinoma had coincident thoracic malignancy, whereas Erkal et al. reported that 7% of patients with cancer in the oropharyngeal/laryngeal site developed metachronous carcinomas of the lungs. Bronchoscopy was suggested in all patients with oral squamous cell carcinoma for detection of possible lung malignancy. In this study, we explored whether prediction of lung cancer in high-risk individuals (smokers) is possible by cytogenetic analysis of healthy-looking oral mucosa.

A direct relationship between smoking status and cellular damage in the oral epithelium as assessed by MN assay was reported. Previous studies demonstrated that the mean MN index of buccal mucosa collected from smokers was significantly higher than those collected from nonsmokers. In concordance, we found a higher frequency of multinucleated oral epithelium in smokers than nonsmokers. However, both of the genes investigated in this study showed a similar frequency of polymorphism in smokers and nonsmokers.

In our study, we demonstrated that compared to those with no lung cancer, individuals with lung cancer had significantly higher frequency of multinucleated buccal cells and higher frequency of p53 and MDM2 polymorphism. Nevertheless, the sensitivity of these assays for detecting lung cancer was not significantly different.
low. Of the cancer patients, only 44% and 34% showed polymorphism for p53 and MDM2, respectively, compared to 12.5% (for both genes) of smokers in the control group. Similarly, Bhutani et al.\(^\text{[12]}\) reported that 30% of the individuals with p16 promoter methylation in bronchial samples showed this methylation in oral samples; and 38% of the individuals with FHIT promoter methylation in bronchial samples showed this methylation in oral samples. Different susceptibility of the individual anatomical sites to the damaging effects of tobacco smoke may be attributable to our results. Compared to the oral cavity, the lungs appear to be more susceptible to the cytotoxic effects of smoke. The percentage of overall methylation of lungs was slightly higher than that in the oral site, indicating greater molecular damage in the lungs.\(^\text{[12]}\) The works conducted by Pyathilake et al. showed that squamous cell carcinoma of the oral cavity and of the lungs displays a different global DNA methylation pattern. Compared to uninvolved epithelium, whereas lung cancer showed hypomethylation,\(^\text{[126]}\) oral cancers showed hypermethylation.\(^\text{[127]}\) Sridhar et al.\(^\text{[128]}\) reported that although gene expression changes occurring in bronchial epithelium in response to cigarette smoke are also reflected in the buccal epithelium, the gene expression consequences of smoking were less pronounced in buccal mucosa. The low sensitivity of the assays used on buccal mucosa assays and wide intersubject variability in buccal mucosa gene expression may also partly be due to the partial degradation of the RNA in the buccal mucosa samples by high concentration of RNAses found in saliva.\(^\text{[28]}\)

This study holds a number of limitations. First, the study was controlled for gender, age, and cumulative smoke exposure (for current and former smokers), but controlling for all other potential confounding factors (such as dietary habits) which may affect genetic stability was not possible. Second, epithelial samples were taken only from buccal mucosa. Although the whole oral mucosa is covered by stratified squamous epithelium, the tissue shows different patterns in different regions. For instance, epithelium that covers the hard palate and the gingiva is keratinized, whereas the lining of the buccal mucosa and the floor of the mouth is nonkeratinized. These various anatomic sites of the oral mucosa may not exhibit the same degree of susceptibility to the carcinogens tobacco smoke contains. Rautava et al.\(^\text{[129]}\) reported that more than half of the squamous cell carcinomas of the mouth were originated from the lateral border of the tongue as opposed to 4% originated from the hard palate. Therefore, other parts of the oral cavity such as the hard palate and the floor of the mouth should be included in future studies to comparably identify genetic alterations.

Taken all together, our findings are the first in the literature showing that oral epithelia of individuals with lung cancer demonstrate a higher frequency of genetic damage compared to those individuals with no lung cancer. However, the MN and genotype assays, we employed in this study have a low predictive value for lung cancer. Validity and reliability of various other genetic assays on oral epithelium as a screening tool to identify increased risk of lung cancer remain to be investigated.

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Conflicts of interest
There are no conflicts of interest.

References
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