ABSTRACT

Introduction: This study reports the changes in serum total antioxidant status and tissue levels of oxidatively modified deoxiribonucleic acid (DNA) lesion (8-hydroxydeoxyguanosine [8-OHdG]) and 8-oxoguanine DNA glycosylase 1 (OGG1) in oral squamous cell carcinoma (OSCC).

Materials and methods: Oral squamous cell carcinoma patients (n = 40) comprising of 80% pathologically well-differentiated and moderately differentiated and 20% poorly differentiated tumors were taken. Besides, blood samples and gingival biopsies (n = 35) from normal individuals were processed accordingly and considered as control. Plasma antioxidant capacity was estimated using ferric reducing antioxidant power (FRAP) assay. Tissue biopsies were processed for ribonucleic acid (RNA) expression of OGG1-specific messenger RNA (mRNA), and DNA samples were isolated for estimation of 8-OHdG levels using enzyme-linked immunosorbent assay.

Results: Results showed that the total antioxidant capacity (TAC) of plasma in OSCC patients was significantly depleted (reduced to about 25%) compared with normal individuals. 8-Oxoguanine DNA glycosylase 1 expression at mRNA levels showed that there is a twofold increase in OGG1 expression in oral cancer tissues compared with normal samples. The 8-OHdG formation in DNA showed that in DNA isolated from tumors, 8-OHdG was also increased (four- to fivefold; p = 0.03) compared with normal samples. However, there was a significant decline in TAC of plasma in one-third of OSCC patients compared with normal individuals.

Conclusion: Overall results suggest that 8-OHdG level and OGG1 expression in OSCC patients are highly associated (r = 0.8; p = 0.001). A decline in FRAP in patients may also suggest that antioxidant factors play a major role in attenuation of DNA damage-repair system leading to progression of OSCC.

Keywords: Antioxidant, Deoxyribonucleic acid repair/damage, Iran, Oral cavity cancer.


Source of support: This study was supported by Tehran University of Medical Sciences and health services Grant Number 92-02-51-23399.

Conflict of interest: None

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies and more than 400,000 of new cases of this type of cancer are diagnosed annually worldwide. Oral cavity cancer has a geographical predilection, the incidence of which is higher in Southeast Asia and Brazil. The risk habits of this type of cancer in these regions are believed to be due to habitual use of chewing tobacco and betel nut. Other etiological factors are alcohol drinking and infection with viruses, particularly human papilloma virus types 16 and 18 that are involved in genital cancers.

An epidemiological study of oral cavity cancers in Iran showed that tongue cancer in nonsmokers is the predominant cancer of oral cavity. The cause of oral cavity cancer among patients who are nonsmokers and are not habituated to alcohol drinking and tobacco/betel nut chewing, other factors may be involved.

Regardless of the risk factors/habits in causation of OSCC, generation of free radicals and oxidative damage to deoxyribonucleic acid (DNA) in the saliva and tissue of these patients is probably common to all the cases. Generation of free radicals, such as reactive oxygen and nitrogen species, by inducing oxidative and nitrative stress plays an important role in causation of OSCC. Factors, such as smoking, alcohol, food, and other volatile sources which enter freely into the oral cavity through the upper aerodigestive tract are major sources of free radicals. The oxidative stress is often associated with the suppression of the antioxidant factors.

Very recently, we reported induced expression of cyclooxygenase (COX)-2 in OSCC tissues from Iranian
patients, which was correlated with H-ras expression, suggesting that together these genes are contributing to cancer progression. Moreover, it was demonstrated that COX-2 is induced in response to proinflammatory factors, such as smoking.\(^8\)

The imbalance of the antioxidant–oxidative stress can contribute to the genome instability and DNA damages. The risk of cancer development and progression relies on the rate of mutations accumulated in critical genes during different stages of cancer.\(^8\) According to Zanaruddin et al.,\(^1\) common oncogenic mutations which are frequently detected in common solid tumors are infrequent in OSCC.\(^9\)

Deoxyribonucleic acid damaging agents, namely sunlight and tobacco smoke, and the efficiency of cellular DNA damage-repair system are major factors of contribution to carcinogenesis.\(^9\) In other words, exposure to a carcinogen in predisposed individuals with defected DNA repair system is important in persistence of DNA mutations. Deoxyribonucleic acid sequence might have a significant role in the etiology of human cancer.\(^9\)–\(^11\) The importance of DNA repair in cancer pathogenesis became clear when it was discovered that several of the mutated genes responsible for hereditary cancer predisposition syndromes encode DNA-repair proteins.\(^12\)

According to Sarasin and Kauffmann,\(^13\) the genetic instability is absolutely necessary to go from normal cells to malignant cells, but one needs some type of genetic stabilization, which can be obtained by overexpressing specific DNA repair genes, in order to produce primary tumor cells that are genetically stable enough to be able to invade and give rise to distant metastasis. Previous studies carried out on Iranian cases showed that increased levels of 8-hydroxydeoxyguanosine (8-OHdG) in saliva samples of OSCC patients were associated with elevation in lipid peroxidation products (malondialdehyde assay).\(^14\) In the repair process, human 8-oxoguanine DNA glycosylase (OGG)\(^1\) plays an important role in removing 8-OHdG from the DNA strand in order to maintain genomic integrity before subsequent replication.\(^15\)–\(^17\) 8-Oxoguanine DNA glycosylase 1 by its glycosylase activity removes 8-OHdG in DNA and further excises a phosphodiester bond at 3’ end of the apurinic site by apurinic lyase activity via a β-elimination reaction.\(^18\)–\(^20\)

### Purpose of Study

The risk factors/habits associated with the pathogenesis of oral cavity cancers in Iranian patients are not well understood. Moreover, the relationship between the risk factors and changes in molecular markers, such as DNA damage and repair system has not been investigated. In the present study, the changes in DNA damage marker and the OGG1 as a DNA repair enzyme as well as antioxidant status in OSCC patients have been compared with normal individuals. Moreover, these changes were evaluated in terms of the type and the tumor stage of oral cavity cancers.

### MATERIALS AND METHODS

#### Patients and Samples

In this study, 40 patients diagnosed with oral cancer in different tissues of the oral cavity were selected. All the patients underwent surgery at the Surgery Department of the Cancer Institute, Imam Khomeini General Hospital, Tehran, Islamic Republic of Iran. The mean age of the patients was 58.9 ± 13.9 (25 males and 15 females). The biopsies comprised 40 tissue biopsies obtained from different parts of the oral cavity, such as tongue, lips, palate, cheeks, and gum.

Based on histological observation, the tumor stage was categorized based on tumor differentiation as well differentiated (WD), moderately differentiated (MD), and poorly differentiated (PD). The number of samples in WD, MD, and PD was 47.5, 32.5, and 20% respectively.

As shown in Figure 1, tongue cancer was the most common cancer among the samples with oral cavity cancer. The rate of tongue cancer was found to be 40% (16/40), and palate is the least common with 5% (2/40) of the cases. The ratio of smokers and nonsmokers in patients was 40/60, whereas in control samples, smokers comprised about 28% of samples.

All the patients were histologically confirmed cases by pathologist as OSCC by hematoxylin and eosin (H&E) staining. Each tissue biopsy (40–50 mg; 10 × 5 mm) was immediately kept in a small tube containing 5 mL of RNAlater buffer and transferred to laboratory. Besides, blood sample from each individual was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes; plasma was separated and kept in freezer for further use.

The patients received no chemotherapy and/or radiotherapy. This study was approved by the Medical Ethics Committee of the Cancer Research Institute and a written consent was obtained from each patient before collection of tissue biopsies and blood sample.

The inclusion criteria of patients in this study were that patients were diagnosed with oral cancer and referred to the surgery department for surgical operation. A group of age- and sex-matched individuals comprising 35 individuals referred to the dental clinics of the Faculty of Dentistry for dental surgery were selected and considered as control samples. These individuals had no sign of infection and chronic diseases. The age of the patients was 48.2 ± 12.9 (18 males and 17 females) years.

---

72
Plasma Total Antioxidant (Ferric-reducing Antioxidant Power [FRAP]) Assay.

Plasma samples from patients and controls were used for determination of total antioxidant capacity (TAC). The assay was performed based on procedure of Benzie and Strain. This assay was modified by scaling down in a microtiter 96-well plate using only 40 μL of plasma. Briefly, 260 μL of working solution was added to each well, then 40 μL of plasma sample was added and vortexed. Thereafter, the plate was incubated for 10 minutes at 37°C before recording the absorbance at wavelength of 595 nm using an enzyme-linked immunosorbent assay (ELISA) reader. The FRAP levels was calculated using the standard curve which was obtained by assaying different concentrations of Fe2+ concentrations. The assay was carried out in duplicate and FRAP is expressed as μmol/L plasma.

Deoxyribonucleic Acid Extraction and Detection of 8-OHdG in DNA Samples

One portion of the tissue biopsy was immediately placed into RNA later for 24 hours before storing at 70°C. The genomic DNA was extracted from tumor biopsies and nontumor tissue using standard method of proteinase K digestion and phenol chloroform extraction procedure. Briefly, tissue biopsy was transferred to 1.5-mL microfuge tubes and homogenization in 400 μL DNA extraction buffer (10 mm NaCl, 20 mm Tris–HCl, 1 mm EDTA, pH 8.0), 50 μL sodium dodecyl sulfate (10% and 25 μL proteinase K (10 mg/mL) was added and gently mixed and incubated for 2 hours at 55°C. Thereafter, 400 μL DNA extraction mixture (phenol:chloroform:isoamyl alcohol; 25:24:1, v/v) was added and mixed. The mixture was centrifuged for 5 minutes at 7000 g, supernatant was separated and transferred to a new microfuge tube. Sodium acetate 3M (50 μL) and cold isopropanol (700 μL) were added, with gentle mixing and centrifugation for 3 minutes at 12,000 g at 4°C. The supernatant was separated and DNA was precipitated by adding 700 μL of cold ethanol (70%). After centrifugation, the DNA pellet was dried by dissolving in 100 μL distilled water. The concentration of genomic DNA in the final eluent was determined using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

An aliquot of the isolated DNA sample was used for estimation of 8-OHdG using HT 8-OHdG ELISA Kit II (Trevigen, Cat. No. 4380-096). The ELISA-based kit detects 8-OHdG by 8-OHdG monoclonal antibody and the immobilized 8-OHdG are detected with a secondary antibody, i.e., horseradish peroxidase conjugate. The assay is developed with tetramethylbenzidine substrate and the absorbance is measured in a microplate reader at 450 nm. The intensity of the yellow color was inversely related to the concentration of 8-OHdG. Then the absorption was recorded at 450 nm using ELISA reader. The level of 8-OHdG was calculated according to the software provided in the assay kit.

Reverse transcriptase-Polymerase Chain reaction Analysis of the OGG1 Transcripts

Total ribonucleic acid (RNA) was prepared from tissue biopsies collected from OSCC patients and gum biopsies from normal individuals by the GeneAll® Hybrid-R™ RNA extraction kit (GeneAll Biotechnology, Republic of Korea, Cat. No. 305-101), according to the manufacturer’s instruction. Total RNA levels in the final eluent were determined using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Then the first-strand complementary DNA (cDNA) was synthesized with 2 μg of DNA-free RNA and oligo (dT) primer using the HyperScript™ RT master mix, first-strand cDNA Synthesis
Kit (GeneAll Biotechnology, Republic of Korea, Cat. No. 601-725) according to the manufacturer’s instructions. The gene expression was performed using the reverse transcriptase polymerase chain reaction (RT-PCR) technique, in which the expression of these messenger RNA (mRNA) of OGG1 was compared with the expression of the mRNA of hypoxanthine phosphoribosyltransferase 1 (HPRT1), a housekeeping gene. For this, approximately 2 mg of total cDNA was used for the RT-PCR, in a 20 μL reaction mixture comprised of 5 pmol of forward and reverse primers suitable for OGG1 and HPRT1 genes, 10 μL of 2× Taq DNA Polymerase Master Mix RED (Ampliqon, Herlev, Denmark). The sequences of the OGG1 primers were: Forward: 5′-CTCCAACAACACATCGCCC-3′ and reverse: 5′-GAGGACTCTCGTAGCTGCTG-3′ and the sequences of the HPRT1 primers were: Forward: 5′-CCTGGCGTCGTGATTAGTG-3′ and reverse: 5′-TCAGTCCTGTCCATAATTAGTCC-3′. The fragment of the OGG1 and HPRT1 genes amplified 210 and 125 bp respectively. The PCR procedure involved preheating at 95°C for 5 minutes, followed by 30 cycles of amplification in a thermocycler (MyCycler, Biorad, USA). Each cycle included a denaturation step at 95°C for 30 seconds, primer annealing step at 58°C for 40 seconds, and chain elongation step at 72°C for 60 seconds, followed by a final elongation step at 72°C for 10 minutes. The PCR products were separated on a 2% agarose gel and visualized by staining using GelRed. The measured intensities of the bands were corrected to the HPRT1 level in each lane and subjected to statistical analyses. The relative mRNA expression of OGG1 in the OSCC was normalized by dividing each value by that of healthy control subjects as a calibrator.

Statistical Analysis

The results of FRAP assay were analyzed by one-way analysis of variance (ANOVA) test. Comparison of the oxidized base 8-OHdG in DNA samples obtained from patients and normal tissues was done by Mann–Whitney test using nonparametric Chi-square test. The results of OGG1 expression by RT-PCR were analyzed by one-way ANOVA test. Correlation between the two variables was estimated by Pearson’s correlation coefficient; p < 0.05 was considered significant.

RESULTS

In this study, 40 tissue biopsies from patients diagnosed as OSCC and 35 gingival biopsies from individuals who had dental surgery with no sign and symptoms of infectious diseases were analyzed. The patients were diagnosed as OSCC in different parts of the oral cavity based on the histopathological examinations. The samples were further categorized according to the grade of the tumor as PD, MD, and WD. The histogram of the representative samples are presented in Figure 1.

Comparison of OGG1 Expression in Malignant and Normal Tissues

As shown in Graph 1, the expression of OGG1 at mRNA levels was significantly increased (p < 0.05) in biopsies of malignant tumors compared with that measured in normal samples. Based on the semi-quantitative RT-PCR, there was a 1.7-fold increase in OGG1 expression in malignant tumors. There was no significant difference in OGG1 expression among the samples with different tumor grades (WD, MD, and PD). Gene expression was done by semi-quantitative RT-PCR. The PCR products were electrophoresed on 2.0% agarose gel and visualized by staining using GelRed. The measured intensities of the bands were corrected to the HPRT1 level in each lane and subjected to statistical analyses. The relative mRNA expression of OGG1 in the cancer biopsies were normalized by dividing each value by that of healthy control subjects as a calibrator. Results are presented as mean ± standard error of mean.

Detection of 8-OHdG in DNA Samples

The level of 8-OHdG measured in DNA samples from malignant tumors and normal biopsies showed that 8-OHdG concentration in DNA samples isolated from normal tissues, malignant tumors was significantly increased (p < 0.05; four- to fivefold) in malignant tumors compared with that measured in normal samples (normal; 5.52 ± 0.85 ng/mL and tumors 23.9 ± 1.2 ng/mL) (Graph 2). Comparative analysis of the data showed that the difference in 8-OHdG levels in different tumor grades was not
significant. There was a significant increase (p < 0.05; four- to fivefold) in tissue levels of 8-OHdG in DNA samples prepared from tumors when compared with samples isolated from normal gingival tissues. The 8-OHdG in DNA samples was measured based on ELISA technique as described in the Methods section. The 8-OHdG is expressed as ng/mL DNA. Results are presented as mean ± standard error of mean.

**Plasma Total Antioxidant Levels (FRAP Assay)**

The plasma levels of TAC (FRAP assay) in normal individuals and patients diagnosed with OSCC were found to be 995.60 ± 88.6 and 228 ± 22.7 μM respectively. As shown in Graph 3, the difference in FRAP levels between controls and cases was statistically significant (p < 0.05).

Details about the blood samples and plasma preparation are as described under Methods section. The assay was performed in ELISA plates and FRAP level was calculated using a standard curve which was obtained by assaying different concentrations of Fe²⁺. Each sample was done in duplicate and FRAP is expressed as μmol/L plasma. Results are presented as the mean ± standard error of mean.

As shown in Graph 4A, the relationship between these parameters in patients revealed that total plasma antioxidant was inversely related with the 8-OHdG levels in tissues with r-value of −0.65 (p = 0.0009). The OGG1 expression was also inversely related to the FRAP concentration in patients (r-value 0.54; p = 0.034) (Graph 4C). Graph 4B shows that there is a good correlation between 8-OHdG and OGG1 expression in tumors obtained from oral cavity cancers (r-value = 0.8; p = 0.001).

**DISCUSSION**

In this study, it was demonstrated that the rate of the DNA damage marker, i.e., 8-OHdG together with the expression of OGG1, a DNA damage-repair marker, is increased in OSCC patients. Increase in DNA damage-repair factors was associated with attenuation of total plasma antioxidant status in the patients suffering from OSCC.

Demographic information showed that this type of cancer in Iran is more common in male (62.5%) compared with the female patients, which were 37.5% of the cases. Based on pathological observations, about two-thirds (80%) of the tumor biopsies were WD/MD types, suggesting that oral cancer is diagnosed at late stages. The risk factors/habits showed that smokers comprised about 40% of the cases. About 60% of the patients were nonsmokers and the cancer originated in tongue was more common in these patients. Among the patients with OSCC, 40% suffered from tongue cancer. Independent reports from Iran show tongue cancer comprised more than 50% of the oral cavity cancers in Iran. This figure was more than 53% in a study carried out in Kerman city (Andisheh-Tadbir et al), whereas a report from Tehran showed that cancer of the tongue tissue comprises 69.9%.

Interestingly, the total antioxidant status (FRAP assay) measured in serum samples was significantly affected in patients compared with a group of normal individuals. The correlation studies analysis presented in Graph 4B showed that in the OSCC patients the level of 8-OHdG is inversely related to the serum levels of TAC (r = −0.65; p = 0.009). Likewise, there was an inverse relationship between total antioxidant levels of plasma and the DNA repair enzyme, i.e., OGG-1 with r-value of 0.3 (p = 0.034) (Graph 4C). The changes in 8-OHdG in OSCC expression patients were highly correlated (r = 80; p = 0.001).

Our results on increased formation of DNA 8-OHdG in oral cavity cancers are in agreement with reports from other laboratories. A four- to fivefold increase in 8-OHdG in tumor tissues compared with normal tissue
(gum tissue from normal individuals) can be due to the multiple genetic alterations during tumor progression and metastasis. According to Paz-Elizur et al, a decrease in 8-OHdG expression is a risk factor of head and neck cancers. On the contrary, there are reports showing the overexpression of 8-OHdG in colorectal carcinoma and melanoma. In most cases, increase in 8-OHdG has been assigned to the metastatic condition of cancers.

A report from Iran shows that lipid peroxidation and 8-OHdG is elevated in saliva samples of OSCC. Increased expression of 8-OHdG in oral cavity cancer compared with normal tissues (~twofold; \( p < 0.05 \)) observed in the present study further confirms the genetic instability and DNA damage in these cases.

The specificity of different DNA repair pathways and genes associated with this system to different tumor types is not well understood. The variations in the DNA repair factors, particularly OGG1, in different tumor types further attest to this finding.

It has been reported that the expression of defense genes increases in adenoma and carcinoma cases of colorectal cancers (CRCs) compared with normal tissues, suggesting that the defense genes are involved in early event of the progression of CRC.

In this study, it was observed that on the one hand there is significant increase in the 8-OHdG levels in DNA of OSCC; on the contrary, the DNA repair gene, i.e., OGG1, is increased in these tissues [DNA repair gene expression (OGG1) is increased]. This finding may suggest that the changes in DNA repair factors depend on the cancer type and progression. In the present study, we showed that the OGG1 expression in oral cavity cancers is a twofold higher tumor tissues compared with control samples \( (p = 0.001) \). However, OGG1 expression was not different among the samples with different pathological grades. Likewise, the oxidized DNA lesion (8-OHdG) as well as plasma antioxidant activity was unaffected by differentiation status of tumors.

These findings suggest that although there is a significant difference in these markers in cancer patients and normal samples, there was no significant difference among the samples collected from patients with different pathological grades of cancer.

**Graphs 4A to C: Correlation coefficient analysis between different parameters measured in oral cancer patients**

(A) Correlation between FRAP and 8-OHdG levels in DNA prepared from tumor tissue; (B) Correlation of OGG1 expression in tumors and 8-OHdG levels; and (C) Correlation between FRAP and OGG1 expression.
Increase in 8-OHdG and OGG1 in the patients’ samples can be explained by showing that most of the tumors in the present study were identified as stage III/IV. However, generally, alterations in the DNA repair system during tumor progress can be explained by the tumor stage/gene as reported by others. It appears that the DNA repair-damage factors are initially suppressed in response to the genetic alterations, whereas expression of these factors is increased in compensation of the multiple genetic and cellular lesions in the tumor.26,27

These data together with our recent work on the correlation between oncogene expression (H-ras) and proinflammatory factors in tumor biopsies from OSCC8 may suggest that environmental/inflammatory factors, such as smoking and alcohol drinking by inducing inflammatory reactions, can contribute to induction of cancer signaling pathways. A positive correlation between DNA damage marker (8-OHdG) and DNA repair enzyme (OGG1) may suggest that the DNA repair system is induced for the compensation of the damages to DNA.

ACKNOWLEDGMENTS

Authors would like to thank the Surgical Department of Imam Khomeini Hospital, Tehran, Iran for providing oral cancer specimens and Dental School for providing normal gum biopsies. Azin Hamidavi and Abdolkarim Moazen-Roodi are graduate students of Tarbiat Modares University.

REFERENCES


10. Roodi are graduate students of Tarbiat Modares University.


