

Immunocytochemical Study of p53 Protein in Exfoliated Cells of Oral Mucosa in Patients With Type 2 Diabetes

Zahra Heidari^{1,2}; Hamidreza Mahmoudzade Sagheb^{1,2}; Azam Asemi Rad^{2,*}; Mahmoud Ali Keikhaee³

¹Non-Communicable Disease Research Center, Zahedan University of Medical Sciences, Zahedan, IR Iran

²Department of Histology, Zahedan University of Medical Sciences, Zahedan, IR Iran

³Department of Internal Medicine, Zahedan University of Medical Sciences, Zahedan, IR Iran

*Corresponding author: Azam Asemi Rad, Department of Histology, Zahedan University of Medical Sciences, Zahedan, IR Iran. Tel: +98-9153490301, E-mail: az_arad@yahoo.com

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Background: There is a potential risk for malignancy in patients with diabetes mellitus. Theoretically, a noninvasive method for detection of precancerous changes can be conducted on oral exfoliated cell smears.

Objectives: In this study, the immunocytochemistry of p53 in exfoliated oral mucosal cells in patients with type 2 diabetes was investigated.

Patients and Methods: Oral mucosal smears were prepared from 50 patients with type 2 diabetes (D) and 50 healthy participants (C). The immunocytochemistry of p53 was assessed according to the kit instructions on oral smears. The Mann-Whitney nonparametric test was used to compare the p53 expression in D and C groups.

Results: The results showed a significant increase in p53 expression in exfoliated cells of oral mucosa in D group compared with C group ($P < 0.05$). There was a significant correlation between the intensity of p53 expression and level of fasting blood sugar (FBS) ($r = 0.816$, $P < 0.001$), as well as between the percentage of p53 positive cells and FBS. The nucleus area (NA) and the cytoplasm to nuclear ratio (CNR) had significant correlations with p53 expression ($r = 0.272$, $P = 0.08$) NA, and CNR ($r = -0.434$, $P = 0.005$).

Conclusions: This study showed that there was a significant expression of p53 in oral mucosal exfoliated cells of group D patients compared with C group. It seems that this noninvasive technique could be useful to detect premalignancy changes of oral mucosa as well as for early detection of malignancies.

Keywords: Diabetes Mellitus; Immunocytochemistry; Buccal Mucosa; p53 Genes

1. Background

Diabetes mellitus is one of the most common endocrine and metabolic diseases; its prevalence is increasing worldwide (1). Oral disorders in patients with diabetes including infection (2, 3), gingivitis, periodontal disease (2-5), increment of severe candidiasis incidence (6), apical root abscess of the tooth, burning mouth syndrome (7), impairment of taste, increment of cell and proliferation of pathogenic microorganisms, incidence of coated tongue, halitosis, cytoplasmic vacuoles, double nuclear and many other cellular lesions caused by hyperglycemia were reported in patients with diabetes (8-10). Sores of oral squamous epithelium and gingivitis are the most common symptoms in patients with diabetes (7). In healthy subjects, the epithelium of oral mucosa creates a protective barrier against carcinogens. Progressive atrophy of oral mucosa in patients with diabetes due to a reduction of pH and the salivary flow can cause reduction of this protective barrier and may increase the risk of malignancy in these patients (1). Associated with malignant disease, the relationship between diabetes, leukoplakia, erythroplakia and lichen planus is considered (2, 11-13). Ahmed et al. reported that the

prevalence of oral lichen planus in patients with diabetes was 6.9% (14). Ujjal et al. showed that the frequency of benign tumors and the precancerous lesions of oral mucosa were 14.5 % and 8% respectively in patients with diabetes (4). It seems that the assessment of cell markers in exfoliative oral cells could be a simple technique in early diagnosis of oral malignant lesions and evaluation of its extent in the route of malignancy treatment (15).

Using the immunocytochemical techniques in evaluation of expression of various proteins in oral lesions and tumors can be very useful (16). A few studies have been conducted about immunocytochemistry on oral exfoliative cell. Using immunohistochemistry on smear of a normal persons, as well as those of cases with benign and moderate dysplasia, Scott et al. showed that minichromosome maintenance proteins (MCM) expression was negative in all the cases (17). Brunotto et al. studied cytomorphometric evaluation of oral smears of patients with lichen planus and squamous cell carcinoma lesions using Bcl-2, p21, Ck14 and p53 as markers. Their results showed that p53 and Bcl-2 were expressed in 45% and 55%

of the samples, respectively (18).

p53 is a tumor suppressor gene located on the short arm of chromosome 17 and its product is a DNA-binding phosphor-nucleoprotein that prevents the progression of cell division from G1 to S (19). This protein is a product of the most common mutated gene in cancers, particularly cancers of oral cavity; it is a tumor suppressor gene (20). Mutations in this gene are very common and can cause a stable protein which does not prevent mitosis and accumulates in the cell nucleus. Using immunohistochemical staining, it is detected in many tumors (19). Increased expression of p53 could be a sign of deactivation of apoptosis, and because of this, the p53 mutation is important in causing cancer, which is frequently seen in various neoplasm such as squamous cell carcinoma (21). Accompanied with the mutation in p53, protein products with long half-lives (4-8 hours) are created which accumulate enough in the cell nucleus and get stained well (22).

2. Objectives

In this study for the first time, we tried to modify the immunohistochemistry technique for detection of p53 protein in smear of the exfoliated oral mucosal cells and then compare its expression in patients with type 2 diabetes and healthy controls to use this noninvasive technique for early detection of oral premalignant and malignant changes caused by diabetes.

3. Patients and Methods

In this case-control study, 50 patients with type 2 diabetes (22 males and 28 females) and 50 healthy controls (23 males and 27 females) were enrolled. Sampling was performed by convenience probability method. The groups were matched in terms of age and gender. The members of the patients group were selected from the patients who referred to the diabetes clinic of Ali Asghar Hospital, Zahedan for their routine checkups. Members of the control group were selected from the patients' fellows. Their nondiabetic states were approved following fasting blood sugar (FBS) test. Subjects with a history of diabetes for 2-8 years were considered as the case group. The inclusion criterion was defined as type 2 diabetes for at least six months from the date of diagnosis. The exclusion criteria were consuming tobacco, alcohol or nondiabetes-related drugs, anemia, any severe oral infection, buccal cancer or other malignancies, systemic diseases such as kidney failure, liver diseases, rheumatic diseases, pregnancy and menopause.

After confirmation of the disease occurrence by an endocrinologist, ethical approvals for recruitment were obtained from the Ethics Committee of Zahedan University of Medical Sciences (No. 90-1761) and written informed consents were taken from all the patients as well as the healthy individuals. The participants' information was registered in prepared data sheets. Three slides from the buccal mucosa of all the participants were prepared and

the immunocytochemistry technique was performed.

3.1. Immunocytochemistry Techniques

Each smear was spread on a prepared glass, clean and dry slide which had previously been numbered and was immediately sprayed by Pathofix (Padtan Company, Iran) from a distance of 25 cm and with a pressure of 2 bar max. The samples were rehydrated by descending alcohol and placed in citrate buffer (pH = 6). After washing with Tris-buffered saline (TBS), the samples were exposed to peroxidase, protein blocker, primary antibody, secondary antibody, and polymer, and then washed with TBS buffer after each stage. At the end, they were stained with chromogen and hematoxylin. The slides were evaluated microscopically for the intensity of p53 expression afterwards. The positive control was a slide of colon tumor and the negative control was a tissue of normal colon adjacent to malignant tissue. Counting started from one side of the slide. Within each slide, 100 intact unfold and unrepeatable cells were evaluated. Based on the staining intensity, the cells were classified in four groups; without expressing p53 (0), mild staining (1), moderate (2), and severe (3). The coded slides were reviewed by two expert histologists blindly. To compare the intensity of p53 expression between the groups, nonparametric Mann-Whitney test was performed. P values less than 0.05 were considered statistically significant. The nucleus area (NA) and the cytoplasmic area (CA) in the cells were estimated using the Cavalieri point counting method (23). Within each slide, 25 intact unfold and unrepeatable cells were evaluated morphologically. The Spearman correlation coefficient was used to investigate the relationship between NA, CA, cytoplasm to nuclear ratio (CNR) and FBS and the staining intensity.

4. Results

Demographic data of diabetic patients was compared with the control group. According to this data and considering the fact that both groups were homogenized in terms of age and gender, there was no significant difference between the two studied groups ($P > 0.05$). Evaluation of p53 immunocytochemistry showed that the intensity of p53 expression in oral mucosal exfoliative cells in diabetic patients significantly increased compared with healthy subjects ($P < 0.05$) (Table 1), (Figure 1, and 2). The mean percentage of p53 positive cells in oral mucosa of diabetic patients was 78.6 ± 6.933 and it was 1.2 ± 0.546 in the control group. Statistical analysis using the nonparametric Spearman's test showed a correlation between the level of FBS and the intensity of p53 expression ($P < 0.001$, $r = 0.816$) (Table 2).

The blood sugar levels and the percentage of p53 expression were analyzed and the correlation between the two variables was statistically significant ($r = 0.833$, $P < 0.001$) (Table 2). The relationship between NA and CNR and p53 expression, using the Spearman correlation coefficients,

were statistically significant ($P < 0.05$). (NA, $r = 0.272$, $P = 0.08$; CNR, $r = -0.434$, $P = 0.005$). The cytoplasmic area and the staining intensity showed no significant correlation ($P > 0.05$).

Table 1. Comparison of p53 Expression Intensity Between the Two Groups (n = 50)^a

Immunocytochemical Parameter	Diabetes Group	Control Group	P Value
p53	1.079 ± 0.144	0.007 ± 0.0159	0.00 ^b

^a Data are presented as Mean ± SD.

^b $P < 0.05$.

Table 2. Correlation Between the p53 Expression Intensity and the Nucleus Area, Cytoplasmic Area and Cytoplasm to Nuclear Ratio^a

	Correlation Coefficient	Probability ^b
p53 and NA	0.272	0.08
p53 and CA		NS
p53 and CNR	-0.434	0.005
p53 and FBS	0.816	0.00

^a Abbreviations: CA, Cytoplasmic Area; CNR, Cytoplasm to Nuclear Ratio; FBS, Fasting Blood Sugar; NA, Nucleus Area; NS, Not Significant.

^b $P < 0.0001$.

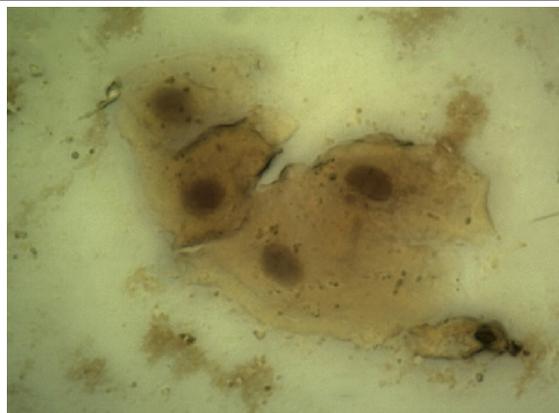


Figure 1. Expression of p53 in Oral Mucosal Cells of Diabetic Patient

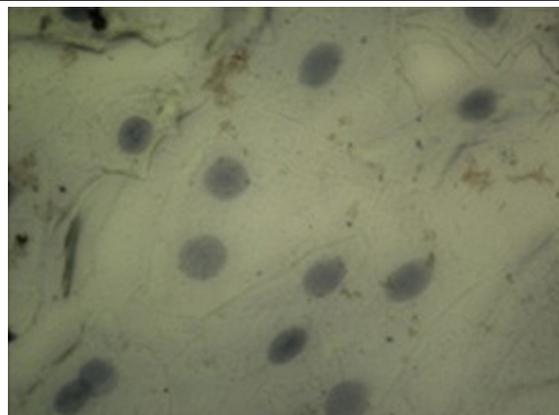


Figure 2. Oral Mucosal Cells of Normal Cases

5. Discussion

The results of the present study showed a significant increase in p53 expression in oral mucosal exfoliated cells in diabetic patients compared to healthy controls ($P < 0.05$). There was a significant correlation ($r = 0.816$, $P < 0.001$) between the intensity of p53 expression and the FBS level as well. Between the percentage of p53-positive cells and the FBS level, a significant correlation was observed ($r = 0.833$, $P < 0.001$). The relationship between NA and CNR with the expression of p53 was significant (NA, $r = 0.272$, $P = 0.08$; CNR, $r = -0.434$, $P = 0.005$).

The results of this study showed that expression of p53 protein in exfoliated cells of oral mucosa in diabetic patients was more than healthy controls. Increments of NA and CNR in exfoliated cells of oral mucosa of diabetic patients compared with healthy controls were observed. In agreement with our results, an experimental study that conducted by Vairaktaris et al. on normal and diabetic rats using biopsy of oral mucosa revealed that p53 expression in oral mucosa of diabetic rats' markers was more than normal rats (24).

An immunohistochemical study by Girtan et al. on oral mucosal biopsies of patients with type 1 diabetes indicated an increased expression of p53 in nucleus of basal cell (25). Agha Hosseini et al. studied the biopsy of oral mucosa in patients with lichen planus. Their results indicated the increment of p53 expression in oral mucosa of patients compared with healthy subjects (26). Abrahao et al. studied the oral mucosal biopsies of patients with leukoplakia, erythroplakia, squamous cell carcinoma and hyperplasia of oral epithelium. They showed that the expression of p53 in these conditions increased (27). Mohtasham et al. studied 27 patients with fibrosis hyperplasia, 40 with leukoplakia and 28 with oral squamous cell carcinoma. The comparison between the groups showed that p53 increased in benign, premalignant and malignant tissues (28).

Atrophy of mouth, leukoplakia and squamous cell carcinoma of oral mucosa are common in patients with diabetes (25). Nylanders showed that the expression of p53 protein in supra basal layers of the dysplastic epithelium mainly in squamous cell carcinoma can be a marker for the progression of these lesions (29). In tumors, loss of wild-type p53 function deactivates the growth control pathway causing uncontrolled growth of the tumor cell (30). In evaluation of exfoliative cells of oral mucosa of diabetic patients, cytomorphometric changes such as increase in size of the nucleus were observed. The cells progress toward malignancy is associated with these changes (12, 13).

The significant correlation between the intensity of p53 expression and NA in diabetic patients may be due to decrease in keratinization followed by decrease in cellular turnover (31). This study showed that by increasing the expression of p53 CNR reduced, which may occur because of an enlarged nucleus in oral mucosal cells of diabetic

patients, while cytoplasm stays unchanged, as has been reported in most studies (1, 3, 31, 32). Preservation of the cytoplasm size is a compensatory mechanism to maintain the cell size in stress condition (1).

Findings of the present study indicated an increased p53 expression in oral mucosal exfoliative cells of diabetic patients; cytomorphometric changes include increment in NA and reduction in CNR. These changes were observed for the first time with modification applying a simple and noninvasive immunocytochemistry method on oral exfoliated cell smears.

Given the high prevalence of premalignant and malignant diseases of oral mucosa in patients with diabetes, further studies are required in this field. This noninvasive and simple method could be used in screening and early detection of oral malignant diseases in these patients.

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References

- Hallikerimath S, Sapra G, Kale A, Malur PR. Cytomorphometric analysis and assessment of periodic acid Schiff positivity of exfoliated cells from apparently normal buccal mucosa of type 2 diabetic patients. *Acta Cytol.* 2011;**55**(2):197-202.
- Ship JA. Diabetes and oral health. *J Am Dent Assoc.* 2003;**134**:45-10.
- Vasconcelos BC, Novaes M, Sandrini FA, Maranhao Filho AW, Coimbra LS. Prevalence of oral mucosa lesions in diabetic patients: a preliminary study. *Braz J Otorhinolaryngol.* 2008;**74**(3):423-8.
- Ujpal M, Matos O, Bibok G, Somogyi A, Szabo G, Suba Z. Diabetes and oral tumors in Hungary: epidemiological correlations. *Diabetes Care.* 2004;**27**(3):770-4.
- Lamster IB, Lalla E, Borgnakke WS, Taylor GW. The relationship between oral health and diabetes mellitus. *J Am Dent Assoc.* 2008;**139** Suppl:19S-24S.
- Sandberg GE, Sundberg HE, Fjellstrom CA, Wikblad KE. Type 2 diabetes and oral health: a comparison between diabetic and non-diabetic subjects. *Diabetes Res Clin Pract.* 2000;**50**(1):27-34.
- Tozoglu U, Bilge OM. Exfoliative Cytology of Type 1 Diabetic Patients. *Eur J Gen Med.* 2010;**7**(3):264-8.
- Shareef BT, Ang KT, Naik VR. Qualitative and quantitative exfoliative cytology of normal oral mucosa in type 2 diabetic patients. *Med Oral Patol Oral Cir Bucal.* 2008;**13**(11):E693-6.
- Negrato CA, Tarzia O. Buccal alterations in diabetes mellitus. *Diabetol Metab Syndr.* 2010;**2**(12):3.
- Shaik NA, Shaik JP, Ali S, Imran A, Rao DK. Increased frequency of micronuclei in diabetes mellitus patients using pioglitazone and glimepiride in combination. *Food Chem Toxicol.* 2010;**48**(12):3432-5.
- Cherry-Peppers G, Ship JA. Oral health in patients with type II diabetes and impaired glucose tolerance. *Diabetes Care.* 1993;**16**(4):638-41.
- Prasad H, Ramesh V, Balamurali P. Morphologic and cytomorphometric analysis of exfoliated buccal mucosal cells in diabetes patients. *J Cytol.* 2010;**27**(4):113-7.
- Bastos AS, Leite AR, Spin-Neto R, Nassar PO, Massucato EM, Orrico SR. Diabetes mellitus and oral mucosa alterations: prevalence and risk factors. *Diabetes Res Clin Pract.* 2011;**92**(1):100-5.
- Ahmed I, Nasreen S, Jehangir U, Wahid Z. Frequency of oral lichen planus in patients with noninsulin dependent diabetes mellitus. *J Pak Assoc Dermatol.* 2012;**22**:30-4.
- Epstein JB, Zhang L, Rosin M. Advances in the diagnosis of oral premalignant and malignant lesions. *J Can Dent Assoc.* 2002;**68**(10):617-21.
- SeyedMajidi M, Shafahi SH, Ehsani H. Immunohistochemical assessment of expression of p53 in dental follicle and dentigerous cyst. *J Isfahan Dent Sch.* 2007;**3**(2):47-52.
- Scott IS, Odell E, Chatrath P, Morris LS, Davies RJ, Vowler SL, et al. A minimally invasive immunocytochemical approach to early detection of oral squamous cell carcinoma and dysplasia. *Br J Cancer.* 2006;**94**(8):1170-5.
- Brunotto M, Zarate AM, Cismondi A, Fernandez Mdel C, Noher de Halac RI. Valuation of exfoliative cytology as prediction factor in oral mucosa lesions. *Med Oral Patol Oral Cir Bucal.* 2005;**10** Suppl 2:E92-102.
- Seifi S, Mahjoub S, SHafigh E, Bijani A, Ahangari MH. Ki67 and p53 proteins in odontogenickeratocyst. *J Babol Univ Med Sci.* 2009;**10**(5):23-9.
- Moshref M, Mehrdad L, Jadali F, Mashhadi F. EVALUATION OF p53, p63 and HPV expression related with clinical stage and microscopic grade of oral squamous cell carcinoma. *J Dent Sch Shahid Beheshti Univ Med Sci.* 2009;**27**(4):205-13.
- Adhami S, Baghaie F, Kakooi S, Raof M. The Expression of P53 in Leukoplakia, Squamous Cell Carcinoma and Normal Oral Epithelium Using Immunohistochemical Staining Method. *J Kerman Univ Med Sci.* 2009;**16**(1):1-11.
- Ansarin H, Daliri M, Salehian Dardashti P. Comparing p53 protein expression in aggressive and non-aggressive Basal cell Carcinoma. *J Iran Univ Med Sci.* 2004;**11**(43):698-704.
- Heidari Z, Mahmoudzadeh Sagheb H, Kohan F. A quantitative and qualitative study of rat testis following administration of methadone and buprenorphine. *Int J high risk behav addict.* 2012;**1**(1):12-5.
- Vairaktaris E, Kalokerinos G, Goutzanis L, Spyridonidou S, Vasiliou S, Derka S, et al. Diabetes alters expression of p53 and c-myc in different stages of oral oncogenesis. *Anticancer Res.* 2007;**27**(3B):1465-73.
- Girtan M, Zurac S, Staniceanu F, Bastian A, Popp C, Nichita L, et al. Oral epithelial hyperplasia in diabetes mellitus. *Rom J Intern Med.* 2009;**47**(2):201-3.
- Agha Hosseini F, Khalili M, Rohani B. Immunohistochemistry analysis of P53 and Ki-67 proteins in oral lichen planus and normal oral mucosa. *Iran J Public Health.* 2009;**38**(2):37-43.
- Abrahao AC, Bonelli BV, Nunes FD, Dias EP, Cabral MG. Immunohistochemical expression of p53, p16 and hTERT in oral squamous cell carcinoma and potentially malignant disorders. *Braz Oral Res.* 2011;**25**(1):34-41.
- Mohtasham N, Saghravanian N, SHarifi N, SHakeri MT, Amelgharib SH, SHojaee S. Evaluation of p53 and PCNA expression in oral Leukoplakic lesions with and without epithelial dysplasia by immunohistochemistry technique. *journal Mashad dental school.* 2009;**33**(2):169-76.
- Nylander K, Dabelsteen E, Hall PA. The p53 molecule and its prognostic role in squamous cell carcinomas of the head and neck. *J Oral Pathol Med.* 2000;**29**(9):413-25.
- Swaminathan U, Joshua E, Rao UK, Ranganathan K. Expression of p53 and Cyclin D1 in oral squamous cell carcinoma and normal mucosa: An Immunohistochemical study. *J Oral Maxillofac Pathol.* 2012;**16**(2):172-7.
- Sonawane K, Jain S, Gupta I, Singaraju S, Singaraju M. Cytomorphometric Analysis of Oral Mucosa in Diabetic Patients in Bhopal Region an In-Situ Study. *Int J Clin Dent Sci.* 2011;**2**(4).
- Suvarna M, Anuradha C, Kumar KK, Sekhar PC. Cytomorphometric analysis of exfoliative buccal cells in type II diabetic patients. *Dr. NTR Univ Health Sci.* 2012;**1**(1):33.