

# Immunohistochemical detection of 8-hydroxydeoxyguanosine: A biomarker of oxidative DNA damage in oral submucous fibrosis

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## Abstract

**Background:** Oral submucous fibrosis (OSMF) is one of the common potentially malignant disorders prevailing in India. The primary etiological factors include tobacco and arecanut, which contain numerous reactive oxygen species (ROS). ROS attack guanine bases in DNA and form 8-hydroxydeoxyguanosine (8-OHdG), which can be detected in patients who have diseases associated with oxidative stress. The oxidative DNA damage produced by oxidative stress may induce malignant transformation.

**Aim:** The aim of the present study is to detect the expression of 8-OHdG in OSMF patients and compare the expression within different grades of OSMF and also normal buccal mucosa.

**Materials and Methods:** A total of 30 samples were examined for the immunohistochemical expression of 8-OHdG. The control group included 10 formalin-fixed paraffin-embedded tissue blocks of the normal buccal mucosa. The study group includes 20 cases of formalin-fixed paraffin-embedded tissue blocks of OSMF (5 cases in each grade of very early, early, moderately advanced and advanced cases of OSMF). Three-micron thick tissue sections were made from each sample and stained with 8-OHdG antibody. The results were statistically analyzed using Kruskal–Wallis and Mann–Whitney U test

**Results:** Statistically significant difference exists in the intensity of 8-OHdG expression between the study groups. The *P*-value obtained was <0.001, which was highly statistically significant.

**Conclusion:** The present study is the first attempt to evaluate the expression of 8-OHdG in tissue samples of OSMF that revealed the role of free radicals and oxidative DNA damage in these patients. Further research with larger sample size, clinicopathologic correlation and long-term follow-up will shed more light on the pathogenesis of OSMF. It will also be useful for the development of new therapeutic strategies targeting treatment modalities for OSMF.

**Keywords:** 8-Hydroxydeoxyguanosine, immunohistochemistry, oral submucous fibrosis, oxidative DNA damage

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**Submitted:** 02-Feb-2020, **Accepted:** 27-Oct-2020, **Published:** 09-Jan-2021

### Access this article online

#### Quick Response Code:



#### Website:

www.jomfp.in

#### DOI:

10.4103/jomfp.JOMFP\_42\_20

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**How to cite this article:** Kulasekaran C, Devi M, Dhivya K, Vijayalakshmi D, Sathishkumar M, Madhanmohan A. Immunohistochemical detection of 8-hydroxydeoxyguanosine: A biomarker of oxidative DNA damage in oral submucous fibrosis. *J Oral Maxillofac Pathol* 2020;24:536-41.

## INTRODUCTION

Oral potentially malignant disorders (OPMD) are described as “Not all lesions and conditions may transform to cancer rather than a morphological alteration among which some may have an increased potential for malignant transformation.” It conveys that not all precancerous lesions and conditions may transform into cancer, rather some may have increased chance for malignant transformation.<sup>[1]</sup> Precancerous lesions like leukoplakia, erythroplakia, palatal changes in reverse smoking and precancerous conditions like oral submucous fibrosis (OSMF), lichen planus and discoid lupus erythematosus that were previously recognized as precancer by the WHO in 1978 are now categorized as potentially malignant disorders.<sup>[2]</sup> In the Indian subcontinent, leukoplakia and OSMF are the most common potentially malignant disorders caused largely by tobacco smoking and areca nut consumption. Areca nut has been declared as a known human carcinogen by the International Agency for research on cancer (IARC) expert group in 2003.<sup>[3]</sup>

OSMF is a premalignant condition and associated with carcinogens in tobacco and arecanut is thought to have some relation with reactive oxygen species (ROS).

ROS are free radicals associated with oxygen and their equivalents. They have stronger reactivity with other molecules than with molecular oxygen.<sup>[4]</sup> ROS usually indicate the following four species: Superoxide anion ( $O_2^-$ ), Hydrogen peroxide ( $H_2O_2$ ), Hydroxyl radical ( $OH^-$ ) and Singlet oxygen ( $O_2$ ). ROS react with the most important structures of cells and particles, altering their biological function.<sup>[5]</sup> It is established that ROS are involved in a variety of biological phenomenon such as mutation, carcinogenesis, aging, atherosclerosis and inflammation.

ROS attack guanine bases in DNA and form 8-Hydroxydeoxyguanosine (8-OHdG) by hydroxylation of guanine base, 8-OHdG can cross the cell membrane and is usually detected in urine, serum or saliva of patients who have diseases associated with oxidative stress. It has widely been used in many studies as a biomarker for the measurement of endogenous oxidative DNA damage, including cancer and other degenerative diseases. The level of 8-OHdG is generally regarded as a biomarker of mutagenesis consequent to oxidative stress.<sup>[6]</sup>

Tobacco-smoking, air-pollution, ultra-violet radiation, mitochondrial damage, chronic inflammation and chronic systemic diseases lead to uncontrolled oxidative damage, which may progress to produce unrepairable DNA damage,

eventually leading to mutation and carcinogenesis.<sup>[7]</sup> The oxidant and antioxidant status in potentially malignant disorders serve as an early biomarker tool to analyze the level of oxidative damage and any further progression of DNA damage.

The most commonly produced base lesion results from oxidation of guanine base, and which is often measured as an index of oxidative DNA damage, is 8-OHdG.<sup>[8]</sup>

Various oxidative stress markers have been studied in OSMF and antioxidants are being routinely used for the treatment of OSMF. With this fact in mind, a more advanced oxidative stress marker 8-OHdG was decided to be studied in OSMF by Immunohistochemistry (IHC) method.

## MATERIALS AND METHODS

### Study design and sample distribution

A retrospective cross-sectional study was conducted on the formalin-fixed, paraffin-embedded tissue blocks obtained from the Department of Oral Pathology, Adhiparasakthi Dental College and Hospital, Melmaruvathur. The study group included 20 cases of histologically diagnosed OSMF (Group 1). Twenty cases of OSMF were divided into four subgroups according to the histopathological grading system by Pindborg *et al.*, group namely-very early, early, moderately advanced and advanced with 5 cases in each subgroup.

The control group included biopsies from normal buccal mucosa adjacent to the site of surgery during surgical removal of the third molar in 10 patients (Group 2). Positive control section included Breast Carcinoma for 8-OHdG and was treated in the same manner as the test groups. Negative control section included one section of the test sample was selected and treated in the same manner as the test groups except that the primary antibody 8-OHdG was omitted.

### Method

Deparaffinized 3- $\mu$  thick tissue sections obtained from archival tissue blocks were immunohistochemically investigated, using Rabbit polyclonal antibody against target protein 8-OHdG (Bioss ANTIBODIES Biotechnologies Private Limited) (IHC-P-1:100-500). With PolyExcel HRP/DAB Detection System (PathnSitu Biotechnologies Private Limited) and method polymer chain two-step indirect technique recommended by Sanderson *et al.*<sup>[9]</sup> The positive expression was seen as light brown or dark brown stain in the cells of the epithelium. Ten Random fields were selected at  $\times 40$ . Sections were scored for staining

intensity and scaled as no stain-0, mild staining-1, moderate staining-2, 3-intense staining.

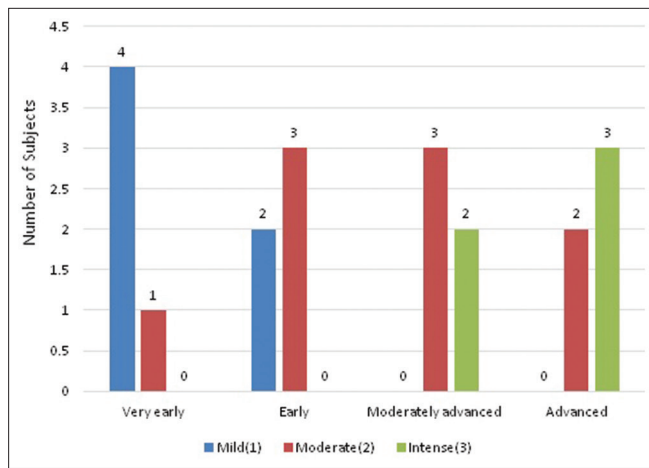
**Statistical analysis**

Median staining intensity and interquartile range were estimated in each of the study groups. The results were statistically analyzed using the Kruskal–Wallis test and Mann–Whitney U test.

**RESULTS**

For evaluation and comparison of staining intensities between different grades of OSMF Kruskal Wallis test was performed. The *P*-value obtained was <0.01, which was statistically significant [Table 1 and Graph 1].

On comparison of 8-OHdG expression in OSMF and normal buccal mucosa, all the OSMF cases showed retention of antigen. Its expression varied from one case to another. Among 20 cases of OSMF, 30% (6 out of 20 cases) showed mild expression [Figure 1], 45% (9 out of 20 cases) showed moderate expression [Figure 2] and 25% (5 out of 20cases) exhibited an intense expression [Figure 3].



**Graph 1:** Evaluation and comparison of staining intensities between different grades of oral submucous fibrosis

In normal buccal mucosa, about 50% (5 out of 10 cases) exhibited mild expression. The remaining 50% (5 out of 10 cases) exhibited no retention of antigen [Figure 4].

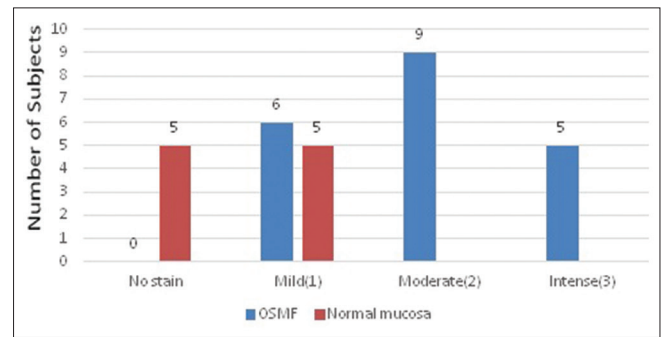
To compare the staining intensity between OSMF group and normal buccal mucosa, Mann–Whitney U test was performed. The *P*-value was <0.001, which was highly statistically significant [Table 2 and Graph 2].

**DISCUSSION**

OSMF, which was previously known as a premalignant condition, is now categorized under OPMD; OSMF has a prevalence rate of 0.2% in Gujarat-0.5% in Kerala,<sup>[10]</sup> with a malignant transformation rate of 7.6%<sup>[11]</sup> among Indian villagers.

Sinor *et al.* suggested that arecanut is the most probable causative agent.<sup>[12]</sup> Numerous ROS and reactive nitrogen species (RNS) in arecanut stimulate the carcinogenic effect in the cells.

Oxidative stress is a process that occurs when there is a failure of body’s endogenous antioxidant defenses to scavenge the free radical species. Oxidative stress can lead to oxidative DNA damage. The consequences of such DNA damage



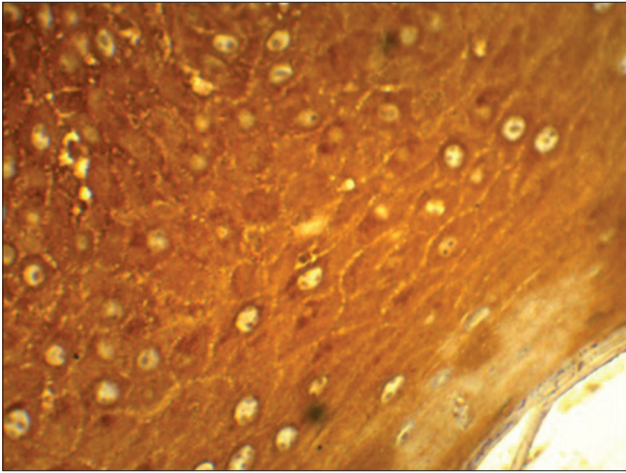
**Graph 2:** Evaluation and comparison of 8-hydroxydeoxyguanosine expression in oral submucous fibrosis and normal oral mucosa

**Table 1: Evaluation & Comparison of staining intensities between different grades of OSMF**

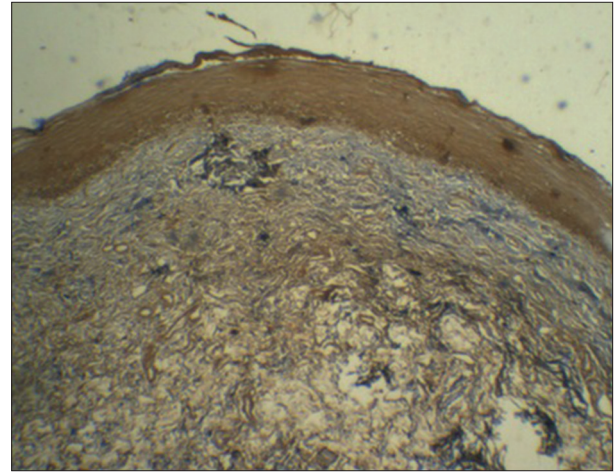
Groups	No of cases (n)	Scores			Median staining intensity	IQR	P
		Mild (1)	Moderate (2)	Intense (3)			
Very early	5	4 (80%)	1 (20%)		1	0.5	<0.01
Early	5	2 (40%)	3 (60%)		2	1	
Moderately advanced	5		3 (60%)	2 (40%)	2	1	
Advanced	5		2 (40%)	3 (60%)	3	1	

**Table 2: Evaluation & Comparison of 8-OHdG expression in OSMF and Normal oral mucosa**

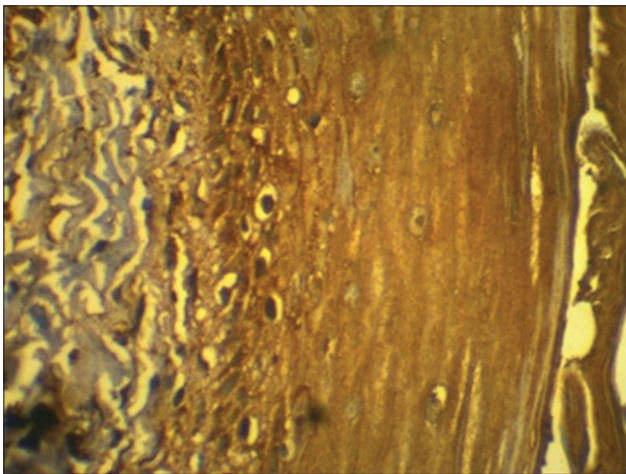
Groups	No of cases (n)	Scores				Median staining intensity	IQR	P
		No Stain (0)	Mild (1)	Moderate (2)	Intense (3)			
OSMF	20		6 (30%)	9 (45%)	5 (25%)	2	1.75	0.001
Normal oral mucosa	10	5 (50%)	5 (50%)			0.5	1	



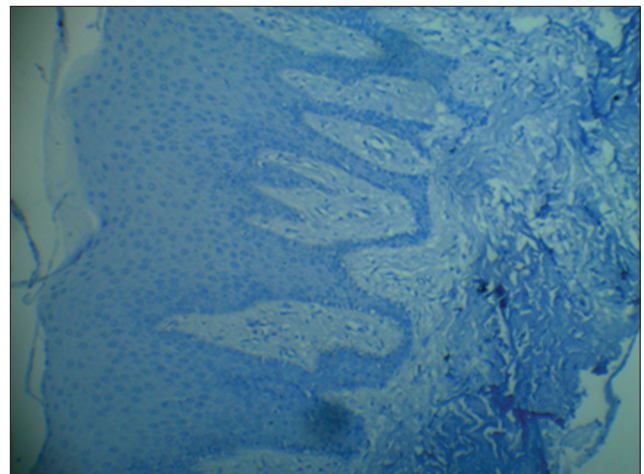
**Figure 1:** High power view (x40) showing mild expression in very early stage



**Figure 2:** Low power view (x10) exhibiting moderate expression of 8-hydroxydeoxyguanosine in early stage of oral submucous fibrosis



**Figure 3:** High power view (x40) showing Intense expression of 8-hydroxydeoxyguanosine in advanced stage of oral submucous fibrosis



**Figure 4:** Low power view (x10) showing expression of 8-hydroxydeoxyguanosine in normal oral mucosa

include cell death, mutagenesis and carcinogenesis. Although it is very difficult to measure these free radical species because of very short half-life, the products of these free radical species cause damage to DNA, lipids, proteins and these are considered good markers of oxidative stress.<sup>[13]</sup> 8-OHdG, a compound formed as a result of reaction between DNA and ROS, is an important biomarker of oxidative DNA damage.

In the current study, out of 20 cases of OSMF, all the cases exhibited positive cytoplasmic expression as in positive control. In OSMF, among very early group, about 80% (4 out of 5) of cases exhibited mild expression of antigen and the remaining one case showed moderate expression. In the early group 60% (3 out of 5) of cases showed moderate expression of antigen and the remaining 40% (2) of cases showed mild expression. In the moderately advanced group 40% (2) of cases exhibited intense expression of antigen and about 60% (3) of cases exhibited moderate expression.

Among the advanced group, 60% (3) of cases exhibited intense expression, 40% (2) of cases showed moderate expression of antigen [Table 1 and Graph 1].

Our study results are in accordance with the study done by Tsai *et al.*<sup>[14]</sup> to evaluate the intensity of cytosol expression of 8-OHdG in renal fibrosis. He concluded that the intensity of 8-OHdG expression was associated with the severity of renal fibrosis.

Kumar *et al.*<sup>[15]</sup> did a study to determine the salivary antioxidant capacity (total antioxidant capacity, glutathione), free radicals (ROS, RNS) and oxidative DNA damage (8-OHdG) to understand the involvement of these biologic indexes in head and neck squamous cell carcinoma. He concluded that increased 8-OHdG in the habituate group (smokers, chewers) along with other oxidative stress markers, suggest a strong contribution toward increased DNA oxidation by free radicals.

The results of the above studies can be correlated with our study that intense expression suggests a strong contribution toward the increased oxidative DNA damage with advanced grades of OSMF.

In the present study, all the cases of OSMF showed immunoreactivity in the cytoplasm, because mitochondrial DNA is not covered extensively by proteins such as Histones, it may be more susceptible to excited oxygen than nuclear DNA. In addition, mitochondrial DNA may be less efficient in repairing DNA damage and replication errors than the nucleus.

The phenomenon of cytoplasmic staining in the present study is similar to results obtained from the study conducted by Nomoto and Tsuneyama<sup>[16]</sup> in nonalcoholic fatty liver disease. They proposed that the cytoplasmic fine granular expression may be a sensitive diagnostic biomarker of early nonalcoholic fatty liver disease events.

In the present study, the expression of 8-OHdG in OSMF was compared with Normal buccal mucosa. Out of 20 cases of OSMF 30% (6) of cases exhibited mild expression, about 45% (9) cases exhibited moderate and the remaining 25% (5) cases exhibited intense expression of 8-OHdG. Among 10 cases of normal buccal mucosa, 50% of cases exhibited mild positive expression, the remaining 50% of cases exhibited negative expression. The reason for the mild expression of 8-OHdG in normal buccal mucosa may be due to the presence of inflammation as the archival tissue blocks were obtained from the buccal mucosa of the impacted third molar region [Table 2 and Graph 2].

The results in the present study are in accordance with the study done by Canakci and Cicek<sup>[17]</sup> to evaluate the levels of 8-OHdG and its relationship with antioxidant enzymes in periodontitis patients. They found higher 8-OHdG levels in periodontitis patients compared to that of healthy controls and concluded that higher 8-OHdG levels reflect increased oxygen radical activity during periodontal inflammation.

Similar findings were also observed by Kaur *et al.*,<sup>[18]</sup> on the evaluation of the salivary 8-OHdG levels in OSMF, OSCC and normal healthy controls. They found significantly higher levels of salivary 8-OHdG in OSMF, OSCC when compared with healthy normals. They concluded that detection of 8-OHdG in oral precancerous lesions and cancer can act as a suitable diagnostic biomarker of oxidative DNA damage.

Tsai *et al.*<sup>[14]</sup> stated that overproduction of ROS and downregulated expression of cellular antioxidant enzymes

impairs the DNA reparative mechanism, which results in the formation of 8-OHdG. The expression of 8-OHdG is an important biomarker of any chronic degenerative disease.

Zimnoch *et al.*<sup>[19]</sup> did an immunohistochemical study on chronic cholecystitis using a Monoclonal antibody against 8-OHdG. Increase in 8-OHdG expression was found in chronic cholecystitis and they concluded that the expression of 8-OHdG is associated with the degree of inflammation and disease duration.

Our present study is the first attempt to evaluate the expression of 8-OHdG in tissue samples of OSMF to know the role of free radicals and oxidative DNA damage, which enhances the initiation and progression of OSMF.

## CONCLUSION

The present study done with 8-OHdG in OSMF is the first attempt on tissue samples by Immunohistochemical investigation to visualize oxidative DNA damage. Its expression can be considered as a biomarker of considerable specificity. The current study revealed the role of free radicals and oxidative DNA damage, which enhances the initiation and progression of OSMF. Further studies with larger sample size, clinicopathological correlation, long-term follow-up will shed more light on the pathogenesis of OSMF and also for the development of new therapeutic strategies targeting treatment modalities for OSMF.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

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