



## ANALYSIS OF ESTROGEN METABOLITES IN ORAL LEUKOPLAKIA AND ORAL SQUAMOUS CELL CARCINOMA

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### ABSTRACT

Oral squamous cell carcinoma is an important malignancy of epithelial origin with high rate of morbidity and mortality. While tobacco and alcohol are the primary risk factors; other factors such as microorganisms, chronic irritation and sex hormones along with underlying genetic factors may also contribute to its pathogenesis. The role of sex hormones like estrogen, progesterone and testosterone are known to influence the behavior and prognosis of OSCC. Various estrogen metabolites may possess clinical significance in OSCC and their precursor lesions. The aim of the present study is to assess the regulation of estrogen metabolites in saliva and serum of patients with oral leukoplakia and oral squamous cell carcinoma. Patients diagnosed with oral leukoplakia (n=21) and oral squamous cell carcinoma (n=22) were compared with normal controls (n=18) using QTOF- liquid chromatography mass spectrometry for the detection of estrogen related metabolites in saliva and serum samples. Statistical analysis was performed employing ANOVA, student t-test and Chi-square test to identify the significance of various estrogen metabolites. Salivary estrone-3-glucuronide, estrone-3-sulfate and serum levels of estrone-3-sulfate, estradiol-17 $\beta$ -3-sulfate were significantly upregulated in oral leukoplakia and OSCC than in normal controls while salivary estradiol valerate was significantly downregulated in the diseased groups. The findings suggest the salivary and serum estrogen related metabolites have a significant role in oral leukoplakia and OSCC and could be applicable in cancer therapy.

**KEYWORDS:** Salivary diagnostics, estrogen receptors, oral leukoplakia, oral squamous cell carcinoma



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## INTRODUCTION

Oral cancer is a significant disease affecting humans and the most common form is the oral squamous cell carcinoma (OSCC) which accounts for over 90% of the reported malignancies of the oral cavity.<sup>1</sup> OSCC is a neoplasm of epithelial origin with high prevalence rate in developing countries of the world. The disease is highly reported to occur in India and possess a multifactorial etiology with tobacco and alcohol being the primary risk factor.<sup>2</sup> The use of tobacco in smokeless and smoking form is one of the important determinants of OSCC with the common intra-oral site being the alveolo-gingivo-buccal complex.<sup>3</sup> In general, OSCC can arise *de novo* or from pre-existing oral lesions such as leukoplakia. OSCC is associated with a high rate of morbidity and mortality. Early detection and prompt diagnosis generally leads to better prognosis and helps in implementation of successful clinical treatment. The difficulties of early detection of OSCC could be attributed to the overlap of clinical symptoms and usual painless presentation along with its presence at inaccessible sites. Hence much of the OSCC lesions are detected at an advanced stage thus delaying treatment and associated morbidity.<sup>4</sup> Saliva is a unique body fluid used for the development of molecular diagnostics as it contains not only components found in serum but also offers several advantages over serum. Saliva collection is cost-effective, safe, easy and noninvasive. Promising new technologies with higher detection sensitivity have met the essential requirements to successfully screen saliva components, especially proteins and nucleic acids.<sup>5</sup> The noninvasive and simple nature of saliva collection allows for repetition and multiple collection that can potentially aid in early diagnosis, monitoring disease progression, or treatment responses with minimally trained personnel. The role of sex hormones was considered as one of the important risk factor in oral carcinogenesis. The sex hormones play an important role in gene expression involved in several biological and neoplastic processes.<sup>6</sup> Cancers of breast, prostate and endometrium have been proven to be strongly associated with the various sex hormones.<sup>7, 8</sup> The important sex hormones of the body are estrogen, progesterone and testosterone which are derivatives of cholesterol and belong to the group of steroid hormones.<sup>9</sup> Estrogen is a sex hormone which exist in three biologically active forms namely estrone, 17- $\beta$ -estradiol and estradiol and 17- $\beta$ -estradiol being the most active among them.<sup>10</sup> Increased levels of estrogen related metabolites have been reported in the literature with respect to OSCC. Endocrine microenvironment of the host could be one of the contributing factors in altering the onset and progression of OSCC. However, the agreement among researchers with regard to its

exact role has been contentious. Thus, the aim of the present study is to evaluate the significance of estrogen related metabolites in saliva and serum of patients diagnosed with oral leukoplakia and OSCC using liquid chromatography/ mass spectrometry analysis.

## MATERIALS AND METHOD

The study participants were divided into three groups namely: Group I (n=18) were normal individuals without any oral lesions, tobacco habits and systemic illnesses; Group II (n=21) included clinically diagnosed cases of oral leukoplakia and Group III (n=22) consisted of clinically and histopathologically diagnosed cases of oral squamous cell carcinoma. The study participants with known history of systemic illness and medications; history of previous therapy for oral leukoplakia and OSCC (surgery, chemotherapy and radiotherapy) and with recurrent oral lesions were excluded from the study. Institutional ethical committee clearance was obtained prior to the commencement of the study (014/10/2013/IEC/SU, dated 15/10/2013). The study details were explained to the participant and written informed consent was obtained. Unstimulated whole saliva was collected by drooling method in a collecting jar preferably in the mornings. The collected saliva was then immediately centrifuged and stored at -80° C before analysis. The obtained samples were centrifuged at 4000rpm for 15 min.<sup>11</sup> For serum collection, 5 ml of blood was collected by venipuncture from forearm region under aseptic precautions and transferred into plain vials. The obtained samples were then centrifuged at 3000rpm for 15 minutes.<sup>12</sup> Protein extraction was done by methanol precipitation method from the supernatant obtained from the saliva and serum samples.<sup>13</sup> The final obtained sample after processing was then analyzed by ultraperformance liquid chromatography coupled with quadrupole time of flight mass spectrometry (UPLC-QTOFMS, Agilent 6550 iFunnel Q-TOF LC/MS). The analysis was performed as per the method given by Yan et al (2008) using XCMS toolbox and software tool MassHunter Qualitative Analysis B.05.00. Statistical analysis was performed using the Agilent G3835AA MassHunter Mass profiler professional (MPP) software. ANOVA analysis to identify the regulation of metabolites between the three groups and chi-square test to indicate the presence or absence of metabolites in the study participants of the three groups was carried out. (SigmaPlot 13.0, Sysat software USA)

## RESULTS

The age distribution of the study participants is given in table 1.

**Table 1**  
Range and median age of the study participants in the normal, oral leukoplakia and OSCC groups

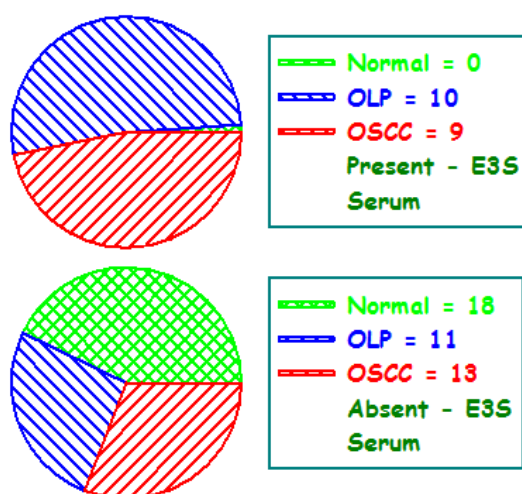
Group	N	Range	Median
Normal	18	21-60	49
Oral leukoplakia	21	29-70	48
OSCC	22	32-70	48

The sex distribution was as follows: normal (Males-12; females-6); oral leukoplakia (males-19; females-2); OSCC (males-18; females-4). In oral leukoplakia 11 participants had tobacco chewing habits (52%), 4 had smoking habits (19%) while 6 others had both chewing and smoking habits (29%). In OSCC group, 18 patients had tobacco chewing habits (82%), making it the predominant form of tobacco use among the study participants. The common clinical presentation of oral leukoplakia in this study was homogenous white keratotic non-scrapable patch (67%) followed by verrucous growth (29%) and red and white lesion (5%). The clinical presentation of OSCC was predominantly as ulcero-proliferative growth (45%), followed by ulcerative

lesion (36%). Analysis of the estrogen related metabolites among the three groups revealed a significant upregulation of salivary estrone-3-sulfate and estrone-3-glucuronide and downregulation of estradiol valerate in oral leukoplakia and OSCC group than in normal controls; while in serum samples, there was a significant upregulation of estrone-3 sulfate and estradiol-17 $\beta$ -3- sulfate in oral leukoplakia and OSCC group. Chi-square analysis indicated a statistical significant difference for serum estrone-3 sulfate (Fig 1) and the difference was not statistically significant for estradiol-17 $\beta$ -3- sulfate (Fig 2) between the three groups.

Figure 1

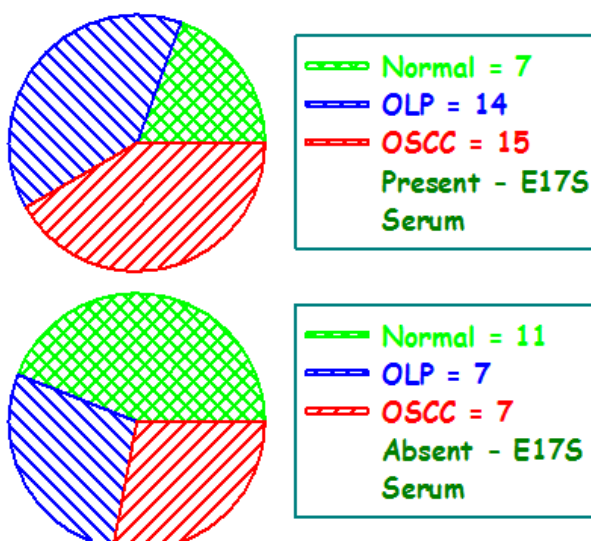
Pie diagram showing the presence or absence of estrone-3-sulfate (E3S) in the serum of normal, oral leukoplakia and OSCC participants.



The first pie-chart indicates the presence of the metabolite in the three groups. The second pie-chart indicates the absence of the metabolite in the three groups. n; Normal=18; oral leukoplakia=21; OSCC=22 Chi- square value= 11.77 P=0.003- the result is statistically significant

Figure 2

Pie diagram showing the presence or absence of estradiol-17- $\beta$ -3-sulfate in serum of normal, oral leukoplakia and OSCC participants



The first pie-chart indicates the presence of the metabolite in the three groups. The second pie-chart indicates the absence of the metabolite in the three groups. n; Normal=18; oral leukoplakia=21; OSCC=22 Chi- square value= 4.287 P=0.117- The difference is not statistically significant.

## DISCUSSION

Sex hormones such as estrogen, progesterone and testosterone have been identified as risk factors in many human malignancies including OSCC. Estrogen is an important sex hormone which exist in three biological forms. The type of estrogen with highest biological activity is 17 $\beta$ -estradiol which is mainly synthesized by sulfatase pathway.<sup>14</sup> The predominant source of estradiol is derived from estrone-3- sulfate (E3S) especially in tumor cells of breast cancer affected individuals.<sup>14</sup> E3S is a circulating inactive plasma estrogen which is desulfated to estrone by estrogen sulfatase which is further converted to estradiol by 17 $\beta$ -hydroxysteroid dehydrogenase.<sup>15</sup> Estrogen metabolites have been associated with carcinogenic potential. The mechanism of carcinogenic activity of estrogen related metabolites is probably by its direct action on DNA by forming adducts and also causing oxidative damage.<sup>16-17</sup> Other possible mechanisms include: multiple estrogen-receptor signal-transduction pathways associated with increased cell proliferation and inhibition of apoptosis;<sup>18</sup> activation of protein kinases and subsequent interaction with other growth factors such as epidermal growth factor;<sup>19</sup> and over-expression of centrosome kinases and centrosome amplification leading to chromosomal instability and subsequent carcinogenesis.<sup>20</sup> Altered estrogen metabolism and its subsequent effect on oral carcinogenesis could also be attributed to the tobacco habits. This inference can be made based on a study finding which showed that the estrogen metabolism pathway is altered in lung tissue following tobacco smoke exposure and similar changes can also be associated with other cancers of the aerodigestive tract.<sup>21</sup> A significant upregulation of estrone -3-sulfate and estradiol-17 $\beta$ - 3 sulfate in saliva and serum samples as observed in the present study suggest their potential involvement in oral leukoplakia and OSCC. A study by Colella et al (2011) showed an increased expression of estrogen receptor  $\alpha$  in mRNA of OSCC tissues than in normal controls thereby suggesting an important role of these sex hormones in OSCC.<sup>22</sup> In another study by Lukits et al (2007) an increased expression of estrogen receptors was observed in the tumor cells of tissue obtained from OSCC patients. While the exact mechanism for this observation is

unconfirmed, it possibly occurs due to deteriorating liver function secondary to alcohol leading to alteration of sex hormone metabolism.<sup>23</sup> In another study by Shatalova et al (2011) a panel of estrogen metabolism genes was expressed in cultured human head and neck cells. Detection of transcripts for these genes in both premalignant lesions and HNSCCs suggests that these enzymes contribute to cellular metabolism throughout tumorigenesis. It was suggested that estrogen may act in association with cytochrome p450 1B1 which may contribute to progression of OSCC.<sup>24</sup> A solitary study showed a positive correlation between estrogen receptor  $\beta$  and improved prognosis in oro-pharyngeal cancers although this was not applicable to other sites.<sup>25</sup> Such findings need careful evaluation and interpretation as they may influence the chemotherapeutic strategies.

## CONCLUSION

To conclude, the data from the current study suggest an alteration of estrogen related metabolites in oral leukoplakia and OSCC based on the significant upregulation of estrone-3-sulfate, estradiol-17 $\beta$ -sulfate and estrone-3-glucuronide in saliva and serum samples. The findings suggest the clinical importance of these metabolites in oral leukoplakia and OSCC. Salivary diagnostics can be applied in clinical practice as an adjunct to serum analysis as well as independently to identify and analyze tumor specific metabolites. Evaluation and validation of the various estrogen related metabolites may aid in successful implementation of various therapeutic regimen aimed at countering oral squamous cell carcinoma and improve prognosis.

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## CONFLICT OF INTEREST

Conflict of interest declared none.

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