ORIGINAL ARTICLE

Molecular screening of lysyl oxidase G473A polymorphism in oral submucous fibrosis

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ABSTRACT

Aim: To investigate the presence of lysyl oxidase (LOX) G473A polymorphism in group 1 {Oral submucous fibrosis (OSMF) patients}, group 2 (betel quid chewers without OSMF) and group 3 (healthy individuals). **Materials and Methods:** A total of 60 patients were taken for the study, which included 20 OSMF patients (group 1), 20 betel quid chewers without OSMF (group 2) and 20 healthy individuals without OSMF and betel quid chewing habit (group 3). DNA was isolated using Qiagen kit. The isolated DNA was quantified using spectroscopic methods. Polymerase chain reaction (PCR) was carried out at annealing temperature of 67°C. PCR amplification was checked on 2% agarose gel. Further, the amplified PCR products were subjected to automated DNA sequencer, to assess LOX G473A polymorphism. **Results:** The gene sequence data generated from the automated DNA sequencer was received as colored electropherograms. These gene-sequencing results did not show LOX G473A polymorphism in any of the 3 groups. **Conclusion:** In our study, gene-sequencing results did not show LOX G473A polymorphism in OSMF patients. Since only one study in the literature has shown the association of *LOX* gene polymorphism and OSMF patients, we conclude that further studies are required to unveil the role of *LOX* gene polymorphism in OSMF.

Key words: DNA sequencing, G473A polymorphism, lysyl oxidase, oral submucous fibrosis

INTRODUCTION

Oral submucous fibrosis (OSMF) is a chronic debilitating disease and a potentially malignant disorder of the oral cavity. The incidence of oral squamous cell carcinoma has been estimated to be 7.6% in OSMF patients after a follow up period of 17 years.[1] There are 200-400 million areca chewers worldwide. Areca-associated oral squamous cell carcinoma (OSCC) is the 3rd most common malignancy in developing countries.[2] OSMF is associated exclusively with areca chewing and is believed to be a homeostatic disorder of extracellular matrix (ECM).[3] Epidemiological evidences strongly indicate the association of the betel quid (BQ) habit and OSMF. The areca nut (betel nut) component of BQ, especially an alkaloid called arecoline, plays a major role in the pathogenesis of OSMF by causing an abnormal increase in the collagen production. The synthesis of collagens is influenced by a variety of mediators, prominent mediator being transforming growth factor-beta (TGF-β). TGF-β1, in particular, seems to be the one that plays a major role in wound repair and fibrosis. It causes the deposition of extra cellular matrix (ECM) by increasing the synthesis of matrix proteins like collagen and decreasing its degradation by stimulating various inhibitory mechanisms.[1] TGF-β has been found to strongly promote the expression of LOX both at the mRNA and protein levels in various cell lines.[1] The LOX is dependent on copper for its functional activity.[1] Areca nuts have been shown to have a high copper content and chewing areca nuts for 5-30 min significantly increases soluble copper levels in oral fluids. This increased level of soluble copper could act as an important factor in OSMF by stimulating fibrogenesis through upregulation of LOX activity.[4] Previous studies have specified the upregulation of LOX expression in normal oral keratinocytes by areca nut extract and its oncogenesis for OSCC.[5] There are seven single nucleotide polymorphism (SNP) sites in LOX coding region, including C225G, G409C, G473A, C476A, G816A, T924G

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and A1135G.[2] Among all SNP sites in LOX coding region, G473A has the highest polymorphic frequency. It causes a change of Arg at residue 158 to Gln (LOX Arg158Gln). The genetic susceptibility or disease nature of OSMF is still largely undefined.[2] The present study investigated the presence of LOX G473A polymorphism in group 1 (OSMF patients), group 2 (betel quid chewers without OSMF) and group 3 (healthy individuals).

MATERIALS AND METHODS

The study was conducted in Department of Oral Medicine and Radiology, from January 2011-June 2012. The subjects for the study were selected by employing 'convenience sampling' method reporting to the Department of Oral Medicine and Radiology.

A total of 60 patients were taken for the study, which was divided as follows: Group 1-20 OSMF patients (clinically and histopathologically diagnosed) with betel quid chewing habit, group 2-20 betel quid chewers without OSMF [Table 1] and group 3-20 healthy individuals without OSMF and betel quid chewing habit.

Inclusion criteria

- Patients with clinically and histopathologically diagnosed OSMF
- Patients with betel quid chewing habit without clinical evidence of OSMF who were undergoing 3rd molar surgery
- Healthy individuals without betel quid chewing habit and having normal oral mucosa who were undergoing 3rd molar surgery
- Above patients who are willing to participate in the study.

Exclusion criteria

- Patients on regular analgesics or anti-inflammatory drugs, which are known to interfere with lysyl oxidase activity
- Patient who already received or on treatment for OSMF.

A detailed case history was taken with an emphasis on the information regarding the habits like betel quid, tobacco chewing, smoking and alcohol consumption. Detailed clinical examination was performed on all the patients. The mean duration of betel quid chewing habit in group 1, group 2 and group 3 was 8.55 years, 8.45 years and 8.50 years, respectively [Table 2]. In group 1, 13 patients had the habit of chewing betel quid 1-10 times/day, 6 patients had the habit of chewing betel quid 11-15 times/day and 1 patient had the habit of chewing betel quid more than 15 times/day. In group 2, 14 patients had the habit of chewing betel quid 1-10 times/day, 6 patients had the habit of chewing betel quid 11-15 times/day.

The study protocol was explained and written informed consent was obtained from all the patients. A wedge biopsy was performed on the patients with clinical suspicion of OSMF and the tissue was divided into 2 halves (one for the purpose of histopathological diagnosis and the other to assess LOX polymorphism). For group 2 and group 3, samples were obtained from patients undergoing 3rd molar extraction for the analysis of *LOX* gene polymorphism.

DNA isolation of the tissue was done using Qiagen kit. Amplicons containing the *LOX* gene were obtained by polymerase chain reaction (PCR) reaction with primers: F-5′-CACTGGTTCCAAGCTGGCTA-3′, R-5′-GGAAGTAGCCAGTGCCGTAT-3'. Genotyping was performed using PCR-based restriction fragment length polymorphism (PCR-RFLP) analysis. 2% agarose gel was prepared. 20 µlt of DNA was loaded along with this 100 bp ladder was loaded and allowed it to run for 30 mins, the bands formed were observed under Gel Documentation system. The PCR amplicons were subjected to DNA sequencing using ABI-PRISM dye terminator at Eurofins Genomics India Pvt Ltd Bangalore. The sequence data generated was received as colored electropherograms.

RESULTS

In the present study, DNA was isolated using Qiagen kit. Quantification of DNA was done by gel electrophoresis and Nanodrop spectroscopy methods. The isolated DNA was made to run in an agarose gel electrophoresis for 30 min. The bands were observed in the gel documentation system [Figure 1]. Ratio of A260/A280 more than 2 ng/µL were treated with proteinase K and subjected to PCR basis. The PCR was carried

Table 1: Distribution of study samples based on duration of betel quid chewing habit

Duration	Group 1	%	Group 2	%	Total
1-10 years	12	60.00	15	75.00	27
11-20 years	6	30.00	3	15.00	9
20 years	2	10.00	2	10.00	4
Total	20	100.00	20	100.00	40
Mean	8.55		8.45		8.50
SD	6.86		5.98		6.35

SD: Standard deviation

Table 2: Distribution of study samples based on frequency of betel quid chewing habit per day

Frequency	Group 1	%	Group 2	%	Total
1-10/day	13	65.00	14	70.00	27
11-15/day	6	30.00	6	30.00	12
>15/day	1	5.00	0	0.00	1
Total	20	100.00	20	100.00	40
Mean	8.50		9.30		8.90
SD	4.59		3.37		4.00

SD: Standard deviation

out at annealing temperature of 67°C. PCR amplification was checked on 2% agarose gel. Further, the amplified PCR products were subjected to automated DNA sequencer to assess LOX G473A polymorphism. DNA sequencing results did not show LOX G473A polymorphism in any of the 3 groups [Figure 2].

DISCUSSION

The present study aimed at investigating the presence of LOX G473A polymorphism in group 1 (OSMF patients), group 2 (betel quid chewers without OSMF) and group 3 (healthy individuals). According to the current literature, only one study has been reported to find the association between LOX G473A polymorphisms and OSMF which was conducted on Taiwan population.[2]

The gene sequencing results did not show LOX G473A polymorphism in any of the 3 groups. Despite the implication of LOX in many diseases including inflammation and inflammatory diseases; fibrosis of distinct organs and fibrotic disorders; and cancer promotion and progression, there are only sparse reports of any mutations or epigenetic alterations in the *LOX* gene.[6]

Shieh *et al*., (2009)[2] conducted a study on Taiwan population to find the association between *LOX* gene polymorphism and OSMF in older male areca chewers. The presence of G to A polymorphism at nucleotide at 473, caused a non-conservative Arg158Gln change in the LOX amino acid sequence. They investigated the relationship between LOX Arg158Gln polymorphism and risk of OSMF. There was a borderline statistical significant difference in Arg158Gln genotype between control and OSMF patients. However, the G/A + A/A of LOX Arg158Gln in OSMF patients older than 50 years was statistically significantly higher than controls below 50 years. In the present study, most of the patients were less than 40 years old, which could be one of the reasons for absence of G to A polymorphism at nucleotide at 473.

In study conducted by Shieh *et al*.,(2009)[2] on Taiwan population, the mean duration of areca chewing habit in patients less than 50 years and more than 50 years was 15 years and 21 years, respectively. In the present study, the mean duration of areca chewing habit in group 1 (OSMF) was 8.5 years, which could also be one of the reasons for absence of G to A polymorphism at nucleotide at 473.

Shieh *et al*., (2007)[5] identified an upregulation of LOX and LOXL2 mRNA expression in areca-associated OSCC tissues and cell lines relative to their normal counterparts. Other previous investigations that focused mostly on breast carcinomas, identified that LOX can induce migration and invasion of malignant breast epithelial cells.

Shieh *et al*., (2009)[2] in his study included sample size of 83 OSMF patients and 216 areca chewers without OSMF as control. In the present study, the sample size included was 20 OSMF patients and 20 areca chewers without OSMF. The absence of *LOX* gene polymorphism in the present study could also be attributed to the smaller sample size. This necessitates further studies with larger sample size.

Another possible explanation for the variation in the *LOX* gene polymorphism between Taiwan population and Indian population could be the difference in genetic makeup.

LOX is a cuproenzyme that is essential for stabilization of extracellular matrixes, specifically the enzymatic cross-linking of collagen and elastin. Copper is essential for organic cofactor formation in lysyl oxidase.[7] Areca products contain high levels of copper (mean 302 nmol/g). Hence local factors such as composition of the quid, consistency of quid, duration and frequency of habit and whether saliva is expelled or swallowed after chewing, can affect the uptake of copper into the oral epithelium.[8] These factors may explain the marked variations seen in the copper levels within the OSMF group which in turn may affect the regulation of *LOX* gene and its polymorphism in OSMF patients. However, in the present study these factors were not considered.

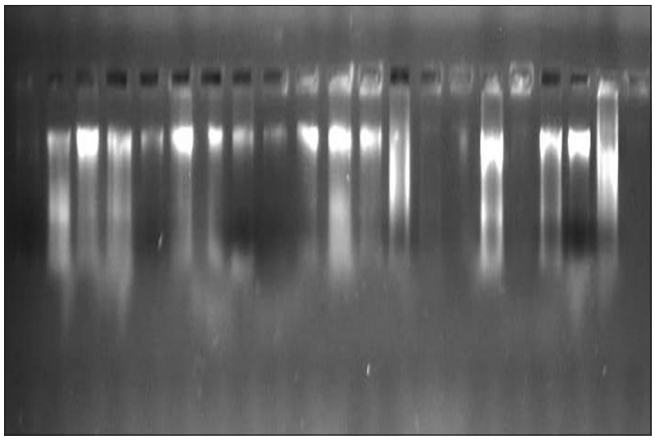

Figure 1: Gel electrophoresis showing DNA bands

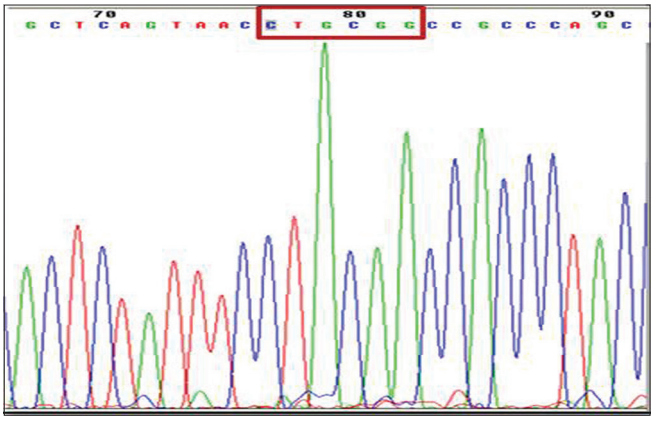

Figure 2: Electrophoregram showing CTGCGG instead of CTGCAG (box) indicating absence of G to A polymorphism

LOX coding region contains seven single nucleotide polymorphism (SNP) sites that includes C225G, G409C, G473A, C476A, G816A, T924G and A1135G. Among all the SNP sites in LOX coding region, G473A has the highest polymorphic frequency.[2] However, in the present study LOX G473A polymorphism was not observed.

SUMMARY AND CONCLUSION

Although LOX is implicated in many fibrotic disorders, there are only sparse reports of any mutations or epigenetic alterations in the *LOX* gene. Only one study has reported the presence of *LOX* gene polymorphism in OSMF patients. The study showed that only elder OSMF patients had significant *LOX* gene polymorphism. In our study, *LOX* gene polymorphism was not observed in OSMF patients which could be attributed to the difference in the genetic makeup between Taiwan and Indian population and on the factors like sample size, frequency and duration of habit, composition and consistency of the quid, which can affect the uptake of copper into the oral epithelium in turn affecting the regulation of *LOX* gene and its polymorphism. Hence, further studies are required to assess the presence of *LOX* gene polymorphism in OSMF.

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