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ORIGINAL ARTICLE

Association of TGF-B1 Gene Polymorphism with Oral Submucous Fibrosis

MUHAMMAD AKRAM[1], SADIQ ALI[2], MUHAMMAD NAUMAN[3], ALI ABU BAKAR[4], ABBAS RAZA SHAH[5], UROOJ KIRMANI[6], NAVAIRA TARIQ[7]

[1]*Consultant Oral and Maxillofacial Surgeon, Zaheer Hospital, Sheikhupura, Punjab*
[2]*Associate Professor, Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, The University of Lahore*
[3]*Assistant Professor, Department of Oral and Maxillofacial Surgery, Faryal Dental College, Sheikhupura, Lahore*
[4]*Consultant Oral and Maxillofacial Surgeon, Doctors Hospital Lahore.*
[5]*Private Dental Practice, Lahore*
[6]*Postgraduate Resident, Department of Orthodontics, Akhtar Saeed Medical College, Lahore*
[7]*Dentist, Derma & Dental Clinic, Lahore*
Correspondence to: Dr. Sadiq Ali, E-mail: sadiqali.glt79@gmail.com, Cell: 0314-4264682

ABSTRACT

Objective: To evaluate the association of TGF-β1 gene polymorphism with oral submucous fibrosis.
Study design: Case-control study
Place and duration of study: Department of Human Genetics and Molecular Biology, The University of Health Sciences Lahore and Department of Oral & Maxillofacial Surgery, Medical University of Montmorency, Lahore.
Methodology: Two hundred participants were divided into two groups of 100 participants, those with normal mucous membranes and those with clinically confirmed oral sub-mucosal fibrosis (OSF). Total genomic DNA was isolated by drawing blood samples.
Results: There was a significant difference in the genotypic frequency distributions of TGF-β1 gene polymorphisms between OSF patients and controls. Oral sub-mucosal fibrosis patients had a higher percentage of the CC genotype (P=0.040) compared to controls. In OSF cases, the proportion of TT, CT, and CC for the TGF-β1 gene was 10.0%, 34%, and 56%, respectively, compared to normal controls which had 16%, 49%, and 35% (P=<0.05) respectively. This suggests a significant relationship between the genotypic distribution and OSF cases (P=0.040). The OR for CC is 2.56 with p-value = 0.040 that indicates that patients with CC gene have 2.56times higher risk for disease as compared to patients with CT and TT genotypes.
Conclusion: The TGF-β1 gene polymorphism was shown to be statistically significantly associated with OSF.
Keywords: TGF-β1 gene, Gene polymorphism, Oral sub mucous fibrosis.

INTRODUCTION

"Oral submucous fibrosis" was coined by Pindborg and Sirsat in 1966 and is in use to this day. They provided a more precise definition as "a slowly progressing chronic condition that can impact any area of the mouth and occasionally the pharynx."[1] In oral submucous fibrosis (OSMF), a chronic premalignant disorder, the lamina propria undergoes fibroblastic transformation, and the oral mucosa becomes rigid. This, in turn, causes a functional impairment of the mouth.[2]

Among the many regions of the globe affected by OSF, South and Southeast Asia rank high. The exact cause of OSF is unknown; however researchers suspect a complex interplay of factors. Among the many contributing elements are areca nut chewing, chilli consumption, genetic and immunologic mechanisms, and dietary deficits.[3] It has been proposed that transforming growth factor-β (TGF-β) is the primary cause of fibrosis, since it is known to play a pivotal role in the development of the disease by increasing collagen formation and decreasing matrix degradation pathways.[4]

The growing factor super-family includes TGF-β. Cells release three distinct forms of TGF-β into the extracellular matrix: TGF-β1, TGF-β2, and TGF-β3.[5] As members of the TGF super-family, they play an important role in regulating gene expression, cell migration, differentiation, and proliferation; as a result, they are involved in fibrotic and reparative responses. Multiple organ fibrosis was shown to be associated with an elevation of TGF-β isoforms, which suggests that it has a strong modulatory influence on fibrosis.[6,7] The fibrotic action of TGF-β1 and TGF-β2 is strong, but the anti-fibrotic impact of TGF-β3 is more pronounced.[8]

According to reports, eating areca nuts controls TGF-β1 signalling in epithelial cells, which impacts surrounding fibroblasts, activates canonical downstream SMAD signalling, causes mesenchymal contact, and ultimately leads to fibrosis.[9] We need scientific study to determine the link between the TGF-β1 polymorphism and OSF at the molecular level, since no studies have been published in our community to far. Hence, future researchers may find this work useful in determining the molecular level involvement of TGFβ1 polymorphism in OSF vulnerability.

MATERIALS AND METHODS

The present investigation included 200 participants, with 100 serving as healthy controls and 100 as patients with clinically confirmed oral sub mucous fibrosis (OSF). The research was conducted at two different institutions: the University of Health Sciences Lahore's Department of Human Genetics and Molecular Biology and the Oral and Maxillofacial Surgery Department at de, Montmorency, Collage of Dentistry Lahore. The study used a sample of 5 ml of blood that included EDTA from patients who had clinically proven oral submucous fibrosis and restricted mouth opening. The DNA was extracted from the blood using the conventional phenol chloroform technique. Electrophoresis was used to quantify the amount of DNA after extraction. The technique relies on measuring DNA and verifying its integrity. Primers were developed for use in PCR-RFLP reactions of the TGF β1 collagen-related gene. A set of primers were prepared and manufactured in the following manner. R.5'.GGTCACCAGAAAGAGGAC.3' and F.5'. CCCGGCTCCATTTCCAGGTG-3'.

For the stock solution, the primers were diluted to 100 pM. Additional dilutions of 5 pM were also made for use. DNA amplification was done by conventional PCR (Polymerase Chain Reaction) with Total reaction volume of 20.3 µl. This total reaction volume constitutes of 12 µl of master mix. 2X PCR Master Mix (Enzynomics) was used comprising PCR buffer, 3Mm Mg, 0.4mM each dNTPs and 0.2 unit/µl Taq polymerases. Reaction mixture was further added by 2.5 µl of DNA, 0.4 µl of each forward and reverse primer equalized up to 5 µl by dH_2O.Denaturation of DNA was initiated at 96°C for 4 minutes. Different annealing temperatures ranging from above to below the Tm of forward and reverse primers were tried in PCR to get a temperature where amplification was optimum and 60.5C temperature was selected to be best/optimum temperature for PCR amplification with this primer pair. Annealing temperatures 60.5C were optimized for timings in ranges of 30-32 seconds. Extension was carried at 72°C

for 1 minute and cycle was repeated. Every PCR reaction has about 35 cycles and each run initiated with starting hold of 45 seconds at 96°C. Comprehensive description of PCR cycles and timings were presented in Fig. 1.

Amplification by PCR: Following tuning of conditions, a typical thermal cycler was used to amplify DNA samples from both patients and controls. A total of 20.3 ml of reaction volume, including 17.8 ml of reaction mixture and 2.5 ml of genomic DNA, was used for amplification.

Genotyping: Electrophoresis using 2.5% agarose gel was performed on the digested Restriction Fragment Length Polymorphism (RFLP) DNA products. The Gel Doc Ez imager (Bio Rod) equipment was used to view the gel. Depending on the genotype of the patient or control sample, three distinct bands were observed: 808bp, 617bp, and 191bp. The sample was classified as TT homozygous when a single band of 808 bp was seen; CC genotype was denoted by two bands, 617 bp and 191 bp. The three bands that were judged to be heterozygous for the CT genotype were 808 bp, 617 bp, and 191 bp (Fig. 2). The data was entered and analyzed through SPSS-25.

Fig. 1: PCR cycling conditions programmed in the thermal cycler

L 1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 2: Agarose gel electrophoresis showing the genotypes for TGF-β1gene polymorphism
L represents the 100 bp ladder Lane 4.11: Homozygous (TT)
Lane 3.9. 10: Heterozygous (CT) Lane 2. 5.6.7.12.13: Homozygous (CC)

RESULTS

Distribution of mouth opening in OSF cases is presented in Fig. 3. The gender distribution for OSF cases included 57 males and 43 females while for controls 68 were males and 32 females (Fig. 4). The eating habit of supari, paan and gutkha were also observed in both OSF patients and controls and found difference between OSF cases and healthy controls. Habit of supari eating 94% seen was more prevalent in OSF cases followed by the habit of pan eating (49%) and gutkha eating (9%) [Table 1].

Genotypic frequency distributions of TGFβ1 gene polymorphisms in OSF patients and controls were considerably different. High percentage of CC genotype was seen in OSF patients (P=0.040) as compared to controls. The proportion of TT, CT, CC, for TGFβ1 gene in OSF cases were 10.0%, 34 %, 56% while for normal controls it was 16 %, 49 %, 35 % (P = <0.05) respectively, suggesting significant relationship between genotypic distribution and OSF cases (p-value =0.040) . The OR for CC is 2.56 with p-value = 0.040 that indicates that patients with CC gene have 2.56times higher risk for disease as compared to patients with CT and TT genotypes (Tables 2-3, Fig.5).

Table 1: Frequency of different variables in patients of OSF

Variable	Yes	No
Supari	94 (94.0%)	6 (6.0%)
Pan	49 (49.0%)	51 (51.0%)
Gutkha	9 (9.0%)	91 (91.0%)

Table 2: Association of TGF-β1 gene between both groups

TGF-β1Gene Allele	OSF Cases	Healthy Controls	OR (95%-CI)	p-value
TT	10	16	Refence	
	10.0%	16.0%		
CT	34	49	1.11	0.821
	34.0%	49.0%	0.45 – 2.74	
CC	56	35	2.56	0.040*
	56.0%	35.0%	1.05 – 6.27	

*Significant

Table 3: Association of TGF-β1 gene allele frequency between both groups

TGF-β1Gene Allele	OSF Cases	Healthy Controls	Total	Frequency
T	54	81	135	33.7%
C	146	119	265	66.25%
CC/TT	200	200	400	

L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 3: L represents the 100 bp ladder
Lane 1, 3: Homozygous (TT)
Lane 2.4.8.10.11.12.14.: Heterozygous (CT)
Lane 5.6.7.9.13.15.16.17.18: Homozygous (CC)

Fig. 4: Bar chart showing gender distribution between study groups

Fig. 5: Distribution of mouth opening in OSF Patients

DISCUSSION

The primary cause of OSMF, a potentially cancerous condition, is eating areca nuts. Burning while eating hot, spicy food, limited mouth opening, and blanching of the oral mucosa are its hallmarks. Increased synthesis of collagen, decreased breakdown of collagen, and aberrant cross-linking are thought to be the main pathophysiological features of this persistent, sneaky illness. Pro- and anti-fibrogenic cytokines, including TGF-β, CTGF, Endothelin-1, and bone morphogenetic proteins 4 and 7, are delicately balanced to regulate collagen formation.[5] While its prolonged expression may result in excessive fibrosis, which is essential for the formation of OSF, the temporary presence of TGF-β aids in tissue healing.[8,10]

The research by Singh et al. found that the intermediate grades of OSMF had the greatest serum TGF-β1 levels. This study's findings are consistent with our own[11] In a number of additional investigations, three of them reported elevated TGF-β1 expression in the advanced stages of OSF. Our results and those of these investigations are rather comparable.[12,13] Our data supports earlier findings that pathological tissue fibrosis, particularly OSF, is associated with elevated TGF-β1 levels.[14,15] Both the illness and control groups in the research had a male majority. The ratio of males to females in the illness group was 2.1:1. Gondivkar et al[16]. also posted similar findings on male predominance. None of the patients belonged to a high status group, and the majority (83%) were from a low income category. According to the findings of Gondivkar et al.[16] The mouth openings of 73% of the research participants were between 11 and 20 mm, whereas 21% and 6%, respectively, had mouth openings between 21 and 30 mm and 1 to 10 mm. Numerous more research corroborate the mouth opening results of this one.[17,18] Our research found that 94% of OSF sufferers had a practice of supari eating, which was followed by pan eating (49%) and gutkha eating (9%).[19,20]

CONCLUSION

The connection between OSF and the TGF-β1 gene polymorphism was noted. The pathophysiology of OSF illness is probably influenced by the polymorphisms in the TGF-β1 gene

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