

DEVELOPMENT OF AN ORAL SUBMUCOUS FIBROSIS-SPECIFIC HAPLOTYPIC SIGNATURE – HOW FAR ARE WE?

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ABSTRACT

Oral submucous fibrosis (OSF) is a scarring pre-malignant condition with the principal causative agent being the chewing of areca and areca-based products and is considered to be a significant, growing public health problem. Not all chewers are afflicted with this condition. Hence, there is a genetic predisposition to this condition. Hence, this review catalogs the genetic polymorphisms that have been specifically reported to be associated with OSF. Also, it underscores the necessity for controlling for ethnicity and chewing history, apart from age and gender, and also reiterates the unmet need for global multi-centric molecular genotyping studies to develop a comprehensive OSF-specific haplotypic signature.

Keywords: Oral submucous fibrosis; Genetic polymorphism; Collagen metabolism genes; Collagen cross-linking genes; Mechanistically-linked polymorphisms, Associative polymorphisms.

INTRODUCTION

OSF is an oral pre-malignant, scarring disorder of the oral cavity affecting people mainly of South Asian or South East Asian origin [1][2]. The principal causative agent is strongly believed to be areca nut in its various processed and unprocessed forms, while other factors like nutritional deficiencies, excessive consumption of chilies, autoimmune factors may play a contributory role [1]. In this regard, the relative risk for this condition (highest for ghutka chewers) increased with an increase in frequency of chewing (15 times daily) upto a duration of 4 years of [3].

Since not all chewers exhibit this condition, a genetic predisposition [4][5], albeit of different degrees, is scientifically plausible and is supported by several papers in the peer-reviewed literature. This review is meant to bring the reader up-to-date on the information pertaining to DNA polymorphisms for the development and/or refinement of the OSF-specific haplotypic signature. Also, the review will underscore the need to further extend the existing knowledge about genetic polymorphisms for the identification of new polymorphic variants and their systematic, concomitant correlation with OSF disease severity, based on clinical and immunohistopathological findings.

Genetic Polymorphisms mechanistically related to collagen turnover and OSF susceptibility

It has been fairly well established that OSF is a disorder involved in collagen turn-over. Hence, genetic variants in genes associated with the synthesis of this extra-cellular matrix molecule and its breakdown would tend to alter the equilibrium between collagen synthesis and breakdown, thereby contributing to an increase in susceptibility to this condition. Among the growth factors involved in this process, TGF- β plays an important role in the dynamics of the process of synthesis and degradation of the matrix [6], of which collagen is an important component as well as for inducing the myofibroblastic phenotype [7][8]. It has been documented that variability in TGF- β 1 protein production is associated with SNPs in the gene encoding for this growth factor. Further, polymorphisms in this gene has been associated with pathological fibrosis [9] which can be inferred from its known ability to regulate pro-collagen-related genes (COL1A2, COL3A1, COL6A1, COL6A3, COL7A1 as well as an increase in the pro-collagen protein levels as well as an up-regulation of lysyl oxidase activity [10].

A comprehensive PCR-RFLP polymorphism study, carried out in Taiwan, has provided evidence for correlating molecular polymorphism-based studies in terms of collagen metabolism and cross-linking pathways with study participant stratification into high and low exposure groups for the identification of the best possible combination of high-risk genotypes relevant

mechanistically to the pathogenesis of this pre-malignant condition. In this regard, the TT, BB, AA, CC, GG, and AA and CC, AA, TT, CC, AA, and AA respectively for the high and low exposure groups were associated with the highest risk for OSF. The genes, respectively for the genotypes listed were collagen1A1, collagen 1A2, collagenase-1, TGF β 1, lysyl oxidase, and cystatin C were, respectively, for the low-exposure group, and for the high exposure group [11]. This approach also provides a basis for the possible effects of areca-nut mediated genetic changes altering the risk profile. Further, this approach may help in identifying the proportion of OSF patients that suffer from this condition as a result of areca nut chewing alone (attributable proportion). Last but not least, this experimental design highlights the importance of community-based apart from the often studied hospital-based ones.

Further, TGF- β 1 -a pleiotropic cytokine, among other functions including immuno-suppression, is associated with an increase in TIMP genes (TIMP-1 and TIMP-2) as well as a Plasminogen activator inhibitor I. Hence, polymorphism-associated changes in this growth factor may alter an important upstream and early event in the fibrogenic process, despite the exact mechanism not being entirely clear since a concomitant increase in MMPs has also been reported. A polymorphism study (TGF- β 1) was done on DNA samples from human blood of OSF patients of the same ethnicity. The polymorphisms studied were in exon-1 (three of them - Arg25Pro(G915C), Leu10Pro(T869C), Glu47Gly(A979G), two each in the 5'UTR (C \rightarrow T(rs13306708) and G \rightarrow A (rs9282871) and promoter regions (C-509T and G-800A) respectively. In the 5'UTR region, the C \rightarrow T transition (rs13306708) was found to be statistically significant in comparison with the results obtained from control samples [9]. Promoter polymorphisms, in MMP-2 (-1306 C/T) and MMP-9 (-1562C/T) (proteases which can cleave TGF- β 1 with different proteolytic cleavage products) potentially altering gene transcription, was found not to be associated with OSF, in comparison with HNSCC, thereby providing a molecular basis for molecular haplotyping as a means for patient/study participant stratification [12]. This lack of association is consistent with the results reported from Taiwan, when similar genotyping studies were performed in the MMP-9 promoter region (-1562 C/T) in genomic DNA from human blood samples drawn from male areca chewers [13]. However, results of this nature provide a basis for extending the haplotype analysis to including other polymorphic sites (MMP-2 promoter region-168G/T; MMP-9 promoter microsatellite -90 (CA)₁₄₋₂₄) [14] for a more thorough evaluation of the experimental data. In the case of MMP-1 promoter-based PCR-RFLP-based analysis for both 1G/1G as well as the 2G/2G genotypes (-1607 1G/2G) in an Indian population, the latter genotype was associated significantly with OSF as well as with an increase in enzymatic activity. Further, this work also provides a scientifically plausible basis for areca nut chewing and alcohol possibly enhancing the expression of this gene in people afflicted with OSF [15].

However, this study is in contrast to the results reported earlier by a group from Taiwan, wherein the 2G genotype (-1607 2G-->1G) was not associated with OSF. However, since the same paper has shown an association with OSCC and NBSCC, this type of functional genotyping would aid in patient/study participant stratification for subsequent suitable therapeutic intervention. In this paper, Genomic DNA was isolated from blood samples, and a PCR-based amplification followed by denaturing high pressure liquid chromatography (dHPLC). was performed. The insertion/deletion (-1306C-->T) MMP-2 promoter polymorphism (thereby possibly affecting transcription, due to an alteration in the binding site of a transcription factor [Sp1]) was studied. Of the genotypes (CC, TT and CT) studied, no association of the CC genotype with OSF was observed [16].

A similar case-control PCR-RFLP genotyping study, involving the promoter region of the MMP-3 promoter region, insertion/deletion polymorphism (-1171 5A->6A) has shown a statistically significant association with OSF with the Odds ratio values indicative of a 2-fold risk for this pre-malignant condition, in the case of the 5A genotype for individuals less than 45 years of age. However, the 6A/6A allele was associated with OSF regardless of the age of the study participants [17]. This work further substantiates the results reported from Taiwan for a similar Insertion/deletion (-1171 5A-->6A) polymorphisms in the promoter region of the same gene in DNA samples from blood with the 5A genotype being significantly correlated with OSF [18].

Apart from genes involved in collagen synthesis and breakdown, lysyl oxidase is considered to be the major enzyme involved in cross-linking processes as well as for the organization of the ECM. Hence, polymorphic variants in the gene coding for this enzyme may be responsible for shifting the equilibrium towards increased collagen deposition. A study reported an association between a non-conservative change (Arg158Gln), due to a G->A polymorphism at the 473rd position of the nucleotide, with a better correlation in individuals older than 50 years, after both G/A and A/A allelotypes were taken into account for the haplotype-association study [19]. Approaches of this nature would also help in better patient stratification.

Loss of heterozygosity hotspots, identified by high resolution genome-wide Affymetrix Mapping Single Nucleotide Polymorphism (SNP)-based studies, were substantiated in around 50% of the OSF cases, which were hitherto identified in HNSCC patients. Hence, studies of this nature, when sufficiently validated in representative populations of different ethnicities, may improve the predictability of the molecular haplotype signature in terms of their relative contribution to the overall genomic instability [20].

Genetic Polymorphisms associated with OSF

Since areca nut is considered to be the principal causative agent for OSF, polymorphisms in genes encoding for enzymes involved in activation and/or detoxification of areca nut and its alkaloids may alter the genetic susceptibility to this condition. Molecular genotyping with PCR-RFLP has shown that polymorphisms in CYP1A1m2 (T3801C); CYP1A1m1 (A2455G) and CYP2E1 PstI site (nucleotide 21259) were associated with OSF as reflected by their statistically significant Odds ratio values in samples taken from an Eastern Indian population [21]. The results reported in an earlier study, in a North Indian population, indicate there was a greater than seven (7) fold increase in risk for OSF for those individuals who had inherited the null genotype (absence of GSTM1 and GSTT1 genotypes). Also, the combination of the null genotype with the presence of the CYP1A1 wild type allele correlated with the group with the highest risk. Hence, it can be inferred that the activation of areca nut compounds into its active metabolites by enzymes including CYP1A1, coupled to the absence of key enzymes involved in detoxification, may have contributed detoxification of areca nut

to the observed increased risk [22]. An earlier study reported from India found a significant correlation between the increased products of oxidative damage (ceruloplasmin and malonaldehyde) and the decreased levels of antioxidant enzymes. The decreased in the levels of the enzymes may be due to the observed polymorphisms in GSTM1 and T1 genes [23]. PCR-RFLP-based methodology, in a North

Indian population, was used to genotype polymorphisms in the same genes and the presence of the null genotype singly or in combination were found to be significant risk factors for OSF. The absence of both GSTM1 and GST T1 conferred a greater than 7 fold risk for this condition [24]. While studies of this nature provide strong evidence for the involvement of GST variants and risk for OSF, more prevalence studies documenting the relative frequencies in control populations [25][26][27]. would enable us to better flag those individuals with an increased risk for OSF that could be attributable, at least in part, to be due to polymorphisms specifically in this gene associated with areca nut detoxification.

A allelotyping-based study (S- short \leq 25 repeats; M - medium 26-30 repeats, L -long \geq 31 repeats) involving (GT)_n repeats in the Heme Oxygenase promoter, (HO-1) has shown that the L allelotype (\geq 31 repeats) had no association with OSF, after adjusting for age. This may be explained by the possibility of the protective functions of this enzyme not being able to counteract the areca nut-mediated oxidative stress. However, this study provides a basis for studies to better understand the mechanisms involved in OSF pathogenesis, apart from dissecting the age-related confounding variables [28].

While the aforesaid polymorphisms pertained to the Phase I category of enzymes, polymorphisms in genes encoding for Phase II enzymes have also been documented. For example, SNP in a gene for N-acetyl transferase (NAT2 gene) can affect detoxification thereby leading to the formation of covalently-linked DNA adducts. Consequently, any aberrations in the genes encoding for repair of bulky adducts of this nature, in the same individual, can have a synergistic effect on the disease condition. This aspect has been demonstrated in a study involving XRCC1, XRCC3 and NAT2, apart from NAT1. Genotyping was done at 7 genetically variant sites (4 in the NAT2 gene - NAT2 481 (C > T; silent), 590 (G > A; Arg197 > Gln), 803 (A > G; Lys268 > Arg), 857 (G > A; Gly286 > Glu), 2 on the XRCC1 gene (18067 (C > T Arg 194 > Trp), 28152 (G > A Arg 399 > Gln) and one in the XRCC3 gene) (26304 (C > T Thr 241 > Met)). The greatest univariate effect, using the multifactor dimensionality reduction approach, was reported for XRCC3 Thr 241 Met, followed by XRCC3 Thr 241 Met - NAT2 A857G. Studies of this nature, involving a combination of SNPs, are very essential to increase the predictive value of the SNP-based signatures. At the same time, this approach is consistent with the known areca nut-mediated mechanism of pathogenesis for OSF [29].

OSF is considered by some to be primarily due to the systemic actions of areca alkaloids on the fibroblast (via the vasculature supplying the buccal mucosa) mainly contributing to the fibrotic changes in the underlying mesenchyme resulting in a decrease in the visco-elastic properties. Others believe that the initial stimulus may be primarily due to the physical and chemical actions of areca and/or its chemical constituents like arecoline, on the keratinocyte contributing to its hypoplasia apart from paracrine signaling with the underlying fibroblasts. In this regard, one of the major enzymes involved in inflammation and pain involves COX-2 due to the production of prostaglandins, prostacyclin and thromboxanes. Hence, polymorphic variants would be expected to alter the inflammatory response, thereby increasing the risk of the individual with OSF. In a study from Taiwan, genotyping of COX-2 (765C allele vs. -765G/G genotype) was found to have an OR of 3.20 in comparison with the controls, after adjustment for possible confounding factors [30].

A Taiwanese study, using the same methodology, followed by logistic regression analysis (multivariate approach), has reported that FAS A(-1377)-G(-670) genotype was associated with a greater than 2-fold increased risk of susceptibility to this condition in comparison with the G(-1377)-A(-670) haplotype [31].

Exon 1 +49 polymorphism analysis by RFLP showed a positive correlation of the G allele for the CTLA-4 gene (encoding for a protein known to be a negative regulator of T cell activation), in contrast with the A/A homozygote, in OSF patients, when compared with control data [32], thereby implying a possible similarity in the mechanisms akin to what is observed in terms of susceptibility to various autoimmune diseases.

A number of research groups in India and overseas are involved in molecular genotyping association studies with clinical and histopathological/molecular [33] correlates. These promising results and the lack of well –designed multi-centric OSF projects, coupled with the variations in ethnicity and chewing habits, further provides a unmet need to systematically analyze known DNA polymorphisms and correlate them with patient stratification based on disease severity (IHC data) . However, issues pertaining to linkage disequilibrium and gene-gene interactions acting as possible confounding factors underscore the need for high resolution genotyping and computational methodologies to be incorporated for OSF-specific genotypic studies, as well as the use of modern SNP discovery tools. Further, haplotypic signatures need to be combined with differential expression-based information [6] for a more meaningful interpretation of the molecular aspects involved in the pathogenesis of OSF. Also, this would help the researchers in the field to possibly arrive at a “minimal haplotype genome set” that would be necessary and sufficient to be better risk predictors for OSF, after taking into account ethnic variations , correlated with the questionnaire-based chewing history and lifestyle-related information. This molecular multi-centric approach would help in flagging individuals for targeted and early intervention and would supplement the concerted efforts of the health care specialists/governmental and non-governmental organizations/other interest groups in terms of public health messages about the adverse effects of chewing areca nut in its processed and unprocessed forms.

Declaration of interest

There is no conflict of interest reported by the author. The author was responsible for this article.

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