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A potential association between mutations in the *iNOS* cDNA 3′ stretch and oral squamous cell carcinoma - A preliminary study

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ABSTRACT

Oral cancers are linked with tobacco, areca nut and alcohol addictive proclivities. The oral squamous cell carcinoma (OSCC) comprises more than 90% cases of oral cancers. The *Homo sapiens* inducible nitric oxide synthase (*iNOS*) gene plays crucial role in OSCC pathologies. The given study analyzed the nucleotide mutations existent within two targets of interest namely *iNOS* cDNA 5′ stretch and *iNOS* cDNA 3′ stretch, using cDNA pool of cancer and pre-cancer oral biopsies employing Q PCR-Tm calling approach. The *iNOS* cDNA 5′ stretch encompassed exons 4 and 5 and *iNOS* cDNA 3′ stretch embodied exons 18, 19, and 20. Total RNA was isolated from oral biopsies: cancer biopsies (OSCC lesions - adjacent normal tissue (ANT) from fifteen OSCC cases), pre-cancer biopsy (ANT of chronic inflamed lesion) and normal oral mucosa (control). The total RNA samples were quantified and PCR based assurance was accomplished for genomic DNA absence. The PCR based integrity assessment was performed using synthesized cDNA samples. The Q PCR-Tm calling was performed using specific primers for normalizer gene (*GAPDH*) and the targets of interest. The amplicons for normalizer gene and *iNOS* cDNA 5′ stretch respectively showed Tm values of ~80 °C and ~81 °C in both control and diseased cDNA pools. On the contrary, the *iNOS* cDNA 3′ stretch showed amplicons with multiple Tm values in diseased (pre-cancer and cancer lesions) cDNA pools, varying significantly from the control (~85 °C). The results indicate existence of highly mutated *iNOS* cDNA 3′ stretches in the cDNA pool of oral cancer and pre-cancer lesions and adjoining visibly normal tissues, thereby raising a need for rigorous experimental efforts for unraveling the functional association(s) between the mutations in *iNOS* cDNA 3′ stretch and oral precancer-cancer pathophysiologies.

1. Introduction

Oral cancer, a complex disease, has shown marked increment in the incidence and mortality rates globally (Shield et al., 2017; Chi et al., 2015). The developing and underdeveloped countries are suffering from raised addictive proclivities for major oral cancer etiologies: tobacco (smokeless and smokable), areca nut and alcohol (Goyal and Bhagawati, 2016). The constituents of tobacco, areca nut and alcohol are teratogenic and/or carcinogenic metabolites, capable of inducing mutations in the genomic DNA of exposed cells (Pirini et al., 2015; Xue et al., 2014; Peng et al., 2016; Pflaum et al., 2016). More than 90% cases of oral cancers are diagnosed as oral squamous cell carcinomas (OSCC) (Lawal et al., 2017). The molecular attributes of inflammatory pathways are major contributors to initiation and progression of oral cancers (Reuter et al., 2010; Choudhari et al., 2014; Patel et al., 2016). The cellular antioxidant machinery, physiologically combating the DNA damages induced by raised oxidative stress, gets weakened during oral carcinogenesis (Juneja et al., 2017; Gokul et al., 2010).

The inducible nitric oxide synthase (*iNOS*) gene comprising 47 k bases (kb) is located at the chromosomal loci 17q11.2 (NCBI). The canonical transcript (4 kb) transcribed from *iNOS* constitutes of twenty seven exons (Ensembl). Under normal physiology, non-canonical transcripts of *iNOS* are produced by alternate splicing of the precursor RNA (Eissa et al., 1996; Kröncke et al., 1998). Eissa et al. have reported the presence of four alternate splice sites functional during post transcriptional modification of the primary transcript (Eissa et al., 1996). The canonical transcript transcribed from *iNOS* encodes a large protoporphyrin IX heme protein, comprising of 1153 amino acid residues (a.a.). The canonical iNOS protein has two distinct domains (i) N-terminus oxygenase domain: 1 a.a. to 504 a.a. (ii) C-terminus reductase domain: 716 a.a. to 1153 a.a. (Alderton et al., 2001). The latter domain contains specific binding regions for coenzymes (a) flavin mononucleotide (FMN): 537a.a. to 687 a.a., (b) flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH): 716 a.a. to 1203 a.a (Alderton et al., 2001). The former domain interacts with the cofactor tetrahydrobiopterin (BH4) (Alderton et al., 2001). The

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residue stretch 509 a.a to 529 a.a., forms binding region for calmodulin causing rapid transfer of electrons from reductase to oxygenase domain, maintaining the active form of iNOS protein (Alderton et al., 2001). The canonical iNOS protein catalyzes NADPH dependent production of nitric oxide (NO) and citrulline using L-arginine (amino acid) and O_2 as substrates (Lind et al., 2017; Lechner et al., 2005; Naureckiene et al., 2008). The iNOS protein mediates innate immune responses against microbial (bacterial and fungal), viral, and parasitic infections (Lind et al., 2017; Lechner et al., 2005). *iNOS* gene over-expression and associated NO built-up have been postulated as the key molecular etiologies for initiation and progression of oral precancer and cancer (Ambe et al., 2016; Rajendran and Varkey, 2007). The saliva of tobacco smokers and chewers has significantly increased NO levels (Preethi et al., 2016; Cooper and Magwere, 2008; Toh et al., 2010). In comparison to rodent cells, human cells hold higher stringency towards cytotoxic aftermaths mediated by NO. In human cells, NO functions as the crucial regulator of various processes namely cell cycle, cell signaling, and apoptosis (Kröncke et al., 1998; Burkart et al., 2000; Traister et al., 2002; Kuzin et al., 1996; Napoli et al., 2013; Villalobo, 2007; Smith et al., 2013; Alland and Chakravorty, 2017). The long term intake of ethanol relates with over-expression of *iNOS* gene and increased activity of the encoded protein (Cooper and Magwere, 2008; Toh et al., 2010; Yuan et al., 2006; Deng and Deitrich, 2007). The enhanced activity of iNOS protein has been reported in oral precancer and cancer pathologies (Rosbe et al., 1995; Brennan et al., 2000). The expression of *iNOS* gene relates directly with OSCC grade and metastasis while inversely with disease outcome prognosis (Yang et al., 2015; Brennan et al., 2001). The increased activity of iNOS protein within normal margins surrounding OSCC lesions implicates to the involvement of *iNOS* gene in tumor microenvironment (Morelatto et al., 2014). In accordance to Le et al. abundant iNOS protein and NO exhibits macrophages mediated anti-tumor activity by inducing growth inhibition and metastasis restriction in cancer cells (Le et al., 2005). Moreover, chemopreventive agents induce production of NO, triggering apoptosis in cancerous cells (Vahora et al., 2016). The cancer cells with reduced intracellular NO show multidrug resistance (Riganti et al., 2005). The perniciously abundant NO inhibit functional attributes of topoisomerases, curtailing DNA repair machinery, thereby making cellular genome more prone to damages (Sinha et al., 2017; Sharma et al., 2015).

The current scenario highlights the blurriness existent between iNOS (both gene and the encoded canonical and non-canonical isoforms) and OSCC pathogenesis (Yang et al., 2015). Moreover, a quintessential need stands for precise scientific efforts targeting the molecular defects or mutations, caused by addictive etiologies of OSCC, in the coding region of *iNOS* gene during oral carcinogenesis (Le et al., 2005; Connelly et al., 2005). In the present work we have performed Q PCR – Tm calling strategy based analyses of the variations existent within *iNOS* cDNA 5′ and *iNOS* cDNA 3′ stretches transcribed in total RNA/cDNA pool of oral precancer and cancer lesions (Table 1). *GAPDH* was used as the normalizer gene and normal oral mucosa was included as the control sample (Khowal et al., 2017). The *iNOS* cDNA 5′ stretch comprised of canonical exons 4th and 5th and the *iNOS* cDNA 3′ stretch composed of canonical exons 18th, 19th and 20th. The evaluated Tm values for amplicons from *GAPDH* and *iNOS* cDNA 5′ stretch were similar in both diseased lesions and control; whereas Tm values evaluated for amplicons from *iNOS* cDNA 3′ stretch showed significant disparities in diseased lesions compared to the control; thereby showing the potential association between genetic variability of *iNOS* cDNA 3′ stretch and oral precancer - cancer pathologies.

2. Methodology

2.1. Ethical statement

The study plan was approbated by the Jamia Hamdard Institutional Ethics Committee (Jamia Hamdard -, INDIA). The study involved cases with oral pathologies: chronic inflammation (one case) and oral squamous cell carcinoma (fifteen cases), attending the Out-patient Department of ENT (HAH Centenary Hospital, HIMSR). The patient's consent for participation was taken prior to sample collection. The OSCC lesions were identified histopathologically as well differentiated (VII_1 to VII_4) and moderately differentiated (VIII_1 to VIII_8). The differentiation pattern of three OSCC lesions (VI_1, VI_2, and VI_3) remained undefined in the histopathological details. The clinical samples contributed by OSCC cases constituted of cancer lesions (patient oral biopsy: POB) and adjacent visibly normal tissues (ANT). The clinical sample contributed by the case I_1 suffering with chronic inflammation of oral mucosa constituted of ANT contiguous to the oral inflammatory lesion. All the cases (except VII_1) showed positive addictive history for tobacco, areca nut and/or alcohol intake. The normal/healthy oral mucosa was included as the control sample. Table 1 showcases the details of participating cases and the control sample.

2.2. Sample preparation

The total RNA isolation from the tissue samples was accomplished using RNA Surespin Kit (Nucleopore). The on-column DNase treatment was included in the total RNA isolation protocol of the RNA Surespin Kit (Nucleopore). The concentration and purity (A_{260}/A_{280}) of isolated total RNA samples were determined using Nanodrop Spectrophotometer (Thermo-fischer scientific) (R Desjardins and Conklin, 2011). The absence of DNA contamination in the total RNA samples was assured by performing PCR using *β-globin* specific primers (Table 2) (Saiki et al., 1988; Naqvi et al., 2004). PCR products were resolved on 2% agarose-1X TAE gel (0.5 μg/ml EtBr). The UV trans-illuminator gel documentation system was used for DNA band visualization. 400 ng total RNA template was subjected to cDNA synthesis using First strand cDNA synthesis kit (Fermantas). The cDNA integrity was checked by performing PCR using *GAPDH* cDNA specific primers (Table 2) (Martínez-Ramírez et al., 2016). PCR products were resolved on 2% agarose-1X TAE gel (0.5 μg/ml EtBr). The UV trans-illuminator gel documentation system was used for DNA band visualization. All PCR reactions were performed in duplicates. The RNA and cDNA samples were stored at −80 °C.

2.3. Q-PCR assay and Tm calling

The *iNOS* cDNA sequence (accession id: *ENST00000313735.10*) was retrieved from Ensembl. The primers for *iNOS* cDNA 5′ stretch and *iNOS* cDNA 3′ stretch were designed followed by *insilico* PCR using MFE primer2.0 (Table 2). The target region *iNOS* cDNA 5′ stretch (120 bp) comprised of nearly complete 4th exon and the 5′ end of 5th exon (Supplementary file 1). The target region *iNOS* cDNA 3′ stretch (101 bp) comprised of 3′ end of 18th exon, complete 19th exon and 5′ end of 20th exon (Supplementary file 1). The Q-PCR assay was performed with cDNA samples and specific primers for the normalizer gene (Wang et al., 2015) and the target of interests. *GAPDH* was used as the normalizer genes while *iNOS* cDNA 5′ stretch (120 bp) and *iNOS* cDNA 3′ stretch (101 bp) were separately analyzed as the targets of interest. The Q-PCR assay was performed on Light Cycler 480 (Roche) using 10 μl reactions constituting of 1 μl cDNA (fivefold dilution), 1 μl of specific forward and reverse primers (0.5 μM), 5 μl of 2× SYBR green master mix (Fermantas) and 2 μl nuclease free water. The reaction configurations were: (i) pre-incubation: 95 °C for 10 min; (ii) amplification: 52 cycles of denaturation (95 °C for 30 s), annealing (60 °C for 30 s), and extension (70 °C for 60 s); (iii) melting: 58 °C to 95 °C; (iv) cooling: 37 °C for 5 min. For assuring reproducibility each Q-PCR assay was performed twice and all reactions were conducted in triplicates. The Light Cycler 480 software version 1.5 was used for performing melting temperature analysis or Tm calling for the amplicons generated during Q-PCR by primer specific amplification reactions.