RESEARCH ARTICLE

A Novel Heterozygous Mutation (F252Y) in Exon 7 of the *IRF6* Gene is Associated with Oral Squamous Cell Carcinomas

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Abstract

Background: Interferon regulatory factor 6 (*IRF6*) is a transcription factor with distinct and conserved DNA and protein binding domains. Mutations within the protein binding domain have been significantly observed in subjects with orofacial cleft relative to healthy controls. In addition, recent studies have identified loss of expression of *IRF6* due to promoter hypermethylation in cutaneous squamous cell carcinomas. Since mutational events occurring within the conserved domains are likely to affect the function of a protein, we investigated whether regions within the *IRF6* gene that encodes for the conserved protein binding domain carried mutations in oral squamous cell carcinoma (OSCC). Materials and Methods: Total chromosomal DNA extracted from 32 post surgical OSCC tissue samples were amplified using intronic primers flanking the exon 7 of *IRF6* gene, which encodes for the major region of protein binding domain. The PCR amplicons from all the samples were subsequently resolved in a 1.2% agarose gel, purified and subjected to direct sequencing to screen for mutations. Results: Sequencing analysis resulted in the identification of a mutation within exon 7 of *IRF6* that occurred in heterozygous condition in 9% (3/32) of OSCC samples. The wild type codon TTC at position 252 coding for phenylalanine was found to be mutated to TAC that coded for tyrosine (F252Y). Conclusions: The present study identified for the first time a novel mutation within the conserved protein binding domain of *IRF6* gene in tissue samples of subjects with OSCC.

Keywords: *IRF6* - *IRF6* mutation - *IRF6* mutation in oral cancer - *IRF6* mutation in Indian cancer

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common forms of cancer, which accounts for over 30% of all cancers that are reported in India (Coelho et al., 2010). Despite the therapeutic advances that have been developed in the recent past, the five year survival rate continues to be ~50% (Warburton et al., 2007). In order to increase the survival rate, an in-depth understanding of the molecular mechanism of carcinogenesis is essential, which in turn would facilitate identification of early cancer detection biomarkers.

Genes involved in the regulation of interferon pathway have recently been identified as potential candidates as early cancer detection biomarkers (Rupesh et al., 2013). The level of messenger RNA expression of the interferon regulated gene-15 (ISG15) was earlier identified by us as a high confident biomarker (Rupesh et al., 2013). ISG15 is an ubiquitin-like molecule, which when conjugates with its substrate proteins, increases their stability by interfering with the ubiquitin/26S proteasome pathway (Zhao et al., 2005; Pitha-Rowe et al., 2007). Though ISG15 has been known to be induced by interferons, its observation in OSCCs suggests that other signaling components of the interferon pathway regulating diverse functions are also expected to contribute towards carcinogenesis. Indeed, genetic anolmalies of the interferon regulatory factors (IRFs), which belong to a family of transcription factors, have been identified in several carcinomas. Loss of expression of IRFs due to epigenetic modification of respective promoter regions have been observed with IRF5/7 in lung cancer (Li et al., 2011), *IRF6* in cutaneous cancer (Botti et al., 2011) and IRF8 in multiple myeloma (Tshuikina et al., 2008), nasopharyngeal, esophageal (Lee et al., 2008) and colon cancers (McGough et al., 2008). Most of the IRFs share among themselves a highly-conserved N-terminal helix-turn-helix DNA-binding domain and a C-terminal protein binding domain called as IRF associated domain (IAD) (Ozato et al., 2007). While the N-terminal DNA-binding domain is essential for IRF proteins to bind with the respective promoter region of genes it regulates, the C-terminal IAD is required for protein-protein interactions (Tamura et al., 2008; Savitsky et al., 2010). Of the nine IRFs (IRF1 to IRF9) that have

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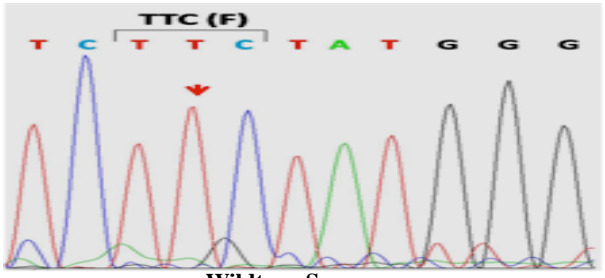
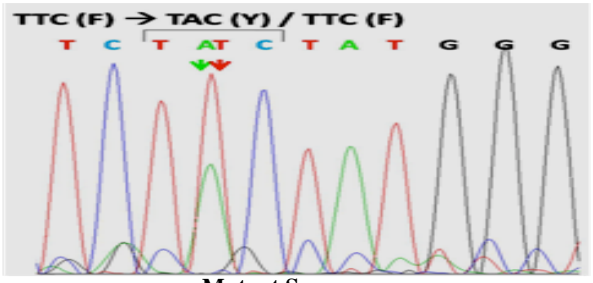


Wildtype Sequence



Mutant Sequence

Figure 1. Genotype of Wild Type and Mutant Sequence of Exon 7 in IRF6 Gene. The wild type sequence TTC that codes for phenylalanine is mutated to TAC coding for tyrosine amino acid, albeit in heterozygous condition. A red arrow indicates the single peak with TTC in wild type sequence, while bold red and green arrows indicate a double peak with TAC and TTC in the mutant sequence

been reported so far, *IRF6* is known to play a vital role in the development of craniofacial region, by regulating cellular differentiation and proliferation (Zucchero et al., 2004). Mutational alterations in the coding regions of *IRF6* gene were first identified in subjects with van der Woude syndrome (VWS), which is known to be the most common of all the Mendelian malformation syndromes (Zucchero et al., 2004). Subjects with VWS have either cleft lip, cleft palate or cleft lip with cleft palate (CL/CP/CLP) and importantly, a labial pit that distinguishes VWS from non-syndromic CL/CP/CLP (Zucchero et al., 2004). The exon 7 of *IRF6* gene encodes for major region of the C-terminal IAD region and hence mutations within this region may be expected cause functional impairment. Indeed, a single point mutation that causes conversion of GTC to ATC creating valine to isoleucine substitution at position 274 in *IRF6* protein has been found to be significantly associated with cleft in several populations (Mangold et al., 2011; Salahshourifar et al., 2011; Stuppia et al., 2011). The identification of loss of *IRF6* expression due to promoter hypermethylation of *IRF6* in cutaneous squamous cell carcinomas and function impairing mutations in exon 7 of *IRF6* in CL/CP/CLP prompted us to investigate, whether such mutations occurred in well differentiated OSCC lesions.

Materials and Methods

Study design and subjects

A cross sectional study was designed to investigate the occurrence of mutation in the conserved protein binding domain of *IRF6* in thirty two OSCC genomic DNA samples that were obtained earlier (Rajendran et al., 2013; Rupesh et al., 2013). The study was approved by institutional ethics committee (IEC) and was conducted at the Human Genetics Laboratory at Sree Balaji Medical and Dental College and Hospital, Bharath University. As mentioned in earlier publication (Rajendran et al., 2013; Rupesh et al., 2013), patients who were found to have lesion associated pain, and/or medical conditions unrelated to OSCC like diabetes mellitus, hypertension or infectious diseases were excluded from the study.

Polymerase chain reaction and direct sequencing

Exon 7 of the *IRF6* gene was amplified with intronic primers that were designed to amplify the entire exon 7 along with splice acceptor and donor sites. The following primer sequences were used: Forward – AGGACTCTCACTGTCAT, Reverse – AGACCAGTACACCTTGCA. The exon 7 was amplified with 25ng of genomic DNA under the following conditions: after denaturing the total genomic DNA at 94°C for 4 min, the exon was amplified for 25 cycles with denaturing at 95°C for 45 sec, annealing at 55°C for 50 sec, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. Subsequently, the amplified region of exon 7 from all OSCC samples were run in a 1.2% agarose gel and eluted with Genelute DNA gel elution kit (Sigma Aldrich, cat# NA1111). 10ng of the eluted PCR amplicon was subjected to direct sequencing (Genetics Lab, Madras Diabetes Research Foundation, Chennai, India) to identify mutations.

Results

The exon 7 of *IRF6* gene encodes for the major protein binding domain called IAD (IRF Associated Domain), with which it interacts to form homo- and hetero-dimers with intracellular proteins. Since mutations within the conserved domain are likely to affect the functional aspect of *IRF6* protein, we investigated for the occurrence of mutations within exon 7 in OSCC tissue samples. Direct sequencing analysis identified a point mutation in 9% (3 of 32) of OSCC samples. The wild type codon TTC at position 252 in *IRF6*, which encodes for phenylalanine amino acid was found to be mutated to TAC coding for tyrosine (F252Y) (Figure 1). The F252Y mutation always occurred in heterozygous condition along with its wild type codon, phenylalanine.

Discussion

Genetic anomalies in the components of the interferon signaling pathway are beginning to be identified as clinically relevant early cancer detection biomarkers. In the present study, we have investigated the chromosomal DNA isolated from tissues that were excised from thirty two OSCC patients at the time of surgery, to investigate the occurrence of mutational alteration in the *IRF6* gene. We analyzed the exon 7 of *IRF6* as it codes for the major region of the C-terminal protein-protein interaction domain. Direct sequencing analysis identified a single point mutation within the coding region of exon 7 in 9% (3/32) of OSCC samples. The mutation resulted in the conversion of phenylalanine amino acid to tyrosine at

position 252 in *IRF6* (F252Y). The occurrence of F252Y mutation in only 9% of OSCC samples suggests that the mutation may not be as prevalent as those that occurs in other genes such as H-ras for example, in which mutations have been in up to 20%, especially in OSCC lesions from India (Murugan et al., 2012; Jayaraman et al., 2013).

Phenylalanine to tyrosine mutation has also been observed in other systems as well; where in the above amino acid substitution has been shown to interfere with the function of the protein. For example, D-Ala-D-Ala ligase (LmDdl) of bacteria L. mesenteroides is a peptide ligase that promotes dipeptide formation by coupling two molecules of D-Ala (D-Alanine) and depsipeptide formation by coupling a molecule each of D-Ala and D-Lac (D-Lactate) (Park et al., 1997). Both D-Ala-D-Ala and D-Ala-D-Lac molecules subsequently get incorporated into peptidoglycans that play a role in determining the sensitivity of the microbe to antibiotics. L. mesenteroides carrying mutant Ddl with substitution of phenylalanine to tyrosine at position 216 (F216Y), fail to couple D-Lac molecules (Park et al., 1997). Hence the *IRF6* mutant (F252Y) identified in the present study is predicted to have an impaired substrate or effector binding ability, which thereby might have played a role in carcinogenesis. However, it is important to note that the above the interpretation is being made solely based on the published evidence, which however, requires an in vivo cell based assay to confirm the functional impairment. Of significant interest is the observation that, mutation of a tyrosine residue within the active site of *E coli* Ddl to phenylalanine caused gain of function of Ddl protein (Park et al., 1997), which clearly reiterates, albeit indirectly, the potential loss of function that is predicted with the F252Y mutation in *IRF6*.

Although cancerous lesions are managed surgically, postsurgical palliative chemotherapy with or without radiotherapy remains to be the preferred line of treatment in routine practice. Interestingly, many chemotherapeutic agents have been observed to cause an increase in the expression of IRFs. For example, treatment of cells with adriamycin, mitomycin C, cisplatin or etoposide causes upregulation of IRF7 (Kim et al., 2000). On the other hand, ectopic expression of IRFs, IRF1 for example in gastric cancer cells have been shown to increase the sensitivity of those cells to 5-fluorouracil relative to untreated control cells (Gao et al., 2012). While these studies clearly highlight the emerging importance of the components of interferon pathway in the development and treatment of carcinomas, it becomes critical to note that our finding of a mutation in *IRF6* carries significant clinical value as incidence of resistance of cancer cells to chemotherapeutic agents have been reported especially in cells carrying mutant genes. For example, cancer cells expressing mutant epidermal growth factor receptor (EGFR) molecules have been found to be resistant to chemotherapeutic drugs (Sigismund et al., 2012). Hence it is possible that the F252Y mutation identified in the three OSCCs in the present study may be expected to confer resistance to chemotherapeutic drugs as well. However, further in-depth explorations are required in order to confirm the above observations. Nevertheless, the finding of a novel mutation *IRF6* F252Y is clinically significant as the mutation is being reported for the first time in OSCC lesions, and that the significance of this may increase when the epigenetic modification of *IRF6* promoter region is also analyzed in the same samples.

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