RESEARCH ARTICLE

Interferon Stimulated Gene - ISG15 is a Potential Diagnostic Biomarker in Oral Squamous Cell Carcinomas

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Abstract

Background: Cancer diagnostic biomarkers have a wide range of applications that include early detection of oral precancerous lesions and oral squamous cell carcinomas, and assessing the metastatic status of lesions. The interferon stimulated ISG15 gene encodes an ubiquitin-like protein, which conjugates to stabilize activation status of associated proteins. Hence a deregulated expression of ISG15 may promote carcinogenesis. Indeed overexpression of ISG15 has been observed in several cancers and hence it has been proposed as a strong candidate cancer diagnostic biomarker. Given the emerging relationship between malignant transformation and ISG15, we sought to examine the expression pattern of this gene in tumor biopsies of oral squamous cell carcinoma (OSCC) tissues collected from Indian patients. Materials and Methods: Total RNA isolated from thirty oral squamous cell carcinoma tissue biopsy samples were subjected to semi-quantitative RT-PCR with ISG15 specific primers to elucidate the expression level. Results: Of the thirty oral squamous cell carcinomas that were analyzed, ISG15 expression was found in twenty four samples (80%). Twelve samples expressed low level of ISG15, six of them expressed moderately, while the rest of them expressed very high level of ISG15. Conclusions: To the best of our knowledge, the results show for the first time an overexpression of ISG15 in up to 80% of oral squamous cell carcinoma tissues collected from Indian patients. Hence ISG15 may be explored for the possibility of use as a high confidence diagnostic biomarker in oral cancers.

Keywords: ISG15 - ISG15 expression in oral carcinoma - over expression of ISG15 - sustained expression of ISG15

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Introduction

Interferons (IFN) belong to a family of multifunctional cytokines that regulates host innate response to contain microbial infections and tumor cells. Secretion of IFNs initiates a cascade of signaling events that result in the upregulation of interferon-stimulated genes (ISG) via JAKs (JAnus Kinases)/STAT (Signal Transducer and Activator of Transcription) mediated pathway (Laurence et al., 2012; Yanai et al., 2012). More than 400 ISGs have been reported so far (Andersen et al., 2006a), of which the IFN-stimulated gene 15 (ISG15) was among the first to be recognized as an ISG (Blomstrom et al., 1986).

ISG15 is a 15KDa protein that belongs to the ubiquitin-like (UBL) superfamily of proteins (Malakhova et al., 2002; Pitha-Rowe et al., 2004), which includes SUMO, NEDD8, HUB1, APG12, APG8 and FAT10 (Boddy et al., 1996; D'Cunha et al., 1996; Kamitani et al., 1997; Liu et al., 1999; Herrman et al., 2007). ISG15 has distinct extra- and intra-cellular regulatory activities. The secreted form of ISG15 exhibits an immunomodulatory function, whereby it stimulates production of IFN-gamma from lymphocytes and augments proliferation of natural killer (NK) cells (Herrman et al., 2007). Within the cells, ISG15 forms covalent conjugates with its substrate proteins by an enzymatic mechanism that is similar to those used by ubiquitin protein conjugation system. This process termed as "ISGylation", involves an ISG15 activating component UBE1L (E1), conjugating component UbcH8 (E2) and ligating component Efp (estrogen-responsive finger

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protein) or Herc5 (E3) (Andersen et al., 2006a). However, unlike the ubiquitination system, where ubiquitin tagged proteins is degraded in a proteasome dependent manner, ISGylation system promotes stabilization of ISG15 conjugated proteins by interfering with the ubiquitin/26S proteasome pathway (Zhao et al., 2005; Pitha-Rowe et al., 2007). For example, ISGylation of the ubiquitin E2 conjugating enzyme UBC13 disrupts its ability to form thioester bond with ubiquitin, which in turn promotes sustained activation of associated proteins (Takeuchi et al., 2005; Zou et al., 2005). It has also been shown that conjugation of ISG15 with proteins other than those involved in the ubiquitin pathway promotes sustained activation of the ISGylated protein. An example for the above is IRF3 (interferon regulatory factor 3), an inducer of antiviral proteins in the host cells. Covalent conjugation of ISG15 with IRF3 antagonizes the interaction of IRF3 with Pin1 (peptidyl-prolyl isomerase1), which otherwise would promote ubiquitination and degradation of IRF3 (Saitoh et al., 2006; He-Xin et al., 2010).

The ISGylation process targets more than 300 substrate proteins that includes a large number of constitutively expressed proteins regulating diverse cellular pathways such as RNA splicing, chromatin remodeling/polymerase II transcription, cytoskeleton organization and regulation, stress responses, and translation (Durfee et al., 2010). Hence a deregulation in the expression of ISG15 may be expected to promote carcinogenesis as ISGylated proteins remain stabilized for a sustained period. Indeed overexpression of ISG15 has been reported in many melanoma cell lines and tumor biopsies from several cancers (Padovan et al., 2002; Iacobuzio-Donahue et al., 2003; Hoek et al., 2004; Andersen et al., 2006b; Bektas et al., 2008; Rajkumar et al., 2011) including those from oral squamous cell carcinoma (OSCC) tissues in American and Chinese patients (Hui et al., 2008; Lang-Ming et al., 2009). Population genetic studies have suggested that the genetic basis of diseases in the Indian population may be different due to differences in the risk allele frequency and pattern of linkage disequilibrium. Further, studies from the Indian Genome Variation Consortium also have suggested that most of the populations in the Indian subcontinent are distinct from HapMap populations (IJVC, 2008). A systematic study by including patients from the same geographical location and race will enable us to gain a better understanding of the expression status of ISG15 in OSCC and its possible implication in the malignant transformation process. Hence the present study was designed to address the above issue by including thirty OSCC patients from the southern part of India who shared a similar genetic background, and the expression of ISG15 was analyzed by semi-quantitative RT-PCR.

Materials and Methods

Clinical samples

Tumor biopsies from thirty OSCC patients with well differentiated oral squamous cell carcinoma, who visited tertiary cancer hospitals during the period February 2011 to August 2012, were included in the study. The study was approved by institutional ethics committee (IEC) and informed consent was obtained from all participating patients. Patients with extreme OSCC lesion associated pain, and medical conditions unrelated to OSCC like hypertension, urinary disorders, endocrine abnormalities and infectious diseases were not included. Also those patients who were unwilling to participate due to personal religious beliefs were excluded from the study.

Tissue storage, RNA extraction and RT-PCR

Tumor tissues resected from cancerous site were washed twice with sterile 1X PBS (Phosphate Buffered Saline) and transferred into a 2ml microfuge tube containing RNA Save reagent (cat # 01-891, Biological Industries). The sample tubes were stored at 4°C for 48h as recommended by the manufacturer, after which the tubes were stored at -20°C until further usage. At the time of RNA extraction, the samples were recovered from RNA Save by washing thrice with cold 1X PBS followed by extraction of total RNA by trizol protocol (TRIzol® Plus RNA Purification System, cat#12183555, Ambion) as recommended by the manufacturer. Following quantification, 500ng of total RNA from each of the sample was reverse transcribed with random primers (High capacity RNA to cDNA kit, cat#4387406, Life Technologies). The polymerase chain reaction was then performed on all samples with the following pair of primers: ISG15F, ISG15R and GAPDH-F, GAPDH-R. Both ISG15 primers and GAPDH primers were multiplexed and samples were amplified with (cat#M0273, New England Biolabs) under the following conditions: initial denaturation at 94°C for 2 min, followed by a 35 cycle reaction consisting of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 45 sec, and a final extension at 72°C for 5 min.

Quantification of ISG15 expression

10μl aliquots of each PCR amplified sample was subjected to electrophoresis in a 1.2% agarose gel. Scanned images of ISG15 and GAPDH bands were analyzed with NIH Image J software under default settings. The scanned values of ISG15 were normalized against GAPDH and plotted with excel software.

Results

To know whether ISG15 was overexpressed in OSCC tissues of Indian patients, we analyzed total RNA isolated from thirty tumor biopsies by semi-quantitative RT-PCR. The total RNA was reverse transcribed with random primers and ISG15 expression was analyzed with primers designed to amplify ISG15 cDNA. The PCR amplification was multiplexed with primers to amplify the cDNA of housekeeping gene – GAPDH, which served as internal control for amplification in each sample.

The results showed overexpression of ISG15 in twenty four of thirty OSCC samples (80%) that were analyzed (Figure 1A). The level of expression, however, varied among the tested samples and hence the gel images of ISG15 were quantified and normalized values expressed in arbitrary units (AU) were plotted (Figure 1B). Analysis of the data showed stratification of the tumor samples into three groups: low expressers – samples that expressed

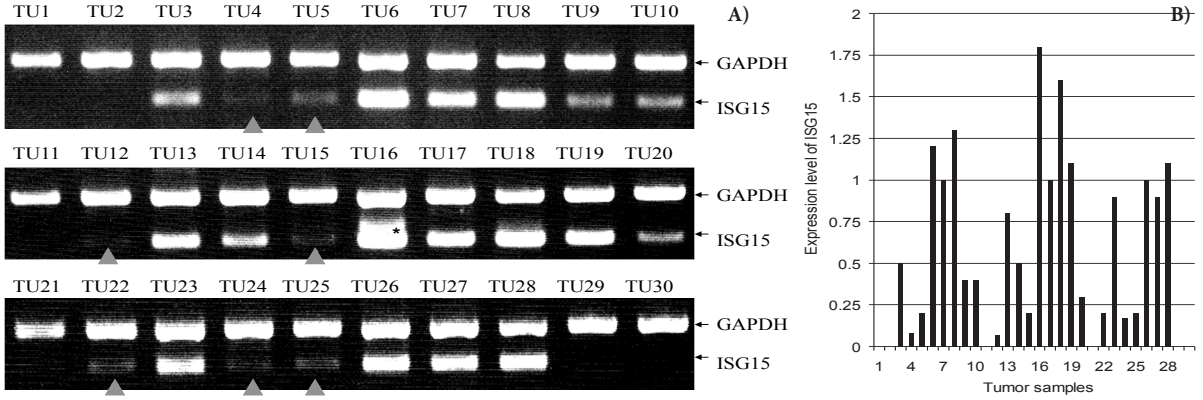

Figure 1. Overexpression of ISG15 in OSCC Tumor Samples. A) Total RNA purified from tumor tissue biopsies were subjected semi-quantitative RT-PCR with ISG15 and GAPDH cDNA specific primers. Twenty four samples showed overexpression of ISG15. B) Graphical representation of varied expression of ISG15 among samples, which were quantified, normalized against internal control GAPDH and expressed in arbitrary units (AU). Every third tumor sample is marked in the bar diagram. Twelve samples showed low ISG15 expression (up to 0.5AU), six showed moderate (between 0.5AU to 1AU) and the rest of the six samples (greater than 1AU) showed high expression. *indicates a higher molecular weight band in tumor sample 16

ISG15 up to 0.5AU (50%), moderate expressers – those that expressed between 0.5AU to 1AU (25%), and high expressers – those with above 1AU (25%). Within the low expresser group, atleast six of the samples showed an expression level that was less than 0.25AU.

Discussion

In the current study, we have analyzed for the status of ISG15 expression by semi-quantitative RT-PCR analysis in a total of thirty OSCC tumor biopsy samples, and found overexpression in twenty four (80%) tumor samples. TU16 sample showed an additional higher molecular weight band just above the ISG15 specific band, which is indicated by an asterix mark in the image (Figure 1A, second panel). Sequencing of this band revealed an unreported isoform (data not shown) not relevant to the present study. The ISG15 specific band in TU16 was indeed confirmed by direct sequencing (data not shown). Interestingly, the level of expression of ISG15 among most of the tumor samples varied in intensity, the reason for which is not clear, and so is its expression in only 80% of tumor samples. A similar observation has been made in other solid tumors such as carcinoma of breast (66-76%) (Betkas et al., 2008) and bladder (93-98%) (Andersen et al. 2006b). Though ISG15 overexpression has also been reported in carcinoma of oral cavity, pancreas and melanoma, the percent overexpression in them was not specifically addressed as the studies included either a very small sample size or were pooled with other genes identified from microarray analysis (Iacobuzio-Donahue et al., 2003; Lang-Ming et al., 2009; Rajkumar et al., 2011).

Besides being induced by IFNs, ISG15 is also transcriptionally upregulated by NFκB (Desai SD et al., 2006) and IRF3 (Pitha-Rowe et al., 2007). While the expression and activation of both NFκB and IRF3 are high in host cells challenged with microbial infections, IRF3 is also induced in response to chemotherapeutic agents (Roberts et al., 2007) independent of IFNs. Since none of the OSCC patients, who were included in the present study had any microbial infections or were under chemotherapy, the possibility of IRF3 mediated overexpression of ISG15 in the twenty four samples may be ruled out. Interestingly, NFκB has also been shown to be highly expressed in carcinomas including OSCC tumor tissues (Desai et al., 2006). Hence it is possible that NFκB may have been active in the twenty four tumor samples that showed overexpression of ISG15. It is also plausible that the level of activation of NFκB varied in these tumor tissues, which in turn reflected in the differential expression level of ISG15. Such differential expression has been observed in other carcinomas as well (Andersen et al. 2006b; Betkas et al., 2008). Nevertheless, the finding is clinically significant as ISG15 overexpression has been confirmed in OSCC patients from India, though their genetic background carries variation.

Studies have revealed differential expression of several genes in OSCC lesions such as cyclin D1, EGFR, Tetranectin, NuclearS100A7, Smad2, Smad6, DeltaNp63 and PCD4, that have been proposed as potential biomarker to predict transforming potential of precancerous lesions and prognosis of well differentiated carcinomas (Ginos et al., 2004; O'Donnell et al., 2005; Saintigny et al., 2009; Arellano-Garcia et al., 2010; Mangone et al., 2010; Reis et al., 2010; Tripathi et al., 2010). However, none of the molecules have had an expression level similar to the findings of present study. It is important to note that the oncogenic potential of ISG15 has been confirmed in vitro by overexpressing or knockdown of ISG15 in a prostrate cancer (PC) cell line that resulted in either an augmented or attenuated growth of PC cells respectively (Satake et al., 2010). As 80% of OSCC tumor tissue samples were found to overexpress ISG15, it is highly likely that the overexpression of this molecule may be used as a clinically relevant OSCC diagnostic biomarker. However, it is suggested that a longitudinal study by involving a statistically significant number of patients with precancerous lesions, early dysplasia and well differentiated OSCC lesions with or without

metastasis is essential to validate and corroborate the clinical application of the ISG15 molecule as an OSCC diagnostic biomarker.

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