RESEARCH ARTICLE

A Novel Mutation in the DNA Binding Domain of NFKB is Associated with Speckled Leukoplakia

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Abstract

Background: Activation and inactivation of nuclear factor of kappa light chain gene enhancer in B cells (NFKB) is tightly regulated to ensure effective onset and cessation of defensive inflammatory signaling. However, mutations within NFKB, or change in activation and inactivation molecules have been reported in a few cancers. Although oral squamous cell carcinoma is one of the most prevalent forms of cancer in India, with a development associated with malignant transformation of precancerous lesions, the genetic status of NFKB and relative rates of change in oral precancerous lesions remain unknown. Hence in the present study we investigated all twenty four exons of NFKB gene in two precancerous lesions, namely oral submucous fibrosis (OSMF) and oral leukoplakia (OL) to understand its occurrence, incidence and assess its possible contribution to malignant transformation. Materials and Methods: Chromosomal DNA isolated from twenty five each of OSMF and OL tissue biopsy samples were subjected to PCR amplification with intronic primers flanking twenty four exons of the NFKB gene. The PCR amplicons were subsequently subjected to direct sequencing to elucidate the mutation status. Results: Sequence analysis identified a novel heterozygous mutation, c.419T>A causing substitution of leucine with glutamine at codon 140 (L140Q) in an OL sample. Conclusions: The identification of a substitution mutation L140Q within the DNA binding domain of NFKB in OL suggests that NFKB mutation may be relatively an early event during transformation. To the best of our knowledge, this study is the first to have identified a missense mutation in NFKB in OL.

Keywords: Oral carcinoma - NFKB expression - NFKB mutation - NFKB signalling pathway - hyperactivation

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Introduction

The NF-kappa-B or NFKB (Nuclear Factor of Kappa light chain gene enhancer in B cells) gene consists of two isoforms namely; NFKB1 and NFKB2 that encodes for p105 and p100 proteins. Upon activation, these proteins are cleaved to produce active forms of NF-kappa-B subunits - p50 and p52, respectively (Khan et al., 2013). Activated NF-kappa-B subunits bind to RelA molecule (ReticuloEndotheLiosis Viral Oncogene Homolog A), which has a molecular weight of 65 KDa, and hence is simply referred to as p65 (Hoesel et al., 2013).

NFKB is activated by a wide range of inducers that includes cytokines like TNFα and IL1β, free radicals, and toxins of bacterial origin like LPS (lipopolysaccharides) (Hayden et al., 2012). Upon its activation, the NFKB-RelA complex translocates into nucleus to transactivate expression of cytokines, chemokines, growth factors and cell adhesion molecules involved in activation of inflammatory mediators associated with targeting and elimination of pathogens including transformed cells (Birbach et al., 2002). Interestingly, constitutively active form of NFKB-RelA complex has been observed in many cancer types and has been associated with its pro-tumorigenic role, especially in patients with chronic inflammatory conditions (Rial et al., 2012). Such constitutive activation of NFKB-RelA complex can occur either due to intrinsic anomaly in the NFKB subunits caused by mutations that impair its ubiquitin mediated degradation by IKB family members or as a result of activating mutation within genes that otherwise activate NFKB signalling pathway (Atsumi et al., 2014). While mutations in coding region of NFKB subunits have so far been reported in breast cancers, mutations in the promoter of NFKB have been shown to increase the cancer risk in some patients (Wang et al., 2011). Besides that, amplifications and point mutations within its dimerization partner, RelA have been reported in lymphoid malignancies such as Hodgkin lymphoma and T-cell lymphomas, and other members of NFKB signalling

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pathway such as c-Rel, Bcl-3, NFKB upstream kinase IKK2, and IκBα and IκBε (Hoesel et al., 2013).

Oral squamous cell carcinomas (OSCC) occur with a very high incidence in India, and the morbidity associated with OSCC remains high due to late diagnosis of the disease (Das S et al., 2015). As OSCCs also arise as a result of malignant transformation of premalignant lesions such as oral submucous fibrosis (OSMF), leukoplakia (OL) and erythroplakia (OEL) (Das et al., 2015) for example, we sought to investigate histopathologically confirmed samples of OSMF and OL for the occurrence of mutation in NFKB coding exons, and associate its presence with the histopathological state of the respective lesions.

Materials and Methods

Study design and subjects

A cross sectional study on histopathologically characterized oral submucous fibrosis (OSMF) and oral leukoplakia (OL) biopsy tissue samples that were obtained from patients visiting tertiary cancer hospitals in Tamil Nadu were included in the study. The study was approved by the institutional ethics committee (IEC).

gDNA extraction, PCR amplification and direct sequencing: A total of 25 each of OSMF and OL samples were included in the study. At the time of biopsy, a part of the OSMF and OL sample was cut and stored in RNA Later reagent (Cat # 76106, Qiagen, USA), while the other section was fixed for histopathological examination. Genomic DNA extraction was processed as described earlier (Jayaraman et al., 2012; Mehta et al., 2014). Intronic primers for all 24 exons for NFKB gene were designed (primer sequences are available on request) so as to detect splice site mutations as well. 50ng of DNA samples were amplified under the following conditions: after an initial denaturation at 94°C for 4 min, the samples were subjected to 30 cycles of denaturing at 94°C for 30 sec, annealing at 59°C for 45 sec, 72°C for 45 sec, followed by a final extension at 72°C for 5 min. The PCR amplicons were run in a 1.5% agarose gel and subjected to SAP treatment before being sequenced.

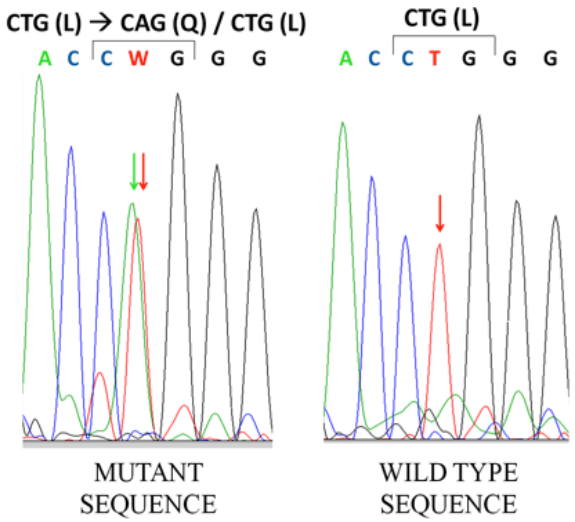

Figure 1. Genotype of Mutant and wild Type Sequence Codon 140 Within Exon 7 of NFKB gene. A copy of the "T" nucleotide within the wild type codon CTG (indicated by a red arrow in wild type sequence) is mutated to "A" (indicated by a green arrow in mutant sequence) in an OL sample

Results

In order to understand whether mutations in NFKB occurred in OSMF and OL, genomic DNA extracted from histopathologically characterized tissue samples were amplified with intronic primers and subjected to direct sequencing. Sequence analysis of the PCR amplicons showed no mutations in OSMF samples. However, an OL sample which was histopathologically characterized as speckled leukoplakia carried a missense mutation, c.419T>A in exon 7 of NFKB gene. The missense mutation occurred in heterozygous state and caused substitution of native leucine residue at position 140 with glutamine (L140Q). None of the other OL samples carried mutation in any other exons. The mutant and wild type codon at c.419 position identified in the sequencing analysis is shown in Figure 1.

Discussion

Following ligand mediated activation of NFKB, the NFKB-RelA complex translocates into the nucleus to transactivate genes involved in regulating and mediating inflammatory response and apoptosis depending on the cell type in which it is activated (Hoesel et al., 2013). Subsequent to this, the NFKB subunits are degraded via ubiquitin mediated proteasome pathway by IKB family members (Kanarek et al., 2012). However, the active form of NFKB can turn pro-tumorigenic, especially when the potentially transforming cells escape the check points. Cytokines secreted within the tumor micro environment further augments the production of activated NFKB within the tumor cells, which then promotes tumor vascularisation by upregulation of VEGF (Xie et al., 2010), epithelial to mesenchymal transition and metastasis via upregulation of MMPs (Wu et al., 2009; Hoesel et al., 2013). While the role of cytokine is indispensable for activation of NFKB and its sustained presence, elevated level of activated NFKB can also arise as a result of genetic aberrations. Indeed such aberrations have been reported in breast cancers, where a missense mutation at c.1594G>A causing valine to isoleucine substitution was identified (Hoesel et al., 2013).

In the present study, we investigated twenty five each of OSMF and OL samples for the occurrence of mutation in NFKB gene. OSMF and OL samples were included as inflammatory cells are frequently found in them, and that NFKB is basically activated in response to pro-inflammatory signals as a cellular defence mechanism (Lawrence et al., 2005). PCR amplification and direct sequencing resulted in the identification of a novel mutation in a sample of OL with features of speckled leukoplakia. The mutation was identified by analyzing twenty five samples of OL, of which two had features of speckled leukoplakia. Finding of L140Q mutation in one out of two speckled leukoplakia samples signifies that the mutation may be an early event during transformation. This finding gains further significance as speckled

leukoplakia has been increasingly associated with malignant transformation. The identification of mutation in OL but not in OSMF samples may be expected, as the later develops fibroelastic change of the lamina propria and epithelial atrophy response to early inflammatory reaction at juxta epithelial junction (Yardimci et al., 2014). During this early period NFKB may have played a critical role, which however, may have ceased following fibroelastic changes.

L140Q NFKB mutation however, occurred in heterozygous condition, which implies the possible role of haploinsufficiency in the malignant transformation process. It is important to note that the substitution of leucine with glutamine has been shown to disrupt the secondary structure of proteins. Hence it is possible that the L140Q NFKB mutation may have altered the secondary structure of NFKB, thereby contributing towards its sustained activation (Gao et al., 2015). This possibility however, requires further in vitro transformation assays to systematically explore its functional contribution in both heterozygous and homozygous condition. The occurrence of mutation in heterozygous condition in OL also raises the question whether the heterozygous L140Q mutant region can undergo further mutagenesis reaction to become homozygous. This hypothesis has to be addressed by sequencing of histopathologically established well differentiated OSCC lesions. Taken together, the finding of a novel L140Q mutation in speckled leukoplakia establishes NFKB as a potential molecule for being explored as a diagnostic biomarker for early detection of potentially transforming lesions.

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