

RESEARCH ARTICLE

Mutation Analysis of the Dimer Forming Domain of the Caspase 8 Gene in Oral Submucous Fibrosis and Squamous Cell Carcinomas

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Abstract

Background: Missense and frame-shift mutations within the dimer forming domain of the caspase 8 gene have been identified in several cancers. However, the genetic status of this region in precancerous lesions, like oral submucous fibrosis (OSMF), and well differentiated oral squamous cell carcinomas (OSCCs) in patients from southern region of India is not known, and hence the present study was designed to address this issue. **Materials and Methods:** Genomic DNA isolated from biopsy tissues of thirty one oral submucous fibrosis and twenty five OSCC samples were subjected to PCR amplification with intronic primers flanking exon 7 of the caspase 8 gene. The PCR amplicons were subsequently subjected to direct sequencing to elucidate the status of mutation. **Results:** Sequence analysis identified a frame-shift and a novel missense mutation in two out of twenty five OSCC samples. The frame-shift mutation was due to a two base pair deletion (c.1225_1226delTG), while the missense mutation was due to substitution of wild type cysteine residue with phenylalanine at codon 426 (C426F). The missense mutation, however, was found to be heterozygous as the wild type C426C codon was also present. None of the OSMF samples carried mutations. **Conclusions:** The identification of mutations in OSCC lesions but not OSMF suggests that dimer forming domain mutations in caspase 8 may be limited to malignant lesions. The absence of mutations in OSMF also suggests that the samples analyzed in the present study may not have acquired transforming potential. To the best of our knowledge this is the first study to have explored and identified frame-shift and novel missense mutations in OSCC tissue samples.

Keywords: Caspase 8 mutation in oral carcinoma - loss of function of caspase 8 - caspase 8 mutation in cancer

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Introduction

Caspase 8 is a cysteine dependent endoprotease that plays a key role in the regulation of apoptosis (also referred as programmed cell death) (Alenzi et al., 2010; Li et al., 2014), an event which is known to be essential in growth and development of embryo, and maintenance of tissue homeostasis (Andon et al., 2013; Weinlich et al., 2013). Caspases are broadly classified as apoptosis or inflammation regulatory caspases based on their role in respective processes. Of those known, caspase - 3, -6, -7, -8 and -9 are categorized as apoptotic caspases, whereas caspase -1, -4, -5 and -12 are grouped as inflammatory caspases. The apoptotic caspases are further classified into initiator caspases (caspase -8 and -9) and effector caspases (caspase -3, -6 and -7) based on their role in transducing the apoptotic signals emanating at the cell membrane (Wallach et al., 2010).

Among the initiator caspases, caspase 8 is activated by

binding of extrinsic apoptosis promoting ligands such as tumor necrosis factor (TNF), TNF-like ligand 1A (TL1A), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and CD95L to a death receptor (Keller et al., 2010; Barca et al., 2013). This ligand-receptor interaction triggers recruitment, dimerization and activation of caspase 8. On the other hand, caspase 9 is activated by molecules released during cellular stress such as cytochrome c for example (Parrish et al., 2013). At the protein level, both activation of caspase -8 and -9 involves cleaving off of the N-terminal prodomain, which is made of two DED domains (Death Effector Domain) in caspase 8, and a single CARD domain (Caspase Recruitment Domain) in caspase 9, followed by dimerization of the larger (p20) and smaller (p10) subunits from each caspase -8 or -9 molecules to form heterotetramer (p20-p10)₂. Subsequent to their activation, both caspase -8 and -9 binds with effector caspases -3, -6 and -7 to cleave, activate and set the apoptosis program in motion (Crawford et al., 2011;

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Dysregulation of the apoptotic process is implicated in various diseases such as cancers, neurodegenerative diseases and autoimmune disorders (Sankari et al., 2012; McIlwain et al., 2013). The dysregulation may be in the form of: 1) insufficient apoptosis, which leads to uncontrolled proliferation and malignant transformation, 2) excessive apoptosis that causes degeneration of neurons, or 3) modification of self-antigen during apoptosis or resistant to apoptosis as observed in autoimmune disorders (McIlwain et al., 2013; Walczak et al., 2013). Faulty apoptotic process is frequently caused by loss of function mutation in various caspases that have been shown to: 1) increase the risk of malignant transformation, and 2) contribute towards progression of carcinomas (Ryoo HD et al., 2012). Caspase 8 being at the top of hierarchical activation flow has been extensively investigated for its association with carcinogenesis in several studies. This includes colorectal carcinomas, gastric cancer, non-small cell lung carcinoma (NSCLC), breast cancer, pancreatic cancer, acute leukemia, glioma childhood neuroblastoma (McIlwain et al., 2013; Zhang et al., 2014) and gingivo-buccal squamous cell carcinomas (GB-OSCC) (IPT-ICGC, 2013). These reports prompted us to investigate whether mutations occurred in caspase 8 gene in tissue samples of patients with oral submucous fibrosis (OSMF) carrying mild dysplastic features and well differentiated OSCC as the genetic status of caspase 8 is not known in south Indian patients. OSMF samples were included as OSCC lesions are also known to occur as a result of malignant transformation of OSMF lesions (Hsue et al., 2007). Of the eight exons that the gene has, exon 7 is considered as the mutational hot-spot as frame-shift and nonsense mutations have been frequently identified in this region, and hence was investigated in the present study.

Materials and Methods

Genomic DNA extraction, PCR amplification and direct sequencing: Patients visiting tertiary cancer hospitals in the state of Tamil Nadu, South India were included in the study after obtaining informed consent and approval from the institutional ethics committee (IEC). Tissue samples were surgically excised from thirty one oral submucous fibrosis and twenty five OSCC lesions and stored in RNA Later (Cat # 76106, Qiagen, USA). Tissues were processed for genomic DNA extraction as described earlier (Jayaraman et al., 2012). Exon 7 of caspase 8 was amplified using intronic primers, Casp8Ex7F (forward primer): CTCTCCAGCTGTGGTCTGTG, and Casp8Ex7R (reverse primer): TGACCTGGGAAATGCAGCTATGTG, using 10ng of genomic DNA. The following conditions was used: after an initial denaturation at 94°C for 2 min, the samples were subjected to 35 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for 45 sec, with a final extension step at 72°C for 5 min. The PCR amplicons were run in a 1.5% agarose gel to confirm for specific size and subjected to SAP treatment before being sequenced.

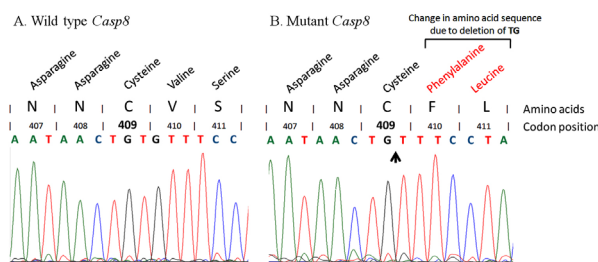


Figure 1. Genotype of Normal (A) and Frame-Shift Mutation (B) in Exon 7 of Caspase 8. The deletion is indicated by a bold arrow in mutant sequence image. Resultant frame-shift and change in amino acid sequences is also indicated

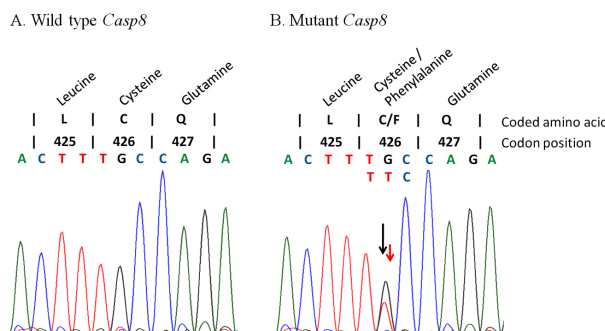


Figure 2. Genotype of Normal (A) and Missense Mutation (B) in exon 7 of Caspase 8. A. The heterozygous missense mutation is indicated by a black arrow for wild type base, and red arrow for mutant base

Results

To know whether caspase 8 was altered in precancerous OSMF lesions with mild dysplastic features and well differentiated OSCC lesions, and to understand its association with carcinogenesis, we screened for mutations in the dimer forming domain coded by exon 7 of caspase 8 gene in thirty one OSMF and twenty five OSCC tissue samples obtained from patients. PCR amplification and direct sequencing resulted in the identification of a frame-shift and missense mutation in two out of twenty five OSCC samples (8%). None of the OSMF samples carried mutations.

The frame-shift mutation was caused by two base pair deletion at position 1225_1226 in the caspase 8 cDNA (c.1225_1226delTG) that resulted in frame-shift of amino acids after codon 409 with an eventual premature stop codon at position 437 (Figure 1A, B). The missense mutation in the other sample was caused by transversion of nucleotide G to T at position 1277 in cDNA (c.1277G>T), which resulted in substitution of its coded amino acid, cysteine with phenylalanine (C426F) at codon 426. This mutation, however, occurred in heterozygous condition (C426C/F) as both wild type (G) and mutant (T) nucleotide peaks were observed at the second position of codon 426 resulting in the occurrence of C426C in one copy of caspase 8 gene and C426F in the other copy (Figure 2A, B).

Discussion

AIN the present study, we have reported the identification

of a frame-shift mutation and a novel missense mutation, C426F in the dimer forming domain coded by exon 7 of caspase 8 gene in two OSCC tissue samples. This finding along with the recent published report on GB-OSCC (IPT-ICGC, 2013) indicates that mutations in the dimer forming domain of caspase 8 may be prevalent in OSCC lesions. The GB-OSCC study investigated fifty tissue samples and identified five nonsense mutations in exon 7, while by investigating twenty five OSCC and thirty one OSMF samples we identified two mutations - a frame-shift and a missense mutation in OSCC samples. It is noteworthy that the samples of GB-OSCC were obtained from north Indian patients, while those that were analyzed in the present study were from south Indian Dravidian race. As studies from the Indian Genome Variation Consortium (IGVC) have established the genetic basis of diseases in most of the populations in the Indian subcontinent to be distinct (IGVC, 2008), it is highly likely that the difference in the race of patients may have influenced the type and prevalence of the exon 7 mutation in the GB-OSCC and present study. Together, however, the data reiterates the fact that the dimer forming domain coded by exon 7 is indeed mutational hot spot in caspase 8 gene as described earlier (Soung et al., 2005). The OSCC samples that were used in the present study constituted a mix of carcinoma of tongue, buccal mucosa, floor of mouth and palatal region. However, this is unlikely to have had influenced the type and pattern of mutation as all these were well differentiated squamous cell carcinomas, histologically very much similar to GB-OSCC samples.

The frame-shift mutation c.1225_1226delTG identified in the present study was similar to the one reported in hepatocellular carcinomas (Soung et al., 2005), while the missense mutation - C426F was novel. Studies have shown that cysteine (C) to phenylalanine (F) missense mutations in other genes generate loss of function mutant proteins, as cysteine residues are required to maintain the tertiary structure of proteins (Prinos et al., 2011). Based on these observations and the fact that the C426F mutation identified in the present study occurred in heterozygous condition, we believe that the C426F mutant may have produced a haplo-insufficiency effect in the malignant cells. Such genetic haplo-insufficiency effect has also been reported in several other disease conditions including carcinomas (Chandrasekharan et al., 2014). As mutations in caspase 8 have been frequently linked with malignant transformations (Podgorski et al., 2006), the identification of caspase 8 mutation in OSCC but not OSMF samples indicates that the OSMF lesions analyzed in the present study may not have acquired transforming potential.

Expression of caspase 8 protein in malignant tissues has been associated with sensitivity of malignant cells to chemotherapy and prognosis. In a study by Kaneda et al. (2006) it was reported that OSCC cell lines with undetectable levels of caspase 8 were resistant to chemotherapeutic agents such as etoposide, cisplatin and 5-fluorouracil relative to those that expressed detectable levels of caspase 8 (Kaneda et al., 2006). In a separate study, Carmen Pingoud-Meier et al found that medulloblastomas (MB) expressing low levels of caspase 8 to be resistant to both chemo- and radio-therapy relative

to those that expressed higher levels (Pingoud-Meier et al., 2003). These findings clearly suggest that the caspase 8 mutants identified in the present and other studies may confer resistance to chemotherapeutic agents. It may also be noted that missense and nonsense mutations have also been identified in other exons of caspase 8 in GB-OSCCs and other carcinomas, but were not explored in the present study. Since these mutants are also expected to contribute towards resistance to chemo- and / or radio- therapy, it is pertinent to have them confirmed independently in *in vitro* studies and then follow it with investigation to identify their prevalence in OSCCs. When accomplished, it will enable us to gain an in-depth understanding of: 1) role of caspase 8 mutations in carcinogenesis, 2) molecular mechanism of chemo -sensitivity / -resistance, and 3) the use of caspase 8 mutants as therapeutic indicator for chemotherapy.

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