RESEARCH ARTICLE

Common Docking Domain Mutation E322K of the ERK2 Gene is Infrequent in Oral Squamous Cell Carcinomas

Gopalakrishnan Mohan Valiathan[1&], Siji Jacob Thenumgal[2&], Bhaskar Jayaraman[1], Arunmozhi Palaniyandi[2], Hemalatha Ramkumar[3], Keerthivasan Jayakumar[4], Sajeev Bhaskaran[5], Arvind Ramanathan[4*]

Abstract

Background: Mutations in the MAPK (Mitogen Activated Protein Kinase) signaling pathway - EGFR/Ras/RAF/MEK have been associated with the development of several carcinomas. ERK2, a downstream target of the MAPK pathway and a founding member of the MAPK family is activated by cellular signals emanating at the cell membrane. Activated ERK2 translocates into the nucleus to transactivate genes that promote cell proliferation. MKP - a dual specific phosphatase - interacts with activated ERK2 via the common docking (CD) domain of the later to inactivate (dephosphorylate) and effectively terminate further cell proliferation. A constitutively active form of ERK2 carrying a single point mutation – E322K in its CD domain, was earlier reported by our laboratory. In the present study, we investigated the prevalence of this CD domain E322K mutation in 88 well differentiated OSCC tissue samples. Materials and Method: Genomic DNA specimens isolated from 88 oral squamous cell carcinoma tissue samples were amplified with primers flanking the CD domain of the ERK2 gene. Subsequently, PCR amplicons were gel purified and subjected to direct sequencing to screen for mutations. Results: Direct sequencing of eighty eight OSCC samples identified an E322K CD domain mutation in only one (1.1%) OSCC sample. Conclusions: Our result indicates that mutation in the CD domain of ERK2 is rare in OSCC patients, which suggests the role of genetic alterations in other mitogenic genes in the development of carcinoma in the rest of the patients. Nevertheless, the finding is clinically significant, as the relatively rare prevalence of the E322K mutation in OSCC suggests that ERK2, being a common end point signal in the multi-hierarchical mitogen activated signaling pathway may be explored as a viable drug target in the treatment of OSCC.

Keywords: Oral squamous cell carcinoma - oral cancer - ERK2 - ERK2 mutation - ERK2 mutation in oral cancer

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Introduction

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that regulates three major groups of signal transduction pathway, which includes ERK1 and ERK2, p38 and JNK/SAPK (McCubrey et al., 2007). Of the above molecules, ERK2 is the founding member of MAPK family and is activated in response to growth promoting and differentiation signals emanating at the cell membrane from receptor tyrosine kinases, G protein-coupled receptors, cytokine receptors and integrins, and is mediated in a Ras/Raf/MEK - dependent pathway (Roberts et al., 2007). ERK2 interacts with its immediate upstream activator MEK through its common docking domain (CD domain), and gets phosphorylated at both threonine (T) and tyrosine (Y) residues in the conserved TEY motif at positions T183 and T185 respectively (Tanoue et al., 2000; Gaestel et al., 2008). Following its activation, ERK2 translocates into the nucleus to activate a host of substrates including ELK1, a transcription factor that promotes expression of cell cycle genes (Yoon et al., 2006). Active ERK2 also promotes upregulated expression of EGFR ligands, thereby setting up a positive feedback loop (Roberts et al., 2007). Because of its crucial role in the promotion of cell division, a prolonged activation of ERK2 as is associated with genetic aberrations in upstream signaling molecules such as activating mutations of EGFR or Ras for example is likely to promote abnormal proliferative signals, and has been reported in several cancers (Schreck et al., 2006). Hence the duration of activation of ERK2 is tightly regulated, with its phosphorylation being principally down

*[1]Department of Periodontia, [4]Human Genetics Laboratory, Sree Balaji Medical and Dental College and Hospital, Bharath University, , Chennai, [3]Department of Pedodontia, S.R.M Dental College and Hospital, S.R.M University, Ramapuram, [2]Department of Periodontia, Rajah Muthiah Dental College and Hospital, Annamalai University, Annamalai Nagar, [5]Department of Conservative Dentistry, Vananchal Dental College and Hospital, Farathiya, Garhwa, India [&]Equal contributors *For correspondence: drarvindram@yahoo.co.in*

regulated by MKP, a dual specific phosphatase. MKP interacts with ERK2 at the common docking (CD) domain located in the C-terminus region of the later (Keyse SM et al, 2008). Mutations in the CD domain disrupt its ability to interact with MKP leading to its constitutive or sustained phosphorylation (Chu et al., 1996). Indeed, in our previous study we have shown that a single point mutation at position one of codon 322 (GAG to AAG) in ERK2 that causes substitution of glutamate with lysine (E322K), impairs its ability to interact with MKP leading to its constitutive phosphorylation in an OSCC cell line (Arvind et al., 2005; Mahalingam et al., 2008). However, the prevalence of this mutation in the genome of OSCC patients remains yet to be investigated. In this present study, we attempted to specifically evaluate the prevalence of E322K-ERK2 mutation in the tissue samples of eighty eight patients with well differentiated OSCC lesion.

Materials and Methods

Study design and subjects

A cross sectional study was designed to investigate the prevalence of E322K mutation in the genome of eighty eight OSCC tissue samples. The study was approved by institutional ethics committee (IEC) and was conducted at the Human Genetics Laboratory at Sree Balaji Dental College and Hospital, Bharath University during the period of October 2011 to September 2012. Biopsy tissue samples were collected from OSCC patients visiting tertiary cancer hospitals in Tamil Nadu. Patients with severe lesion associated pain, and medical conditions unrelated to OSCC like diabetes, hypertension and infectious diseases and those who were unwilling to participate due to religious beliefs were excluded from the study.

Genomic DNA extraction

Biopsy tissues collected were transported in RNA Later (Cat# 76106, Qiagen, USA) and were stored at -20°C until being processed. Tissues were washed once with cold 1X PBS (Phosphate Buffered Saline) and were lysed as follows. Briefly, 20 mg of tissue was lysed with 1000μl of lysis buffer (0.1% SDS, 25 mM EDTA, 75 μg/100μl Proteinase-K and 200 mM Tris-Cl at pH 8 [Sigma-Aldrich, St.Louis, MO, USA]) at 55°C overnight with intermittent agitation. On the following day, the cell lysates were spinned at 12,000 rpm for 5 min to clear undigested cells. Cell lysates were then extracted equal volumes of Tris-saturated phenol and 24:1 Chloroform/ Isoamyl alcohol to remove proteins, residual phenol and detergents. Following the extraction steps, 1/10th volume of Sodium Acetate pH 5.2 and 0.6 volume of isopropanol was added to the aqueous phase, vortexed vigorously centrifuged at 12,500 rpm for 15min at room temperature to precipitate the genomic DNA.

Polymerase chain reaction and direct sequencing

Exon 7 of the ERK2 gene was amplified with specific primers as described earlier (Arvind et al., 2005), using 100 ng of genomic DNA under the following conditions: after an initial denaturation at 94°C for 4 min, the exons were amplified for 35 cycles with denaturing at 95°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. Subsequently, the amplified exon 7 was run in a 1.5% agarose gel and eluted with Genelute DNA gel elution kit (Sigma Aldrich, cat# NA1111). 10 ng of the eluted PCR amplicon was subjected to direct sequencing (Genetics Lab, Sankara Nethralaya, Chennai, India) to identify mutations.

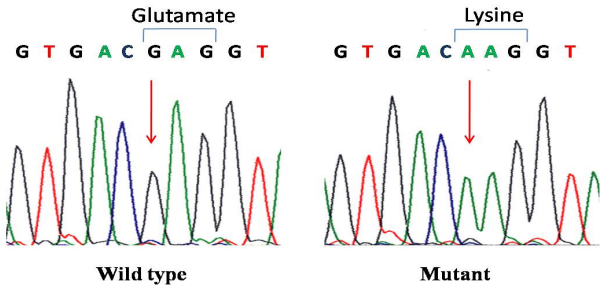

Figure 1. Genotype of Wild Type and Mutant Sequence of Exon 7 of ERK2 Gene. The wild type sequence GAG that codes for the amino acid glutamate is the last codon in exon 7, which is mutated to AAG in the mutant OSCC sample. Red arrow indicates the first base of the codon in the wild type and mutant chromatogram

Results

To understand the prevalence of E322K mutation in the common docking domain of ERK2 in OSCC tissues, we have screened genomic DNA isolated from biopsy tissue of 88 OSCC patients. PCR amplification and direct sequencing of ERK2 exon 7 identified the E322K mutation in a single OSCC sample (1 of 88, 1.1%) (Figure 1).

Discussion

In the present study, we have investigated the genomic DNA extracted from tissue biopsy samples that were excised from eighty eight OSCC patients for the prevalence of the E322K-ERK2 mutation. From our analysis of the samples by PCR amplification and direct sequencing, we identified E322K-ERK2 mutation in one of eighty eight (1.1%) samples, suggesting a relatively rare occurrence of this mutation in OSCC.

Genetic aberrations and differential expression of MAPK genes that encode molecules functioning upstream of ERK2 in the signaling cascade have been observed in well differentiated OSCC lesions. Mutations in EGFR (Hsieh et al., 2011; Szabó et al., 2011; Van Damme et al., 2010) and Ras (Hsieh et al., 2011; Murugan et al., 2012) and over expression of EGFR (Laimer et al., 2007), adaptor protein SHC (Plyte et al., 2000), G protein Rap (Mitra et al., 2003), and guanine nucleotide exchange factor SOS (Severino et al., 2008) have been reported in OSCC lesions. While none of the studies had investigated on the activating mutations in ERK2 gene, we observed and reported a single point mutation in the common docking domain of ERK2 (E322K) in an oral squamous cell carcinoma cell line in our previous study (Arvind et al., 2005). This prompted us to investigate the prevalence of E322K mutation in the tissue samples of patients with

well differentiated OSCC lesions, which showed a low prevalence of this mutation in OSCC. Nevertheless the finding is clinically significant, as drugs targeting ERK and other components of MAPK are being increasingly explored in treatment of cancers (Friday et al., 2008; Montagut et al., 2009; Chappell et al., 2011; Steelman et al., 2011). Though the MAPK/ERK signaling pathway is activated in response to a wide variety of activated receptors including receptor tyrosine kinases (RTKs), the specific components that regulate the signaling events in each of the cascade vary greatly among different stimuli. However, the cascades share a common architecture that includes a set of adaptors, which recruits G proteins to the receptor via a guanine nucleotide exchange factor (Roberts et al., 2007), and hence drugs targeting this pathway are under evaluation and development for the therapeutic intervention of a wide range of cancers.

It is important to note that most of the drugs are developed for wild type molecules, which are either over expressed or are key mediators of over stimulated signaling events. Incidence of drug resistance due to mutation in signaling molecules have indeed been reported. For example, cells expressing EGFR with activating site mutation has been found to confer resistance to gefitinib, an inhibitor of EGFR's tyrosine kinase domain (Greulich et al., 2005; Wang et al., 2012). It is possible that the E322K-ERK2 mutant, which also remains constitutively active similar to the above EGFR mutant, may similarly display resistance to inhibitors of EGFR and MEK. Interestingly, an orally consumable MEK inhibitor has recently been developed and found to be effective in treating melanoma patients (Falchook et al., 2012). This drug may eventually find useful in the treatment of early OSCC and/or precancerous lesions. Hence our finding of the low prevalence of E322K-ERK2 mutation in OSCC lesions is clinically significant, as it suggests that drugs targeting the MAPK/ERK signaling pathway may be developed and explored for their efficacy in treating OSCC.

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