RESEARCH ARTICLE

Lack of RING Finger Domain (RFD) Mutations of the c-Cbl Gene in Oral Squamous Cell Carcinomas in Chennai, India

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Abstract

Background: In normal cells, activated epidermal growth factor receptor (EGFR) molecules are subjected to ubiquitination-mediated proteasome degradation pathway by c-Cbl, an ubiquitin ligase that checks uncontrolled proliferation. Hence expression of wild type c-Cbl molecule is essential to keep this degradation machinery in a functional state. Loss of expression or function of c-Cbl may consequently lead to sustained activation of EGFR and promote carcinogenesis, loss of function mutations in the c-Cbl gene already being reported in lung and hematopoietic cancers. However, the genetic status of c-Cbl in oral squamous cell carcinoma (OSCC) is not known. Hence in the present study we investigated the genomic DNA isolated from OSCC tissue biopsy samples for mutations in the RING finger domain coding region of c-Cbl gene, which has also been reported to be most frequently mutated in other cancers. Materials and Methods: Total genomic DNA isolated from thirty two post surgical OSCC tissue samples were amplified using primers flanking the exon 8 of c-Cbl gene that codes for the RING finger domain. The PCR amplicons were then resolved in a 1.2% agarose gel, purified and subjected to direct sequencing to screen for mutations. Results: The sequencing data of the thirty two OSCC samples did not identify mutations in the RING finger domain coding region of c-Cbl gene. Conclusions: To the best of our knowledge, this is the first time that the genetic status of c-Cbl gene in OSCC samples has been investigated. The present data indicates that genetic alteration of RING finger domain coding region of c-Cbl gene is relatively infrequent in OSCC samples.

Keywords: RING finger domain mutation - RING finger domain - c-Cbl mutation in oral cancer - Chennai

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Introduction

The epidermal growth factor receptor (EGFR) belongs to a family of receptor tyrosine kinases (RTKs) that includes HER2/neu (erbB2), HER3 (erbB3), and HER4 (erbB4) (Fekrazad et al., 2011; Jiang et al., 2012). Binding of its cognate ligand – epidermal growth factor (EGF) to the extracellular ligand-binding domain of EGFR promotes homo- and hetero- dimerization between receptors of the same family, which is followed by auto-phosphorylation and transactivation via its intracellular tyrosine kinase domain (Bazley et al., 2005). Activation of specific tyrosine kinase residues of EGFR creates binding sites for a series of proteins, and triggers activation of diverse signal transduction pathways. For example, phosphorylation at Tyr845 residue in the activation loop of EGFR stabilizes and maintains EGFR in an active state. Phosphorylation at tyrosine residues 992 and 1173 or 1068, however, creates binding motifs for phospholipase C-γ (PLC-γ) or Grb2/SH2 adaptor proteins and enables the activation of ERK2 cascade. Besides the above, additional phospho-tyrosine residues on the EGFR (pY1148, pY1086, pY1101) enable differential activation of a number of other proteins in response to EGF stimulation (Papagiorgis et al, 2012).

ERK2 is the founding member of MAPK family, the activation of which is mediated via Ras/Raf/MEK kinase pathway (Arvind et al., 2005; Mahalingam et al., 2008). Activated ERK2 translocates into the nucleus to transactivate cell cycle promoting genes. A sustained activation signal emanating from the EGFR is likely to promote constitutive activation of ERK2 and consequently carcinogenesis (Arvind et al., 2005). Hence in physiologically normal cells, the activation and inactivation of EGFR molecules is a tightly regulated event (Visser et al., 2005). For example, following activation of EGFR the cytoplasmic domain of receptor is tagged with multiple mono-ubiquitin molecules by an ubiquitin ligase – c-Cbl (de Melker et al., 2004), which promotes endocytosis (Mosesson et al., 2003; Visser et al., 2009) and subsequent degradation of the receptor (Rubin et al., 2005; Glogowska et al., 2012). c-Cbl

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belongs to RING finger domain family of E3 ubiquitin ligases (Swaminathan et al., 2006), which has a tyrosine kinase binding (TKB) domain and a RING finger domain linked by an intervening linker sequence in the N-terminus followed by an extended C-terminal region that includes proline-rich domain, ubiquitin associated–leucine zipper domain and tyrosine residues (Nau et al., 2003; Ryan et al., 2010). Phosphorylation of these tyrosine residues upon stimulation by growth factors such as EGF is essential for functional activation of c-Cbl molecules (Sorkin et al., 2009). Of the above domains, the RING finger domain primarily confers the E3 ubiquitin ligase activity to c-Cbl, by recruiting the E2 conjugating enzyme and ubiquitin monomers at the linker-RING finger domain interface of c-Cbl bound to activated EGFR (Sorkin et al., 2009; Ryan et al., 2010). Hence deregulation of c-Cbl function even within the RING finger domain alone may result in sustained activation of EGFR for a prolonged period such as those that occur in mutated EGFR molecules, and consequently may promote carcinogenesis. Indeed deregulating mutations specifically in the RING finger domain of c-Cbl have been frequently reported in hematopoietic (Muramatsu et al., 2010; Ogawa et al., 2010) and lung carcinoma (Tan et al., 2010). However, the occurrence of this mutation in the genome of OSCC tissues remains yet to be investigated. Hence in the present study, we investigated the sequence profile of the RING finger domain coding region of c-Cbl gene in thirty two patients with well differentiated OSCC lesion.

Materials and Methods

Study design and subjects

In order to investigate the occurrence of mutations in RING finger domain coding region of c-Cbl gene, a cross sectional study was designed by including tissue samples from OSCC lesions obtained post-surgically from patients visiting tertiary cancer hospitals in Tamil Nadu. 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 during the period of October 2011 to December 2012.

Genomic DNA extraction, polymerase chain reaction and direct sequencing

Biopsy tissues collected were transported in RNA Later (Cat # 76106, Qiagen, USA) and were processed as described earlier (Jayaraman et al., 2012). Exon 8 of the c-Cbl gene was amplified with following set of intronic primers, c-CblF (forward primer): cccagactagatgctttctggtt, and c-CblR (reverse primer): aagaagcaaaagatagtaacagatgc, using 70 ng of genomic DNA. PCR amplification was performed as follows: after an initial denaturation at 94°C for 4 min, 35 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min was followed to amplify the exon 8 region. The PCR amplicon was run in a 1.5% agarose gel and eluted with Genelute DNA gel elution kit (Sigma Aldrich, cat# NA1111), which was then subjected to direct sequencing to screen for mutation.

Results

To know whether the RING finger domain coding region of c-Cbl is mutated in OSCC tissues, we screened the DNA samples obtained from 32 OSCC patients. The entire exon 8 of c-Cbl gene, which encodes for RING finger domain of c-Cbl protein, was amplified with exon specific primers, which was followed by gel purification and direct sequencing. The results showed no mutations in the amplified fragments of exon 8 of c-Cbl gene in the thirty two samples that were analyzed.

Discussion

In the present study, we have investigated genomic DNA from thirty-two OSCC samples for the occurrence of mutations in exon 8 of c-Cbl gene that encodes for the RING finger domain in c-Cbl protein. Sequencing analysis did not identify mutation in the thirty two samples that were analyzed, indicating that the occurrence of mutations in the RING finger domain to be relatively rare.

RING finger domain is essential for E3 ubiquitin ligase activity of c-Cbl, and mutations in this domain have been reported in hematopoietic (Muramatsu et al., 2010; Ogawa et al., 2010) and lung carcinomas (Tan et al., 2010). Though the c-Cbl protein structure has TKB, RING finger and proline rich domains along with an alpha helical linker region and tyrosine phosphorylation sites in C-terminal region, mutations within the RING finger domain have been reported to occur preferentially in acute myeloid leukemia (AML) (Caligiuri et al., 2007) juvenile myelomonocytic leukemia (Muramatsu et al., 2010) and non-small cell lung carcinoma (Tan et al., 2010). These mutations include deletion of entire exon 8, substitution and nonsense mutations. Mutations within the TKB region, linker region, intron 8 splice acceptor site, proline rich domain and extended C-terminus region have also been reported albeit at a relatively lower prevalence.

A comparative study of all exons of c-Cbl in patients with non-small cell lung carcinoma biopsy tissues from three different races, Caucasians, African Americans and Asians (Taiwanese) showed distinct mutation profile among them (Tan et al., 2010). While the RING finger domain mutations were found with higher prevalence in Caucasians and African Americans, none of the patients from Taiwanese Asian group carried the mutation. Furthermore the distribution of mutation profile along the entire domain region was found to vary among the Caucasians and African Americans. Whereas Caucasian patients carried mutation in the TKB and proline rich domains besides the RING finger domain, African American patients were found to carry no such mutations in the TKB and proline rich domains. They however carried mutations in extended C-terminal region, which was not prevalent in Caucasians and African Americans. In addition to the above genetic race based variations, cancerous lesion based variation in the mutation profile within the RING finger domain has been reported. While V391I mutation was found to occur in non-small cell lung carcinomas, C401S, Y371H and inframe deletions within the RING finger domain coding region was found

in juvenile myelomonocytic leukemias (Muramatsu et al., 2010). Given the fact that such variations are common in cancers affecting patients from diverse racial background, it is possible that the patients that we analyzed either harbored mutation in other regions of c-Cbl or did not carry any mutation at all especially since the patient population that we analyzed were of a mix of individuals from North-Eastern region and South Indian Dravidian population. It is noteworthy that the genetic background of Indian population has been found to be divergent based on genome-wide polymorphism analysis (Reich et al., 2009; Gupta et al., 2012). Hence the lack of mutation in the RING finger domain of samples analyzed in the present study does not rule out for the presence of mutation in other regions of c-Cbl gene and remains to be investigated by including a larger number of OSCC samples from one single race to determine the actual c-Cbl mutation prevalence.

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