RESEARCH ARTICLE

Tyrosine 1045 Codon Mutations in Exon 27 of *EGFR* are Infrequent in Oral Squamous Cell Carcinomas

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Abstract

Background: The activation and inactivation of receptor tyrosine kinases are tightly regulated to ensure faithful replication of cells. After having transduced extracellular growth activating signals, activated EGFR is subjected to downregulation either by clathrin mediated endocytosis or c-Cbl mediated proteasome degradation depending on the ligand concentration. c-Cbl is an ubiquitin ligase which requires a phosphorylated tyrosine residue at position 1045 in the cytoplasmic domain of EGFR to interact and add ubiquitin molecules. While activating mutations in exons 19 and 21 have been associated with the development of several cancers, the status of mutations at tyrosine 1045 coding exon 27 of EGFR remain to be investigated. Consistently, defective phosphorylation at 1045 has been associated with sustained phosphorylation of EGFR in non-small lung carcinomas. Hence in the present study we investigated the genetic status of the tyrosine 1045 coding site within exon 27 of EGFR gene to explore for possible occurrence of mutations in this region, especially since no studies have addressed this issue so far. Materials and Methods: Tumor chromosomal DNA isolated from thirty five surgically excised oral squamous cell carcinoma tissues was subjected to PCR amplification with intronic primers flanking the tyrosine 1045 coding exon 27 of EGFR gene. The PCR amplicons were subsequently subjected to direct sequencing to elucidate the mutation status. Results: Sequence analysis identified no mutations in the tyrosine 1045 codon of EGFR in any of the thirty five samples that were analyzed. Conclusions: The lack of identification of mutation in the tyrosine 1045 codon of EGFR suggests that mutations in this region may be relatively rare in oral squamous cell carcinomas. To the best of our knowledge, this study is the first to have explored the genetic status of exon 27 of EGFR in oral squamous cell carcinoma tissue samples.

Keywords: *EGFR* expression in oral carcinoma - tyrosine 1045 mutation - tyrosine 1045 phosphorylation

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Introduction

Epidermal growth factor receptor (EGFR) belongs to the Erb family of proteins that also includes HER2/neu, HER3/ErbB2 and HER4/ErbB4. EGFR is a transmembrane receptor tyrosine kinase (RTK) and is expressed in multiple cell types (Castillo et al., 2004; Soonthornthumet al., 2011). The molecular structure of EGFR comprises of an extracellular ligand binding domain, a single transmembrane section and an intracellular tyrosine kinase domain (Leahy et al., 2004), which plays an important role in cell proliferation and differentiation upon its activation (Voldborg et al., 1997; Ogiso et al., 2002). EGFR is activated when its cognate ligand epidermal growth factor (EGF) binds to its extracellular domain, which initiates homo or hetero dimerization between the members of the Erb family (Burgess et al., 2008). The ligand binding and dimerization is followed by the activation of cytoplasmic kinase domain by autophosphorylation (Rubin et al., 2005). The autophosphorylation of EGFR forms docking sites for cytosolic proteins containing the SH2 (Src Homology 2) domain (Fukazawa et al., 1996), on whose interaction a series of intracellular signaling cascade is activated (Normanno et al., 2006). Ligand binding not only plays a role in activating a cascade of intracellular pathways but also activates a negative regulatory pathway in order to degrade the receptor by endocytosis. Endocytosis plays an important role to stop the growth signal transduction by removing activated receptors from the cell surface and replacing them with active receptors when required (de Melker et al., 2001).

Degradation of EGFR is regulated by two processes, (*a*) by clathrin mediated endocytosis (CME) pathway and (*b*) by non-clathrin mediated endocytosis (NCE) pathway (Woelk et al., 2007). The pathway involved for degradation depends largely on the concentration of the ligand in the extracellular region. At lower concentrations of EGF the degradation is mediated by CME where ubiquitination is not required before internalization whereas at higher concentrations of EGF, CME is also active but a considerable number of activated EGFR molecules are

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degraded by NCE (non-clathrin endocytosis). In NCE pathway the degradation takes place in three steps that involves ubiquitination, internalization and proteasome mediated degradation, whereas in CME pathway the ubiquitination step is omitted (Huang et al., 2007).

Ubiquitination of EGFR is carried out by a ubiquitin ligase, c-Cbl (de Melker et al., 2004), which interacts with EGFR either by direct interaction of c-Cbl's PTB (phospho tyrosine binding) domain to a phosphorylated tyrosine (Y1045) present on the cytoplasmic domain of EGFR or indirectly by binding to Grb2, an adaptor molecule, which then binds to phophorylated tyrosine (Y1068 or Y1086) with its SH2 domain (Lipkowitz et al., 2002; Pennock et al., 2008). It is also found that ubquitination of EGFR is not as much poly-ubiquitination as mono-ubiquitination at multiple sites has also been observed to be sufficient to promote degradation (de Melker et al., 2004). The failure of c-Cbl binding to EGFR leaves the EGFR in active state for a prolonged period, which leads to continuous activation of components of MAPK (Mitogen Activated Protein Kinase) pathway – H-Ras, cRaf and ERK2. Constitutive activation of EGFR, H-Ras, cRaf and ERK2 has been shown to be carcinogenic (Jayaraman et al., 2012). Multiple tumor cases in the past have shown a correlation between prolonged activation of EGFR and cancer (Kim et al., 2002; Normanno et al., 2006). Mutations at tyrosine 1045 codon of EGFR have been shown to interfere with its phosphorylation and ability to interact with c-Cbl (Kirisits et al., 2007). This was also confirmed in a recent case control study, where in phosphorylation at tyrosine 1045 residue was observed only in 52% of 218 cases of non-small cell lung cancers (NSCLCs) that were analyzed (McMillen et al., 2010). The lack of phosphorylation in rest of the tumor samples may have been due to a mutation at the tyrosine 1045 region, but was not analyzed. This prompted us to investigate whether oral squamous cell carcinomas (OSCCs) harbored mutations at the tyrosine 1045 site of EGFR by direct sequencing of genomic DNA obtained from tissue biopsies of patients with well differentiated OSCC, as constitutive activation of EGFR has been observed in OSCCs (Valiathan et al., 2012).

Materials and Methods

Study design and subjects

The occurrence of mutations in the c-Cbl interacting site tyrosine 1045 of EGFR was investigated in a cross sectional study on well differentiated oral squamous cell carcinoma tissue samples that were obtained post-surgically from patients visiting tertiary cancer hospitals in Tamil Nadu. The study was approved by the institutional ethics committee (IEC) and was conducted at the Molecular Medicine Research Division, Enable Biolabs, Chennai, Tamil Nadu, India.

Tumor gDNA extraction, PCR amplification and direct sequencing

Surgically excised biopsies of cancerous tissues were collected and transported in RNA Later (Cat#76106, Qiagen, USA). Genomic DNA extraction was processed as described earlier (Jayaraman et al., 2012). The exon 27 of the EGFR gene was amplified using intronic primers, Y1045F (forward primer): gcctgcccaacctactaat, and Y1045R (reverse primer): gacactggagactggctgc, using 25ng of genomic DNA. The exon 27 region was amplified under the following conditions: after an initial denaturation at 94°C for 2 min, the samples were subjected to 35 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 45 sec, 72°C for 45 sec, which was then 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.

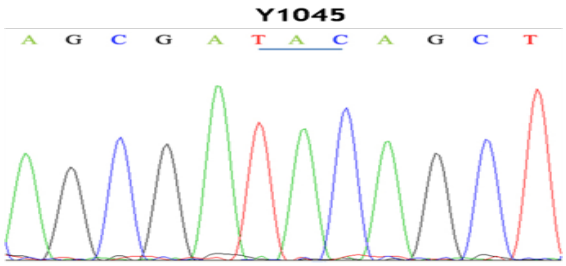

Figure 1. Genotype of Wild Type Sequence of Tyrosine 1045 Codon within Exon 27 of EGFR Gene. The wild type sequence TAC (indicated by a blue underline) that codes for the amino acid tyrosine remained unchanged in the thirty five oral squamous cell carcinoma tissue samples

Results

Ligand binding to EGFR causes dimerization and activation of EGFR that mediates transduction of mitogenic signals to the nuclei. Subsequently, the ligand-receptor complex is endocytosed and degraded via ubiquitin mediated proteasome pathway, which results in attenuation of the mitogenic signal. This process requires phosphorylation at tyrosine 1045 residue of EGFR that provides docking site for the ubiquitin ligase c-Cbl. We speculated that mutations within the tyrosine 1045 codon, if present, may interfere with the degradation machinery thereby causing sustained activation of EGFR in the tumors. To test our hypothesis, the genomic DNA extracted from thirty five oral squamous cell carcinoma tissue samples was amplified with primers flanking exon 27 of EGFR containing tyrosine 1045 codon and subjected to direct sequencing. Sequence analysis of the PCR amplicons showed no mutations in the 1045 codon region or other codons within exon 27 in the thirty five samples that were analyzed. The wild type codon at 1045 region identified in the sequencing analysis is shown in Figure 1.

Discussion

Following ligand mediated activation of EGFR, the ligand-receptor complex is principally degraded via ubiquitin mediated proteasome degradation pathway especially when the concentration of the ligand is high. This requires binding of the ubiquitin ligase c-Cbl at phospho-tyrosine 1045 in the cytoplasmic domain of EGFR to facilitate ubiquitination of EGFR molecules (Rubin et al., 2005; Visser Smit et al., 2009; Glogowska

et al., 2012). Hypophosphorylation at tyrosine 1045 residue reduces ubiquitination and downregulation of EGFR, which indeed have been reported in NSCLC (Han et al., 2007; Sigismud et al., 2012) and glioblastomas (Abella et al., 2009). However, it is not known whether the tyrosine 1045 codon carried mutations in the NSCLC and glioblastomas. Similarly, constitutive activation of EGFR has been observed in OSCC both in in vitro studies involving cell lines and tissue samples of patients (Valiathan et al., 2012). While constitutive activation of EGFR has been linked with gene amplification in some OSCCs, the reason for constitutive activation in OSCCs without EGFR gene amplification has not been investigated. Since, mutations within the tyrosine 1045 codon are likely to interfere with its phosphorylation and subsequent binding of c-Cbl and ubiquitination of EGFR, we have investigated, in the present study, for the presence of mutations within exon 27 of EGFR that carries the codon for tyrosine 1045 in thirty five oral squamous cell carcinoma biopsy tissues. Direct sequencing analysis showed mutations in none of the thirty five samples that were analyzed neither at the 1045 site or in other codons within exon 27, which indicated that mutations within this region may be infrequent.

Though no mutations were identified within exon 27, the data does not rule out the possibility of mutations at other locations within EGFR, which may have acted in concert to deter the interaction of c-Cbl with activated EGFR. For example, activating mutations within exon 19 of EGFR have shown to deter interaction of c-Cbl with activated EGFR and its subsequent ubiquitination and degradation (Hosaka et al., 2007). The mutation in the codon for tyrosine 1045 also prevents the phosphorylation of tyrosine 1045 residue, which is critical for interaction of c-Cbl. Besides, mutations in exons 18 and 21 have also been shown to prolong the duration of activation of EGFR in in vitro studies, however without affecting the tyrosine 1045 phosphorylation (Hosaka et al., 2007). Hence, it is possible that mutations may exist in either exon 18, 19 or 21 in the OSCC samples that were analyzed in the present study. It is important to note that the present data does not address whether the expressed EGFR molecules in the analyzed OSCC tissues were constitutively activated or hypophosphorylated at tyrosine 1045 residue. The fact that overexpression and constitutive activation of EGFR, and sustained activation and mutations in other downstream molecules of EGFR – H-ras, c-Raf and ERK1/2 have been observed in several carcinomas including OSCC (Valiathan et al., 2012), prompted us to investigate for the occurrence of mutations in the tyrosine 1045 codon of EGFR. Hence, further investigations on the genetic status of exon 18, 19 and 21 in these OSCC samples are essential to understand and correlate the role of EGFR in the development of OSCC lesions.

The SH2 domain at the N terminus of c-Cbl, named as TKB (Tyrosine Kinase Binding) domain, is essential for its interaction with phospho-tyrosine 1045 residue of activated EGFR, to ubiquitinate and degrade. Mutations within the TKB and associated linker domain have been reported in somatically acquired malignancies (Martinelli et al., 2010). Ubiquitination of activated EGFR by c-Cbl is also promoted by its indirect interaction to EGFR via Grb2 bound to phophorylated tyrosine 1068 or 1086 (Lipkowitz et al., 2002; Pennock et al., 2008). Hence, mutations within the adaptor Grb2 interacting domain of c-Cbl or c-Cbl interacting domain of Grb2 are likely to promote sustained activation of EGFR. However, these possibilities remain to be addressed. It is important to note that mutations within the RING finger domain coding region of c-Cbl, which is required for functional activation of c-Cbl, were not observed in an earlier study in OSCC tissue samples (Rajendran et al., 2013). This strongly suggests the existence of aforementioned possibilities with respect to defects either within EGFR or its other associated molecules that promotes its ubiquitination. Besides being tagged for ubiquitin mediated degradation by c-Cbl, the ligand activated EGFR molecules are also degraded by lysosomal proteases, which is mediated via clathrin-coated pits involving endosomal pathway. Mutations and expression anomalies of genes regulating both proteasome and lysosomal degradation pathways have been reported in several carcinomas (Kirkegaard et al., 2009).

The lack of identification of mutations within the tyrosine 1045 coding region suggests that this specific mutation may be infrequent in OSCCs, unlike mutations in other genes such as H-ras for example, which has been found to occur in up to 20% of OSCCs from India (Murugan et al., 2012). The finding also carries clinical significance as glioblastomas carrying a deletion mutant of EGFR - EGFRvIII, which has an in-frame deletion in its cytoplasmic domain, are either hypophosphorylated or not phosphorylated at tyrosine resistant 1045 (Sigismund et al., 2012), are not degraded upon treatment with tyrosine kinase inhibitors and are resistant to the EGFR inhibitor drug, gefitinib (Sigismund et al., 2012). As the usage of several EGFR inhibitor drugs are in clinical trials for therapeutic management of OSCCs (El-Rayes et al., 2004), the absence of mutation at tyrosine 1045 codon suggests that OSCCs may respond favorably to therapeutic management.

References

Abella JV, Park M (2009). Breakdown of endocytosis in the oncogenic activation of receptor tyrosine kinases. *Am J Physiol Endocrinol Metab*, **296**, 973-84.

Burgess AW (2008). EGFR family: structure physiology signaling and therapeutic targets. *Growth Factors*, **26**, 263-74.

Castillo L, Etienne-Grimaldi MC, Fischel JL, et al (2004). Pharmacological background of EGFR targeting. *Ann Oncol*, **15**, 1007-12.

de Melker AA, van der Horst G, Calafat J, Jansen H, Borst J (2001). c-Cbl ubiquitinates the EGF receptor at the plasma membrane and remains receptor associated throughout the endocytotic route. *J Cell Science*, **114**, 2167-78.

de Melker AA, van der Horst G, Borst J (2004). Ubiquitin ligase activity of c-Cbl guides the epidermal growth factor receptor into clathrin-coated pits by two distinct modes of Eps15 recruitment. *J Biol Chem*, **279**, 55465-73.

El-Rayes BF, LoRusso PM (2004). Targeting the epidermal growth factor receptor. *Bri J Cancer*, **91**, 418-24.

Fukazawa T, Miyake S, Bandi V, Band H (1996). Tyrosine

phosphorylation of Cbl upon epidermal growth factor (EGF) Stimulation and its association with EGF receptor and downstream signaling proteins. *J Biol Chem*, **271**, 14554-9.
Glogowska A, Stetefeld J, Weber E, et al (2012). Epidermal growth factor cytoplasmic domain affects erbb protein degradation by the lysosomal and ubiquitin-proteasome pathway in human cancer cells. *Neoplasia*, **14**, 336-409.
Han W, Zhang T, Yu H, Foulke JG, Tang CK (2006). Hypophosphorylation of residue Y1045 leads to defective downregulation of EGFRvIII. *Cancer Biol Ther*, **5**, 1361-8.
Hosaka T, Inoue F, Ando K, et al (2007). Mutant epidermal growth factor receptor undergoes less protein degradation due to diminished binding to c-Cbl. *Anticancer Res*, **27**, 2253-64.
Huang F, Sorkin A (2005). Growth factor receptor binding protein 2 mediated recruitment of the RING domain of Cbl to the epidermal growth factor receptor is essential and sufficient to support receptor endocytosis. *Mol Biol Cell*, **16**, 1268-81.
Huang F, Goh LK, Sorkin A (2007). EGF receptor ubiquitination is not necessary for its internalization. *PNAS*, **104**, 16904-9.
Jayaraman B, Valiathan GM, Jayakumar K, et al (2012). Lack of mutation in p53 and H-ras genes in phenytoin induced gingival overgrowth suggests its non cancerous nature. *Asian Pac J Cancer Prev*, **13**, 5535-8.
Kim YT, Park SW, Kim JW (2002). Correlation between expression of EGFR and the prognosis of patients with cervical cancer. *Gynecol Oncol*, **87**, 84-9.
Kirisits A, Pils D, Krainer M (2007). Epidermal growth factor receptor degradation: an alternative view of oncogenic pathways. *nt J Biochem Cell Biol*, **39**, 2173-82.
Kirkegaard T, Jaattela M (2009). Lysosomal involvement in cell death and cancer. *Biochim Biophys Acta*, **1793**, 746-54.
Leahy DJ (2004). Structure and Function of the Epidermal Growth Factor (egf/erbb) family of receptors. *Adv Protein Chem*, **68**, 1-27.
Lipkowitz S (2002). The role of the ubiquitination–proteasome pathway in breast cancer Ubiquitin mediated degradation of growth factor receptors in the pathogenesis and treatment of cancer. *Breast Cancer Res*, **5**, 8-15.
Martinelli S, De Luca A, Stellacci E, Rossi C, Checquolo S et al (2010). Heterozygous germline mutations in the CBL tumor-suppressor gene cause a Noonan syndrome-like phenotype. *Am J Hum Genet*, **87**, 250-7.
McMillen E, Ye F, Li G, Wu Y, Kin G et al (2010). Epidermal growth factor receptor (EGFR) mutation and p-EGFR expression in resected non-small cell lung cancer. *Exp Lung Res*, **36**, 531-7.
Murugan AK, Munirajan AK, Tsuchida N (2012). Ras oncogenes in oral cancer: the past 20 years. *Oral Oncol*, **48**, 383-92.
Normanno N, De Luca A, Bianco C, et al (2006). Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene*, **336**, 2-16.
Ogiso H, Ishitani R, Nureki O, et al (2002). Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. *Cell*, **110**, 775-87.
Pennock S, Wang Z (2008). A tale of two Cbls: interplay of c-Cbl and Cbl-b in epidermal growth factor receptor downregulation. *Molecular and Cell Biology*, **28**, 3020-37.
Rajendran S, Muthupalani RS, Ramanathan A (2013). Lack of RING finger domain (RFD) Mutations of the c-Cbl gene in oral squamous cell carcinomas in Chennai, India. *Asian Pac J Cancer Prev*, **14**, 1073-5.
Rubin C, Gur G, Yarden Y (2005). Negative regulation of receptor tyrosine kinases: unexpected links to c-Cbl and receptor ubiquitylation. *Cell Res*, **15**, 66-71.
Sigismund S, Confalonieri S, Ciliberto A, et al (2012). Endocytosis and signaling: cell logistics shape the eukaryotic cell plan. *Physiol Rev*, **92**, 273-366.
Soonthornthum T, Arias-Pulido H, Joste N, et al (2011). Epidermal growth factor receptor as a biomarker for cervical cancer. *Ann Oncol*, **10**, 2166-78.
Valiathan GM, Thenumgal SJ, Jayaraman B, et al (2012). Common docking domain mutation E322K of the ERK2 gene is infrequent in oral squamous cell carcinomas. *Asian Pac J Cancer Prev*, **13**, 6155-7.
Visser Smit GD, Place TL, Cole SL, et al (2009). Cbl controls EGFR fate by regulating early endosome. *Sci Signal*, **2**, 86.
Voldborg BR, Damstrup L, Spang-Thomsen M, Poulsen HS (1997). Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. *Annals of Oncology*, **8**, 1197-206.
Woelk T, Sigismund S, Penengo L, Polo S (2007). The ubiquitination code: a signalling problem. *Cell Division*, **2**, 11.