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EGFR over-expression in non-small cell lung cancers harboring EGFR mutations is associated with marked down-regulation of CD82

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ABSTRACT

Epidermal growth factor receptor (EGFR) gene mutations are strongly associated with lung adenocarcinoma and favorable response to EGFR tyrosine kinase inhibitor. The mutated EGFR proteins (EGFRs) are hyper-phosphorylated and refractory to receptor down-regulation. To address the discrepancy between hyper-phosphorylation and lack of down-regulation of mutant EGFRs, we have examined the expression of EGFR negative regulators in non-small cell lung cancer (NSCLC) cell lines. We found that NSCLC cell lines expressing mutant EGFRs often had low expression of various negative regulators for EGFR. Among them, tumor suppressor CD82 was up-regulated by wild type (WT) EGFR but down-regulated by mutant EGFRs. Reconstitution of CD82 exerted stronger suppressive effects on mutant EGFRs than on WT EGFR. Active exportation of CD82 through the exosome was one of the mechanisms involved in achieving the overall CD82 down-regulation in mutant EGFR-expressing lung cancer cell lines. Over-expression of mutant EGFR protein frequently occurred in the lung cancer tissues of mutant EGFR-transgenic mice and also associated with CD82 down-regulation. Immunoblot analyses on the tumor tissues from 23 lung adenocarcinoma patients (12 with WT EGFR, and 11 with mutant EGFRs) also identified significantly stronger down-regulation of CD82 in tumors with mutant EGFRs than WT. Our data indicate that CD82 down-regulation could be a critical step involved in the EGFR over-expression and the stronger tumorigenic activity triggered by EGFR mutations. Up-regulation of the CD82 level may become a promising new treatment strategy for lung adenocarcinoma.

1. Introduction

Epidermal growth factor receptor (EGFR) participates in various cellular functions and abnormal EGFR signaling is involved in many malignant diseases [1,2]. EGFR mutations in the kinase domain frequently occur in lung adenocarcinoma (LAC) among East Asians [3,4]. The L858R mutation and deletions (Del) in exon 19 are the major EGFR mutations [3–7]. Mutant EGFR proteins (EGFRs) are strongly tyrosine-phosphorylated in the absence of ligands [8,9]. Phosphorylation of wild type (WT) EGFR leads to subsequent internalization, ubiquitination, and degradation [1]. In contrast, mutated EGFR is resistant to down-regulation albeit their hyper-phosphorylation status [8,10,11]. Agreeing with these observations, EGFR mutations are closely associated with EGFR over-expression in LAC tissues [3]. Thus, mutant EGFRs are supposed to exert stronger tumorigenic activity than WT EGFR [12]. However, the detailed molecular mechanism of mutant EGFRs' stronger tumorigenic ability remains to be elucidated.

To address the discrepancy between hyper-phosphorylation and lack of down-regulation of mutant EGFRs, we have examined the expression of various negative regulators for EGFR in lung cancer cell

Abbreviations: EGFR, epidermal growth factor receptor; LAC, lung adenocarcinoma; Del, deletion; WT, wild type; c-Cbl, casitas B-lineage lymphoma; GAK, cyclin G-associated kinase; NSCLC, non-small cell lung cancer; PKC, protein kinase C; SP-A, surfactant protein A; SP-C, surfactant protein C; BGH, bovine growth hormone; 5-aza-dC, 5-aza-deoxy-cytidine; TsA, trichostatin; Flot 1, Flotillin 1

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lines expressing mutant EGFRs, which include casitas B-lineage lymphoma (c-Cbl, the EGFR ubiquitin ligase) [13], cyclin G-associated kinase (GAK, an endocytosis regulator) [14], and CD82 [15–18]. We found that lung cancer cell lines expressing mutant EGFRs frequently had low expression of one or several of the above three proteins. Among them, the tumor suppressor CD82 was up-regulated by WT EGFR but down-regulated by mutant EGFRs, which is intriguing.

CD82, a member of the teraspanin family, is a cancer metastasis suppressor and is often down-regulated in cancers [15–18]. Moreover, CD82 expression is correlated with a good prognosis in non-small cell lung cancer (NSCLC) patients [19]. CD82's anti-metastatic ability is attributed to its interaction with integrin molecules [20–23]. CD82 interacts with Duffy antigen receptor for chemokines (DARC) on endothelial cells and causes the growth inhibition of the CD82-expressing tumor cells [24]. Additionally, CD82 interacts with EGFR and c-Met to suppress the receptor-induced lamellipodia formation [25]. CD82 enhances EGF-induced EGFR internalization, which is mediated through protein kinase C (PKC)α and ganglioside-dependent mechanisms [25–27]. A recent study shows that CD82 reduces the level of ubiquitylation of EGFR upon heparin-binding EGF (HB-EGF) or amphiregulin stimulation and alters the postendocytic trafficking of EGFR [28].

In this study, we found that EGFR over-expression was associated with a marked reduction of CD82 in (1) NSCLC cells expressing mutant EGFRs, (2) NSCLC cells with endogenous EGFR mutations, (3) lung cancer tissues derived from mutant EGFR-transgenic (Tg) mice, and (4) tumor tissues of LAC patients with EGFR mutation. Our data indicated that down-regulation of CD82 could be a mechanism involved in the mutant EGFR over-expression in LAC cells.

2. Material and methods

2.1. Cell cultures and transfection

Five human NSCLC cell lines (H1299, HCC1975, HCC1650, HCC827 and PE089) were used in this study. The H1299 cell line is established from a patient with lung carcinoma. The PE089, H1650, H1975, HCC827 cell lines (the latter three were provided by Dr. Tsu-An Hsu from the Institute of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taiwan) are derived from patients with LAC. H1299 cells express wild type (WT) EGFR; the H1975 cell line has L858R/T790M double mutations; and H1650, PE089, and HCC827 cells all have EGFR exon 19 deletion (Del). H1299 derivatives were established by transfection with an empty pcDNA4/TO/myc-His vector (Invitrogen; Carlsbad, CA) or with a vector containing WT-EGFR, EGFR-L858R, or EGFR-Del coding sequence. The transfected cells were subjected to 500 μg/ml of zeocin (Invitrogen) selection for one week to establish permanently transfected cells as previously described [8]. H1299-EGFR, H1299-L858R, and H1299-Del cells were combined in pools of drug-resistant colonies expressing the respective receptor, as confirmed by immunoblotting assays, to avoid clonal variations. H1299 and its derivatives were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) plus penicillin and streptomycin [8]. H1650, H1975, and HCC827 cells were cultured in the same media as H1299 cells. PE089 cell line was cultured in DMEM basal medium supplemented with 10% FCS plus penicillin and streptomycin [29]. Transient transfections of cells were performed using the Lipofectamine-2000 reagent (Invitrogen) according to the manufacturer's instruction.

2.2. Construction of DNA vectors

CD82-coding sequence was PCR-amplified using a cDNA pool prepared from H1299 cells. The DNA fragment was enzyme-digested and inserted into a pCR3.1 vector (Invitrogen) between Hind III and Xho I sites with a carboxy-terminal Flag tag. To construct the vectors for Tg mice with mutated EGFR, the backbone plasmid used was the pcDNA4 mutant EGFR-expressing vector described previously [8]. Surfactant protein A (SP-A) promoter was PCR amplified using the genomic DNA of H1299 cells as the template. The bovine growth hormone (BGH) poly-adenylation sequence was PCR amplified using the pCR3.1 vector as a template. The chicken β-globin insulator sequence was kindly provided by Dr. Felsenfeld (National Institute of Diabetes and Digestive and Kidney Disease, MD, USA) [30]. All the PCR-derived DNA fragments inserted into the Tg vector were sequence-verified. The sequences of the oligonucleotides used in this study were listed in the Table 1.

2.3. Establishment of Tg mice with mutant EGFRs

The 8.9 kilo-based Tg cassette was released from the construction vector between Sca I and Pme I sites for mouse pronuclear injections. The micro-injections of C57BL/6 fertilized eggs were performed in the Level Biotechnology Inc. (Taipei, Taiwan). Successful injections were verified by positive identification of the human EGFR gene in the Tg mouse tail DNA through Southern blot analyses and PCR genome-typing. Mutant EGFR-Tg mice were sacrificed at indicated ages and were examined for the presence of any tumor-like lesions. Portions of the lung tissues, either with or without tumor formation, were snap frozen and stored at −80 °C deep freezer for future DNA, RNA and protein extractions. The remaining lung tissues were fixed in neutral formalin for paraffin section preparations. All experimental procedures using animals were approved by the Institutional Animal Care and Use Committees of National Health Research Institutes (NHRI).

2.4. Patient materials

Fresh frozen tumor and paired non-tumor lung tissue specimens of 23 LAC patients receiving surgical resection at Chang Gung Memorial Hospital (CGMH) and with signed informed consent were obtained from the tissue bank of CGMH. All of the specimens were snap frozen soon after resection and stored at −80 °C. Only tumor tissues with higher than 70% tumor content were included for this study. The specimens were used for protein extraction and immunoblot analysis to examine the expression of EGFR and CD82 in these specimens. The study protocol has been reviewed and approved by the Institutional Review Boards of CGMH and NHRI.

2.5. RNA extraction and complementary DNA (cDNA) synthesis for quantitative polymerase chain reaction (Q-PCR)

Fresh frozen tumor and paired non-tumor lung tissues from Tg mice, control non-Tg mice, or LAC patients were used as starting materials for

Table 1
DNA and RNA oligonucleotides used in this study.

No.	Name	Oligonucleotides sequences
1	CD82 cloning	5′-GCGAAGCTTACCATGGGCTCAGCCTGTATCAAAGTCAC & 5′-GCGCTCGAGATGTACTTGGGGACCTTGCTGTAG
2	SP-A promoter	5′-GCGCACGCGTGGACACTATTGGGGCATTGGGTAC & 5′-GCGAAGCTTCAGAGCCTCCAGCTGCTTGGGTCTC
3	BGH poly A seq	5′-GCCGCTAGCTCAGCCTCGACTGTGCC & 5′-GCGTCTAGACTCAGAAGCCATAGAGCC
4	Insulator I	5′-CGCGGGCCCGGGACAGCCCCCCCCCAAAG & 5′-GCGTCTAGAGACTCCGTCCTGGAGTTGG
5	Insulator II	5′-GGTCCGCGGGGGACAGCCCCCCCCCAAAG & 5′-CGCGGGCCCGACTCCGTCCTGGAGTTGG
6	CD82 qRT-PCR	5′-GTGAGGAAGGGCTTCTGCGAG & 5′-GTACTTGGGGACCTTGCTGTAG
7	GAPDH qRT-PCR	5′-CGGAGTCAACGGATTTGGTCGTAT-3′ & 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′
8	Actin qRT-PCR	5′-CCTGGCACCCAGCACAAT & 5′-TCCTGCTTGCTGATCCACATC
9	CD82 siRNA	5′-GUGUAUCAAAGUCACCAAA & 5′-UUUGGUGACUUUGAUACAG