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Gefitinib resistance of cancer cells correlated with TM4SF5-mediated epithelial–mesenchymal transition

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ABSTRACT

Although cancers can be initially treated with the epidermal growth factor receptor (EGFR) inhibitor, gefitinib, continued gefitinib therapy does not benefit the survival of patients due to acquired resistance through *EGFR* mutations, *c-MET* amplification, or epithelial–mesenchymal transition (EMT). It is of further interest to determine whether mesenchymal-like, but not epithelial-like, cancer cells can become resistant to gefitinib by bypassing EGFR signaling and acquiring alternative routes of proliferative and survival signaling. Here we examined whether gefitinib resistance of cancer cells can be caused by transmembrane 4 L six family member 5 (TM4SF5), which has been shown to induce EMT via cytosolic $p27^{Kip1}$ stabilization. Gefitinib-resistant cells exhibited higher and/or sustained TM4SF5 expression, cytosolic $p27^{Kip1}$ stabilization, and mesenchymal phenotypes, compared with gefitinib-sensitive cells. Conversion of gefitinib-sensitive to -resistant cells by introduction of the T790M *EGFR* mutation caused enhanced and sustained expression of TM4SF5, phosphorylation of $p27^{Kip1}$ Ser10 (responsible for cytosolic location), loss of E-cadherin from cell–cell contacts, and gefitinib-resistant EGFR and survival signaling activities. Additionally, TM4SF5 overexpression lessened the sensitivity of NSCLC cells to gefitinib. Suppression of TM4SF5 or $p27^{Kip1}$ in gefitinib-resistant cells via the T790M *EGFR* mutation or TM4SF5 expression rendered them gefitinib-sensitive, displaying more epithelial-like and less mesenchymal-like characteristics. Together, these results indicate that TM4SF5-mediated EMT may have an important function in the gefitinib resistance of cancer cells.

Introduction

Cancer cells can be initially treated with tyrosine kinase inhibitors (TKIs), such as epidermal growth factor receptor (EGFR) inhibitor gefitinib [1,2]. Most lung cancer patients with activating *EGFR* mutations such as exon 19 deletion and the exon 21 L858R substitution were significantly sensitive to EGFR-TKIs [3]. However, continued gefitinib therapy does not benefit the survival of patients with *EGFR*-sensitive mutations, due to acquired resistance caused by the secondary mutations such as T790M in the EGFR kinase domain [4].

The T790M mutation occurs in approximately 50% of lung cancer patients with mutant EGFR, causes steric hindrance to TKI binding and enhances EGFR affinity for ATP with competitively decreased binding to gefitinib, an ATP-competitive kinase inhibitor [5,6]. Other mutations of *EGFR* such as D761Y and L747S also lead to gefitinib resistance during TKI therapy [7,8]. Among NSCLC, representatively gefitinib-sensitive (with an IC_{50} less than 0.025 μM) HCC827 cells have a deletion mutation in EGFR (from E746 to A750 in exon 19), whereas gefitinib-resistant (with an IC_{50} higher than 10 μM) NCI-H1975 cells have mutations in EGFR of T790M (within exon 20) and L858R (within exon 21) [9,10].

However, acquired resistance to gefitinib in NSCLC can also be attributed to amplification of c-*MET*, a gene that encodes a receptor tyrosine kinase for hepatocyte growth factor [11]. A subpopulation from gefitinib-sensitive NSCLC HCC827 cells (derived from the selection of resistant cells by culturing the cells to increasing gefitinib concentrations) demonstrates TKI resistance that maintains ErbB3/PI3K/Akt pathway activation and c-*MET* amplification, and inhibition of c-MET restored the sensitivity to gefitinib [12]. A gefitinib-resistant subpopulation of gefitinib-sensitive A549 cells shows activated PI3K activity and IGF1R pathway and inhibition of IGFIR restored the sensitivity to gefitinib [13]. Thus, bypassing EGFR signaling for cell growth and proliferation appears to be another mechanism for gefitinib resistance.

Additionally, acquired TKI resistance of NSCLC appears to correlate with epithelial–mesenchymal transition (EMT) that affects cell–cell contacts [14]. TGFβ1-mediated EMT causes resistance to gefitinib in addition to loss of E-cadherin and cytokeratin expression [15]. Thus, mesenchymal-like, but not epithelial-like, NSCLC cells may likely be

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resistant to TKI inhibition such that mesenchymal-like cells bypass EGFR signaling and acquire alternative routes of proliferative and survival signaling. For this to occur, membrane receptors may be cooperative to specify the alternative signaling pathway(s) or organized to allow synergistic or antagonistic relationships between them during communications of cancer cells with diverse extracellular cues.

EMT enhances cell proliferation, migration, and invasion [16]. In addition to the TGFβR and c-MET pathways, tetraspan TM4SF5 also induces EMT with persistent proliferation even under confluent conditions [17] and accelerates S-phase entry [18]. TM4SF5 expression decreases expressions of E-cadherin and ZO1, and increases expression of α-smooth muscle actin (α-SMA) leading to a loss of cell–cell contacts, depending on cytosolic $p27^{Kip1}$-mediated RhoA inactivation and morphological changes; the TM4SF5-mediated EMT is blocked by a suppression of TM4SF5 or $p27^{Kip1}$ [17]. Even hepatocyte growth factor (HGF)-mediated EMT of endogenously TM4SF5-expressing hepatocytes was also blocked by TM4SF5 suppression [17]. The tetraspan TM4SF5 homologous to the lung cancer antigen L6 forms the transmembrane 4 L six family with L6, IL-TIMP, and L6D [19]. This family shares four-membrane spanning membrane topology with genuine TM4SF (transmembrane 4 superfamily) or tetraspanins [20]. TM4SFs are known to regulate the integrity of a membrane receptor network, known as a 'tetraspanin-web' or 'tetraspanin-enriched microdomain (TERM)' where they collaboratively perform their biological function [21]. TM4SF5 collaborates with integrins or growth factor receptors for cellular function [22,23].

Thus, we rationalized that gefitinib-resistant cells may adapt an alternative signaling pathway to bypass EGFR-dependent proliferation and survival signaling pathways via integrative roles by (an)other membrane receptors such as tetraspanins. In the current study, we explored whether TM4SF5 was involved in gefitinib resistance of cancer cells, because TM4SF5 has been shown to induce EMT and collaborate with other receptors on the cell membrane surface [22,23]. We examined the expression and activity levels of signaling molecules in gefitinib-sensitive HCC827 and -resistant NCI-H1975 [9,10] cells to determine whether TM4SF5-mediated signaling and cellular function correlate with resistance to gefitinib. We found that TM4SF5-mediated effects, including cytosolic $p27^{Kip1}$ stabilization and EMT, appeared to be involved in gefitinib resistance of NSCLC cells.

Materials and methods

Cells

HCC827 (with EGFR mutation of deletion from E746 to A750), NCI-H1975 (with EGFR mutation of L858R and T790M), NCI-H358 (with K-Ras mutation of G12C) lung carcinoma and MKN45 (with *c-MET* amplification) gastric adenocarcinoma cells (ATCC, Manassas, Virginia) were maintained in RPMI-1640 with 10% FBS, 10 U/ml penicillin, and 10 μg/ml streptomycin at 37 °C and 5% CO_2. HCC827 cells stably-expressing mock or T790M EGFR or FLAG-TM4SF5 were established via transfection and G418 (500 μg/ml, A.G. Scientifics) selection and maintained in RPMI-1640 containing 10% FBS and 250 μg/ml G418.

Standard Western blots

Cells were transiently transfected with shRNA against control sequence or TM4SF5 for 24 h, or infected with adenovirus encoding for siRNA against control or $p27^{Kip1}$ sequence [17] overnight, before treatment with DMSO or gefitinib (LC Laboratories) for 24 h at different concentrations. Cells were then harvested for whole cell lysates with RIPA buffer (50 mM HEPES, pH 7.5, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM NaF, 1 mM Na_2VO_4, 1 mM nitrophenylphosphate, and protease inhibitors). The lysates were normalized and processed for standard Western blots using antibody against E-cadherin (#610182, BD Bioscience; sc-8426, Santa Cruz Biotech.), active EGFR (#610025), $p27^{Kip1}$ (#610241, BD Transduct. Lab.), $pS^{10}p27^{Kip1}$ (#AP3191a, ABGENT), β-catenin (sc-7963, Santa Cruz Biotech.), pY^{1173}EGFR (#4407), phospho-Y^{992}EGFR (#2235), phospho-Y^{845}EGFR (#2231), phospho-Y^{1045}EGFR (#2237), phospho-Y^{1068}EGFR (#2234), Erk1/2 (#9102, Cell Signaling), phospho-Erk1/2 (#9101s, Cell Signaling), FLAG (#2368), phospho-S^{473}Akt (#9272, Cell Signaling), Akt (sc-7985R, Santa Cruz Biotech.), α-smooth muscle actin (SMA, #A2547, Sigma), vimentin (#V5255, Sigma), α-tubulin (#T5168, Sigma), EGFR (sc-03, Santa Cruz Biotech.), or TM4SF5 [17].

Cell imaging

Cells in normal culture media were imaged using a camera-equipped CX41 microscopy (Olympus).

MTT assay

Cells (3000 cells/well) were seeded in 96 well plates and 24 h later DMSO or gefitinib was added at different concentrations (0 to 10 μM) for additional 72 h. Standard reading of MTT (Sigma) metabolites was performed for OD_{540} and mean ± standard deviation values were graphed.

Coimmunoprecipitation

Whole cell lysates from stably FLAG-mock or FLAG-TM4SF5 expressing HCC827 cells under a subconfluent normal culture condition were incubated with anti-FLAG M2 sepharose beads (Sigma, 1 mg protein/40 μl of 50% slurry/condition) overnight at 4 °C, washed twice with lysis buffer, and then twice with cold PBS. Immunoprecipitates were boiled within 2× SDS-PAGE sample buffer, and immunoblotted with anti-FLAG M2 (#2368, Sigma) or -EGFR (sc-03, Santa Cruz Biotech.) antibody.

Indirect immunofluorescence

Cells were replated on fibronectin (10 μg/ml)-precoated coverslips and incubated within normal serum-containing media overnight at 37 °C. The cells were then treated with DMSO or 0.01 μM gefitinib for additional 18 h, before immunostaining [17]. Antibodies for immunostaining include anti-$p27^{Kip1}$ (#610241, BD Transduction. Lab), or E-cadherin (#610182, BD Biosource) antibody. DNA in nucleus was stained by DAPI (Molecular Probes). Mounted samples were visualized using a fluorescent microscope (BX51, Olympus).

Results

Gefitinib-resistant NCI-H1975 cells efficiently grow in a scattered pattern despite decreased EGFR signaling activity

While trying to understand the mechanisms underlying gefitinib resistance of cancer cells, we hypothesized that resistance might be caused by alternative signaling pathways emanating from membrane receptors that closely collaborate with EGFR. To test this hypothesis, we first compared the characteristics of gefitinib-sensitive HCC827 and -resistant NCI-H1975 cells [9]. To first confirm their susceptibility to gefitinib, we performed MTT assay using the cell lines treated with vehicle or gefitinib at various concentrations (0 to 10 μM). As expected, HCC827 cells were sensitive to gefitinib with an IC50 of approximately 0.02 μM, whereas NCI-H1975 cells were resistant with an IC50 ≥ 10 μM (Fig. 1A). Immunoblotting for EGFR and survival signaling molecules from subconfluent cells cultured under normal culture conditions showed that HCC827 exhibited higher EGFR phosphorylation and activity than NCI-H1975 cells, whereas phosphorylation of Akt and Erk1/2 was slightly higher in the NCI-H1975 cells (Fig. 1B, middle). NCI-H1975 cells were more efficiently proliferative under