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TM4SF5-mediated Resistance against EGFR Kinase Inhibitors Involves an IGF1R Cooperation.

JUNGEUN CHOI
Interdisciplinary Program in Genetic Engineering
The Graduate School
Seoul National University

Transmembrane 4 L6 family member 5 (TM4SF5) is a membrane protein similar to the tetraspanins highly expressed in Hepatocellular carcinoma (HCC), and causes enhanced migration and invasion. TM4SF5 collaborates with other membrane proteins during its protumorigenic roles, presumably at tetraspanin-enriched microdomain (TEM). Here, we explored the TM4SF5-mediated resistance against the clinically important EGFR kinase inhibitors, with regards to cooperation with another membrane protein especially Insulin-like growth factor 1 receptor (IGF1R). In this study, therefore, I explored
resistance against anti-tumor drugs using non-small cell lung carcinoma (NSCLC) cell lines that are ectopically overexpressing TM4SF5 or have suppressed IGF1R levels. When the NSCLC cells were tested with diverse anti-cancer drugs, TM4SF5-positive and/or IGF1R-positive cell lines showed more resistance against EGFR kinase inhibitors (TKIs), Erlotinib or Gefitinib, but not PDGFR/Raf kinase inhibitor, sorafenib. The levels of TM4SF5 and IGF1R modulated each other. Meanwhile, either suppression in EGFR TKI-resistant NCI-H1299 cells caused sensitizations to the EGFR kinase inhibitors, whereas expression of either of TM4SF5 and IGF1R caused activation of its downstream signaling molecules like ERKs, AKT, and S6K leading to resistance against the TKIs. Furthermore, TM4SF5 and IGF1R formed a complex each other, and even further, the complex included EGFR. The complex of EGFR, IGF1R, and TM4SF5 would be sustained in EGFR TKIs treated conditions. In addition to these 2 dimensional (2D) condition, the cells were examined for the drug resistancy when they were embedded in 3D collagen I gels. TM4SF5 and/or IGF1R expression in HCC827 and NCI-H1299 cell lines gave them compact spheroid formation ability in a less-adhesive aquaoues cell culture system. Further, the spheroids with high expression of TM4SF5 and IGF1R were resistant against EGFR TKIs, gefitinib and erlotinib. Thus, this study evidences that TM4SF5 and/or IGF1R appeared to collaborate to cause survival signaling, forming with a triple complex with EGFR, even with EGFR TK
Keywords: TM4SF5, IGF1R, EGFR, Erlotinib, Gefitinib, Drug resistance, 3D culture

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<tr>
<td>Col</td>
<td>Collagen</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epithelial Growth factor receptor</td>
</tr>
<tr>
<td>EGFR TKI</td>
<td>EGFR tyrosine inhibitor</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
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<tr>
<td>TM4SF5</td>
<td>Transmembrane 4 L Six Family Member 5</td>
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INTRODUCTION

Among many lethal cancers, especially lung cancer causes the most deaths.\(^1\) According to the national cancer registration and statistics system, the number of new lung cancer patients was 20,711 in 2011, which is equivalent to 10% of the value of the total cancer 202,053. All cancer deaths from lung cancer deaths were the highest mortality rate not only in Korea but also worldwide.

Based on pathological classification, Lung cancer divided into non-small lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC cell falls into three categories, such as adenocarcinoma (40%), squamous cell carcinoma (30%), and large cell carcinoma (15%). For unknown reasons, EGFR kinase domain mutations seem to be restricted to a subset of NSCLC, although very rare mutations have also been reported in SCLC, cholangiocarcinoma, ovarian, colorectal, head and neck, oesophageal and pancreatic cancers.\(^2\) EGFR is often amplified, and/or mutated in cancer cells\(^3\). EGFR expression and activation are reported in 70% of NSCLC. EGFR activation is related with poor prognosis, since it activates cell proliferation, cell survival, angiogenesis, metastasis through mitogen activated protein
kinase (MAPK) and AKT.

Generally, cancer cells can be initially treated with tyrosine kinase inhibitors (TKIs), such as epidermal growth factor receptor (EGFR) inhibitor gefitinib or erlotinib. 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. However, continued EGFR TKI 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.

However, there are many researches concerning about EGFR TKI resistance which is mediated by other signaling pathways. A gefitinib-resistant subpopulation of gefitinib-sensitive A549 cells show activated PI3K activity and IGF1R pathway, and inhibition of IGF1R restores the sensitivity to gefitinib. Furthermore, there is a previous report on TM4SF5-mediated resistance against gefitinib in NSCLC. Thus, bypassing EGFR signaling for cell growth and proliferation appears to be another mechanism for gefitinib resistance.

IGF1R is a receptor for IGF1, IGF2 and insulin. It consists of 2
alpha subunits and 2 beta subunits. Ligand (e.g. IGF1, IGF2, insulin) binds alpha subunit and this binding leads autophosphorylation of beta subunit. Eventually it lead its downstream activation.\(^1\) There are many reports about IGF1R expression and/or its activation lead cancerous characteristics like uncontrolled proliferation and anti-cancer drug resistance\(^2\). The insulin-like growth receptor pathway regulated by IGF-1R and IGF-2R is critically important for numerous hallmarks of neoplasia\(^3\), which has been recognized as an attractive target for anticancer therapies. A number of clinical trials are under ways to test two major anti–IGF-1R strategies for TKIs\(^4\), \(^5\). Interestingly, without the secondary EGFR mutation of T790M mutation, IGF1R activation is shown as a molecular mechanism that confers acquired resistance to erlotinib in lung cancers with the wild-type EGFR\(^6\).

TM4SF5, a tetraspanin protein, can localize at the tetraspanin-enriched microdomain (TERM) of cell membrane surface\(^7\), where the tetraspanins and other membrane receptors including growth factor receptors and integrins are associated and specify unique signal transduction toward the cytosol\(^8\). TM4SF5 is highly expressed in cancers including hepatic cancer and other
cancer types, and TM4SF5 expression in HCC correlates with epithelial-mesenchymal transition (EMT)\(^1\)\(^8\).

In this study, since TM4SF5 and IGF1R separately showed to cause EGFR TKI-resistance in NSCLC, we tried to understand how the both molecules could collaborate each other for TKI-resistance using stable HCC827 cells overexpressing TM4SF5 and H1299 cells with stable IGF1R-\(\beta\) suppression. We found that TM4SF5 and IGF1R-\(\beta\) regulated expression bound each other, collaborated to transduce intracellular signaling for growth and survival leading to drug resistance. The TM4SF5/IGF1R expression and interaction mediated resistance against EGFR-TKI, but not to PDGFR-TKI, in 2D normal culture, 3D spheroid culture system, and 3D extracellular matrix-surrounded culture conditions.
MATERIALS AND METHODS

1. Cells

HCC827 (with EGFR mutation of deletion from E746 to A750), NCI-H1299 were maintained in RPMI-1640 with 10% FBS at 37 and 5% CO2. HCC827 cells stably expressing mock of FLAG-TM4SF5 were established via transfection and G418 (500μg/ml, A.G. Scientifics) selection and maintained in RPMI-1640 containing 10% FBS and 250 μg/ml G418. NCI-H1299 cells stably expressing shscramble and shIGF1R were established by transfection. And these cell were selected and maintained by puromycin.

2. Cell lysate preparation and western blots

Cells were harvested for whole cell lysate with RIPA (50mM HEPES, pH 7.5, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 150mM NaCl, 50mM NaF, 1mM Na3VO4, 1mM nitrophenylphosphate, and protease inhibitors). The lysate were normalized and processed for standard Western blots using
antibody against EGFR (sc-03, Santa Cruz Biotech.), active EGFR (#610025), p27 (#610241, BD transduct. Lab.), pS10p27 (#AP3191a), Erk1/2 (#9102, Cell Signaling), phosphor-Erk1/2 (#9101s, Cell signaling), Flag (#2368, Cell Signaling), phospho-S473Akt (#9272, Cell Signaling), Akt (sc-7985R, Santa Cruz Biotech.), alpha-tubulin (#T5168, Sigma), p70 S6 Kinase Antibody (#9202, Cell Signaling), Phospho-pT389 70 S6 Kinase Antibody (#9205, Cell Signaling).

3. RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), and complementary DNA (cDNA) was synthesized using and amfiRivert Platinum cDNA synthesis master mix (GenDEPOT) according to manufacture’s instructions, cDNA was subject to reverse-trasnscription polymerase chain reaction with Dream Taq Green PCR master mix (Thermo scientific).

4. MTT assay

Cells (2-3 X 103 cells/well) were seeds in 96 well plates and 24hr
later DMSO, erlotinib, gefitinib were treated at different concentrations (0 to 10uM) for additional 72hr. After reaction, cells were incubated with 20uM MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (sigma) solution for 4hr at 37 and added 100ul MTT solvent. Cover with tinfoil and agitate cells on orbital shaker for 5 min and read absorbance at 540nm.

5. Co-Immunoprecipitation

Whole cell lysate from stably expressing 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/20 ul of 50% slurry condition) overnight at 4, washed twice with lysis buffer, and then twice with cold PBS. Immununoprecipitated were boiled within 2X SDS-PAGE sample buffer, and immunoblotted with anti-FLAG M2 (#2368, Sigma), IGF1R-β (cell signaling) and EGFR (sc-03, Santa Cruz Biotech.) antibody.
6. Immunofluorescence

Cells were seeded on precoated cover slips. The cells were incubated with normal conditions (10% FBS in RPMI 1640). Cells were fixed in MetOH for 20 min. After being thoroughly washed with PBS, the cells were blocked with 3% BSA in PBS, and stained with anti-Flag (#2368, Cell Signaling), anti-IGF1-β (Cell Signaling). DNA in nucleus was stained by DAPI (Molecular Probes). Epifluorescence Microscope image were acquired on a microscope (BX51TR, Olympus) using 406/0.75 or 1006/1.3 (oil) NA U plan semi Apochromat objective lens (Olympus). The filters for GFP or Red fluorescent protein (RFP) were XF105-2 or XF115-2, respectively. AJVC KY-F75U CCD digital camera with 1394 interface was used. The TOMORO image analyzer software (Techsan Community Co., Korea) was used for image analysis.

7. Spheroid formation and image

Spheroid were made from Hanging drop plate, and petri dish (in shaking incubator). 24 hr later, spheroids are harvested and embedded in ECM collagen matrix. Cells in hanging drop plates
were imaged using a camera equipped CX41 microscopy (Olympus) Radius of spheroid were measured by NIS software or IMARIS (Bitplane AG, Zurich, and Swiss). Volume of spheroids and area of spheroids were calculated by NIS software.

8. Time-lapse imaging

Time-lapse cell images in 3D type 1 collagen gel were captured with IX81-ZDC (Olympus) for 48 hr. The cells embedded in 3D collagen I gels were cultured in the DMSO, erlotinib 10uM or gefitinib 10uM in PDMS glass coverslips. All microscope were equipped with Chamlide incubator systems (LCI live cell instrument, Korea), and an environmental chamber mounted on the microscope maintained constantly at 37, 5% CO2 and 95% humidity.

9. 3D Immunofluorescence

Spheroids and single cells were cultured within Polymethylsiloxane prepolymer (PDMS) glass coverslip and fixed directly with 4% formaldehyde for 30 min at room temperature
(RT), and subsequently treated with 100mM glycine to quench residual aldehyde groups. After PBS washing, cells were permeabilized for 30 min with 0.5% Triton X-100 at room temperature (RT) and blocked for 2hr with PBS in 3% BSA. Nuclei were stained with diaminophenylindole (DAPI; Molecular Probes). Confocal images were captured using a confocal microscope with a Nikon Plan-Apochromat 40X and analyzed using the NIK software of IMARIS imaging software (Bitplane AG, Zurich, Swiss)
RESULTS

TM4SF5 and IGF1R regulates the expression one another and its downstream signaling pathway.

TM4SF5 overexpression in non-small lung cancer cell lines (NCI-HCC827, NCI-H23, CorL23, NCI-H1703, NCI-H1755) induce IGF1R-β expression (Fig. 1A). Not only lung cancer cell lines but also TM4SF5 overexpression in hepatocellular carcinoma (HCC) induced IGF1R-β expression (Fig. 1B). When TM4SF5 overexpression was induced, TM4SF5 downstream signaling molecules like FAK and SRC were activated. Furthermore, expression of IGF1R and activation of IGF1R downstream signaling molecules including Akt and S6K were activated too (Fig. 1B). Vice versa, when IGF1R was overexpressed, expression of TM4SF5 and activation of its downstream signaling molecules increased (Fig. 1C). Through these expression patterns, we speculated that there were certain connections between TM4SF5 and IGF1R expression. To investigate further relationship between TM4SF5 and IGF1R, cell line screening was conducted. We could
eventually as expected from many previous studies found out that HCC 827 is sensitive to and NCI-H1299 is resistance against EGFR TKIs (gefitinib and erlotinib, Fig. 1D). Using TM4SF5-flag tagged plasmid and shIGF1R RNA, we made stable cell lines suitable for our study. Their characteristics like intracellular signaling and mRNA expression patterns were confirmed (Fig. 1E). When expression of TM4SF5 and IGF1R increased, their downstream effectors were activated like previous experiment (Fig. 1B and 1C). Their transcription levels affected each other expression level.
Figure 1. TM4SF5 and IGF1R regulates the expression one another.

(A, B, and C) Positive correlation between TM4SF5 and IGF1R-β in hepatic (SNU449 or Chang cells) or NSCLC (HCC827, or NCI-H23, NCI-H1703, NCI-H1755 and CorL23 cell lines) was observed. Subconfluent cells were harvested for whole cell lysate before western blots for the indicated molecules. Cells were stably transfected with mock, TM4SF5, or IGF1R plasmids, or transiently transfected with control siRNA, siRNA against (siTM4SF5), control shRNA against a scramble sequence, or shRNA against IGF1R (shIGF1R) for 48 h. In case, cells were treated with PBS control or TGFβ1 (1 ng/ml) for 24 h. (D) HCC827 or NCI-H1299 cells were analyzed for cell viability in the absence or presence of treatment with erlotinib and Gefitinib at different concentrations (0 to 10 μM) via MTT assay. (E) Subconfluent HCC827 cells stably expressing mock or TM4SF5-Flag plasmid and NCI-H1299 cells stably expressing shScramble (control) or shIGF1R were proceeded to RT-PCR.
TM4SF5 and/or IGF1R induced EGFR TKIs specific resistance.

Drug resistance characteristics of HCC827 and NCI-H1299 cell lines were verified using MTT assay. Using these cell lines, the assays were performed to find out whether expression of TM4SF5 and/or IGF1R induced drug resistance, and this resistance was abolished in their absent. As a result, overexpression of TM4SF5 caused HCC827 cells which were sensitive against EGFR TKIs to be resistant against EGFR TKIs (Fig. 2A). In vice versa, knock down of IGF1R in NCI-H1299 cells led for them to be resistant against EGFR TKIs (Fig. 2B).

In figure 1, we found out that either expression of TM4SF5 or IGF1R induced expression each other and activated their downstream signaling molecules. Furthermore, in figure 2, we could conclude that expression of TM4SF5 and/or IGF1R not only induced expressions each other, but also conferred specific resistance against EGFR TKIs.
Figure 2. Survival of EGFR TKI resistant cells in the presence of EGFR TKI correlated with sustained TM4SF5 and IGF1R- β expression.

(A, B) HCC827 cells stably expressing Mock or TM4SF5-Flag plasmids (A) and NCI-H1299 cells stably expressing shScramble or shIGF1R (B) were processed to MTT assay after treatment with either DMSO, gefitinib, erlotinib, sorafenib at different concentrations (0.001 to 10 μM in DMSO). Serial diluted anticancer drugs were directly added to culture media. Each value was averaged from triplicate in one experiment out of 3 independent MTT assay (i.e., triplicate X 3 times).
TM4SF5 and IGF1R-β induced EGFR TKI resistance through focal adhesion, p27 stabilization and PI3K signaling pathway.

Previous study, it was verified that p27 stabilization in cytosolic region induced resistance against EGFR TKI in NSCLC. In this research, we also tried to verify whether p27 stabilization was induced by expression of TM4SF5 and/or IGF1R, and whether this p27 stabilization eventually induced EGFR TKI specific resistance. To find out these, we treated EGFR TKIs, gefitinib, or erlotinib to HCC827-TM4SF5 positive, -negative, NCI-H1299-IGF1R-positive, or negative stable cells for 24, 48 hr. As a result, we could find out that higher expression of these two proteins induced stabilization of p27 in cytosolic region in EGFR TKI treated condition. Also cells bearing high expression of TM4SF5 and IGF1R showed highly activated MAPK, PI3K and focal adhesion signaling pathway (Fig. 3). Therefore, overexpression of TM4SF5, IGF1R and activation of its downstream signaling molecules were induced and sustained.
Figure 3. Interaction between TM4SF5 and IGF1R-β correlated with EGFR TKI resistance through focal adhesion, p27 stabilization and PI3K signaling pathway.

EGFR TKI inhibitor-resistant NCI-H1299 cells stably expressing shScramble or shIGF1R-β and HCC827 cells stably expressing mock of TM4SF5-Flag were treated with either DMSO, erlotinib, gefitinib, or sorafenib at 1 μM for 48 hr, before preparation of whole lysates for immunoblotting.
TM4SF5 and IGF1R are located in cell membrane and form complex with EGFR

There was a report about crosstalk between TGF-β and EGFR signaling pathways induce TM4SF5 expression, which eventually leads to EMT. Thus, the relationship between TM4SF5 and EGFR induced cancerous action like EMT-mediated proliferation and metastasis. Since the both signaling pathways have common signaling activities like phospho-Akt, they may form binding complex. Additionally there was a report about binding of EGFR and IGF1R. These three proteins (i.e., TM4SF5, EGFR, and IGF1R) are located in cell membrane commonly. By combining the previous results of the above, we speculated that they could bind one another. To investigate their binding, we conducted Immunofluorescence and Immunoprecipitation approaches. During immunofluorescence, TM4SF5 and IGF1R were co-located in plasma membrane region (Fig. 4A). In addition to this co-localization result their binding were checked via an immunoprecipitation. As a result, TM4SF5 formed a complex with IGF1R and EGFR as well (Figs. 4B and C). Being consistent, there was a report about IGF1R and EGFR dimerization in EGFR TKI resistance condition. Altogether, it is likely that the complex consisting of TM4SF5, IGF1R and EGFR would be important for EGFR specific resistance.
Figure 4. TM4SF5 and IGF1R are located in cell membrane and form complex with EGFR.

(A) HCC827 stably Mock and TM4SF5 flag cells were seed on glass slide. After 24hr, cells were fixed with 70% EtOH. After being thoroughly washed with PBS, the cells were blocked with 3% BSA in PBS, and stained with anti IGF1R-β and flag antibodies. DNA in nucleus was stained by DAPI. Epifluorescence Microscope image were acquired on microscope. (B) NCI-H1299 cells stably expressing shScramble or shIGFR-β and HCC827 cells stably expressing mock of TM4SF5-Flag were harvested for whole cell lysates. The H1299 lysates were pulled-down using streptavidin-coated beads, and HCC827 cell lysates were pulled-down using beads coated with anti-Flag M2 antibody to eliminate nonspecific binding proteins. Then, the immunoprecipitates were proceeded to standard western blots against EGFR and IGF1R-β. The data shown represent 3 different experiments.
Correlation between TM4SF5 and IGF1R confers EGFR TKI resistance in the spheroid system.

Unlike conventional two-dimensional cell culture environment, cells are normally exposed to microenvironment like ECM *in vivo*. Therefore, a sophisticated imitation of microenvironment for researches gets the limelight for cancer research. Three dimensional (3D) culture environment surrounded with extracellular matrix (ECM) is thought to have an important to more mimic the *in vivo* microenvironment. Two experimental systems were chosen for EGFR TKI specific resistance which was caused by TM4SF5 and/or IGF1R. First one was hanging drop assay (i.e. aqueous 3D culture). Second one is full-embedding of cells in 3D ECM gel system.

When HCC827 stable cells with expressing or non-expressing TM4SF5 were seeded on hanging drop plates for 24 hr, they formed spheroids. Especially, cells expressing TM4SF5 maintained spheroid forms. In vice versa, spheroids made from TM4SF5-negative cells formed less compact spheroids and their boundaries of the spheroids were collapsed depending on drug treatment (Fig. 5A). Cell viability assay using CCK-8 showed that
TM4SF5 induced EGFR TKI resistance in aqueous 3D spheroid culture system (Fig. 5B). In addition, they kept sustained spheroids even after the EGFR TKI treatment, unlike TM4SF5-negative spheroid (Fig. 5C).
Figure 5. Correlation between TM4SF5 and IGF1R confers EGFR TKI resistance in Hanging drop system.

(A) HCC827 cells stably expressing Mock of TM4SF5-Flag were processed to hanging drop system for spheroid formation starting with 500 cells/well. During the hanging drop, media were treated with either DMSO, Erltonib (1 μM), or Gefitinib (1 μM). (A) One or two day later after seeding, phase-contrast images of each well were imaged. Radius of each spheroid was measured by NIS software using a microscope. (B) Relative volume of each spheroids in experimental conditions were calculated using the radius of each spheroid measured. At each condition, average volume at the 2nd day were divided by average volume at the 1st day. (C) Two days after cell seeding into a hanging drop plate, CCK-8 was added to each well to assess cell viability.
Collaboration between TM4SF5 and IGF1R confers EGFR TKI resistance in 3D collagen gels.

In aqueous 3D spheroid culture system (Fig. 5), a kind of aqueous cell culture method, was performed to mimic 3D culture systems. In order to mimic in vivo more-delicate manner, embedding of cells into 3D ECM gels was adopted. Cells in spheroids have different drug penetration depending on their location, so the spheroids less than 70 μm were embedded in ECM collagen.

Cells with TM4SF5 and/or IGF1R showed sustained spheroid form in EGFR TKI treated condition. Even HCC827 TM4SF5 positive spheroid were getting bigger over time. Spheroid made from TM4SF and/or IGF1R negative cells were collapsed, and disseminated. To confirm whether this phenomena induced by cell death or not, DNA staining with DAPI was performed. As a result, DNA cleavage and dismantled cell membrane was observed in TM4SF5 and/or IGF1R negative cells (Figs. 6A and B). Additionally when single cells which were more sensitive to microenvironment were embedded in the same ECM 3D situation, single cells were more exposed to the extracellular environment. Therefore, cell death of TM4SF5 and/or IGF1R-negative cells
occurred more quickly than TM4SF5 and/or IGF1R positive cells. DNA cleavage pattern after DAPI staining was observed valid like spheroid embedding experiment (Figs. 6C and D). Altogether, these results suggested that EGFR TKI resistance mediated by TM4SF5 and/or IGF1R were reproducible in 3D ECM gel culture systems.
Figure 6. Collaboration between TM4SF5 and IGF1R confers EGFR TKI resistance in 3D collagen gels.

(A) Spheroids of HCC827 cells stably expressing mock or TM4SF5-Flag (TM4SF52-9) were embedded in 3D collagen I gels. After embedding, media containing DMSO, Erlotinib (1 μM), Gefitinib (1 μM) were added to each condition. Two days after the treatment and DAPI staining, the spheroid images were saved and representative image for each condition were shown. (B, C) HCC 827 cells stably expressing mock of TM4SF5-Flag and H1299 stably expressing shScramble or shIGF1R were embedded into 3D collagen I gels. After embedding, media containing DMSO, Erlotinib (1 μM), Gefitinib (1 μM) were added to each condition. After the treatment for 10 (B) or 24 hr (C) together with DAPI staining, spheroid images were saved and representative images were shown. Please note that broken nucleus (via splitted DAPI stains) indicate dead cells.
DISCUSSION

With the development of science, there have been many efforts to overcome cancer. As their characteristics became defined more, many cancer treatments became possible to be developed. The cancer has characteristics like uncontrolled proliferation, insensitivity to anti-growth signals, limitless replicative potential, tissue invasion and metastasis, evading immune surveillance, sustained angiogenesis, evading apoptosis, and insensitive to stress from DNA damage, mitosis, proteotoxic, metabolism and oxidative. These characteristics are certainly induced by highly activated growth factors receptors, especially epithelial growth factor receptor (EGFR). EGFR is receptor for epithelial growth factor, and its binding leads to EGFR activation, and eventually to induce uncontrolled cell proliferation, and anti-apoptosis.

Focused on this characteristics, many anti-cancer drugs targeting EGFR have been developed. The representative drugs targeting EGFR, such as gefitinib or erlotinib, have been used for treatment for a decade. These drugs inhibit EGFR tyrosine phosphorylation. Through it, these drugs inhibit EGFR downstream signal molecules. But therapeutic effect of these drugs was lower than expected.

Through the effort to find the cause of the failure of the EGFR TKI drugs, the causes were identified. Especially, some proteins which induce
EGFR TKI specific resistance were revealed. Non-small cell lung cancer was reported for high activation of EGFR. Focused on these characteristics, EGFR TKI anti-cancer drugs were widely used for NSCLC treatment. But chronic treatment of many EGFR TKIs cause resistant phenomena. Some researches reveal that these EGFR TKI resistance correlate with activation and/or expression of IGF1R.

TM4SF5 is a kind of tetraspanin, which can be located in cell membrane, and cross talks with extracellular molecules like ECM through integrins. Especially, TM4SF5 senses ECM like collagen and induces focal adhesion signaling pathway activation. Also uncontrolled proliferation and metastasis through p27Kip1 stabilization in cytosolic region are reported previously. Recently it were revealed that overexpression of TM4SF5 leads to a resistance against EGFR TKI in NSCLC, and mitotic inhibitor in HCC. With these researches concerning about TM4SF5 or IGF1R, both TM4SF5 and IGF1R lead uncontrolled proliferation and anti-cancer drug resistance. Therefore, I want to understand the relationship between IGF1R and TM4SF5 known to induce EGFR TKI resistance.

In NSCLC or HCC model, expression of TM4SF5 and IGF1R activated not only its downstream signaling molecules but also relative downstream signaling molecules. Based on these features, we speculated a functional connection between TM4SF5 and IGF1R. So
finding their correlation would be valuable for cancer treatment.

We made stable cell lines of HCC827 Mock (TM4SF5 negative), HCC827-TM4SF5 (TM4SF5 positive), NCI-H1299 with endogenous IGF1R, and NCI-H1299 with IGF1R suppression for further study. With these stable cell lines, positive relationship between of TM4SF5 and IGF1R in protein level and at the transcriptional level were revealed. Using indirect immunofluorescence, we could observe that they are located commonly at the plasma membrane regions, being consistent with the observation by immunoprecipitation for their binding. Recently it is previously reported that binding of IGF1R and EGFR is sustained when EGFR TKI resistance was induced in NSCLC. Also there are reports about binding of TM4SF5 and EGFR, EGFR and IGF1R. Altogether, we could speculate that these three proteins, TM4SF5, IGF1R, and EGFR are located together at plasma membrane regions. This complex then affects cell signaling by which EGFR TKI resistance occurred.

So far, we found out that expression of TM4SF5 and IGF1R is mutual supplementation, and their relationship induces EGFR TKI specific resistance in 2D cell culture conditions. EGFR TKI resistance by the expressions of TM4SF5 or IGF1R is involved in activation of cell proliferation protein like Erk or S6Kinase. Based on these data, we could speculate that overexpression and/or activation of TM4SF5 and IGF1R
induce activation of cancer signaling pathway leading to EGFR TKI resistance.

Advanced endometrial cancers often show resistance to clinical chemotherapy, although potencies of anti-cancer drugs in vitro are promising. The disparity suggests that in vivo microenvironments are not recapitulated by in vitro models used for preclinical testing, although spheroids replicate some important properties of tumours in vivo. Meanwhile, in particular, the centers of the cell clusters lead to transformation by reducing the supply of oxygen and nutrients, and this characteristic leads cells more cancerous. The cells of the core have small external environmental exposure area, but cells of surface have large area. These characteristics also lead different drug activity between in 2D and in 3D culture system. Because 2D culture system and 3D culture system have different features, we conducted 3D spheroid cultures in 3D ECM gel systems to determine the reproducibility.

During assays at the aqueous 3D spheroid culture system or embedding into 3D ECM gels, cells bearing TM4SF5 and/or IGF1R (HCC827-TM4SF5 positive, NCI-H1299-IGF1R positive) showed stained spheroids in aqueous or ECM-surrounded 3D culture systems. Based on the overall results, TM4SF5 and IGF1R were expected to form a complex. Further the complex could show another complex component
of EGFR, leading to the resistance against EGFR TKIs. Cell line model for this experiment showed no EGFR mutations, so correlation between TM4SF5, IGF1R and EGFR would be new direction for cancer treatment.
요약(국문초록)

암은 epidermal growth factor receptor (EGFR)을 억제하는 gefitinib와 같은 tyrosine kinase inhibitors (TKIs)의 복용에 의한 치료가 이루어진다. 대부분의 폐암 환자들은 19번 exon 결실 및 21번 exon의 L858R 대체와 같은 돌연변이를 갖고 있으며, EGFR-TKIs에 민감하게 반응한다. 하지만 지속적인 gefitinib의 투여는 T790M과 같은 2차 돌연변이(second mutations)을 일으켜 내성을 유발한다. TM4SF5는 the Tetraspanin-Enriched Microdomain (TERM)에 위치하고 있는 tetraspanin 단백질로서, 많은 암세포에서 높은 발현이 보고된 바 있다. 특히 간암세포에서 TM4SF5의 발현은 epithelial-mesenchymal transition (EMT)와 관련되어 있다는 보고가 있다. 또한 폐암세포 주에서 TM4SF5의 발현에 의한 EGFR-TKI 내성현상 역시 관찰된 바 있다. Insulin-like growth receptor pathway는 IGF-1R와 IGF-2R에 의해 그 활성이 조절되며 neoplasia의 hallmark로 알려져 있다. 이러한 성질로 insulin-like growth receptor pathway는 각광받는 항암치료제 타겟이다. 상당수의 임상시험에서 EGFR의 주가 돌연변이가 없더라도 IGF1R의 활성이 나타나면 EGFR-TKI인 erlotinib에 저항성을 나타내는 현상이 관찰되었다. 이와 같은 TM4SF5, IGF1R이 각각 EGFR-TKI 내성을 유발한다는 기존의 보고에
기반하여 TM4SF5와 IGF1R의 상관관계를 파악하기 위한 실험을 수행하였다. 그 결과 TM4SF5와 IGF1R은 단백질과 mRNA 수준에서 서로의 발현을 조절하였다. 또한 TM4SF5와 IGF1R의 과발현은 NSCLC에서 EGFR TKI 특이적인 내성현상을 나타내었다. 이러한 현상은 세포 내 생존과 생장에 관여하는 세포신호전달 과정을 수반하였다. 간접적, 혹은 직접적인 결합 확인 실험 결과 TM4SF5, IGF1R 그리고 EGFR는 복합체를 형성하고 이를 통해 EGFR TKI 내성 모델에서 세포 내 신호전달 과정을 함께 조절하는 것으로 추측된다. TM4SF5와 IGF1R의 상호작용에 의한 EGFR TKI 특이적 내성현상은 3차원적 세포배양 환경에서도 역시 동일하게 재현되었다. 따라서 이들의 관계를 명확하게 파악하고, 내성을 유발하는 단계를 발굴하는 것은 EGFR TKI 내성연구에 주요한 역할을 할 것으로 기대된다.

표제어: TM4SF5, IGF1R, EGFR, Erlotinib, Gefitinib, Drug resistance, 3D culture
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