Effect of Cetuximab and EGFR Small Interfering RNA Combination Treatment in NSCLC Cell Lines with Wild Type EGFR and Use of KRAS as a Possible Biomarker for Treatment Responsiveness

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ABSTRACT

Background The epidermal growth factor receptor (EGFR) is a therapeutic target for patients with non-small cell lung cancer (NSCLC). Cetuximab is an anti-EGFR monoclonal antibody that inhibits EGFR signaling and proliferation of colorectal cancer and head and neck cancers. Since only few NSCLC patients benefit from cetuximab therapy, we evaluated a novel combination treatment using cetuximab and EGFR small interfering RNA (siRNA) to strongly suppress EGFR signaling and searched for a biomarker in NSCLC cell lines harboring wild-type EGFR.

Methods Alterations in EGFR and its downstream genes in five NSCLC cell lines (A549, Lu99, 86-2, Sq19 and Ma10) were assessed. The expression levels of these molecules were assessed through western blotting. The effect of combination treatment was determined through cell proliferation assay, caspase-3/7 assay, invasion assay, and migration assay.

Results All cell lines were harboring wild-type EGFR, whereas KRAS, PTEN, TP53 and LKB1 were mutated in A549 and Lu99; Lu99 and Sq19; Lu99, 86-2, Sq19 and Ma10; and A549, 86-2, and Sq19 cell lines, respectively. PTEN was not expressed in Sq19, and LKB1 was not expressed in both A549 and Sq19. TP53 was not expressed in both A549 and Lu99. The combination of cetuximab and EGFR siRNA significantly suppressed cell proliferation in 86-2, Sq19 and Ma10, which express wild-type KRAS. It induced apoptosis in A549, 86-2 and Ma10 cells, which express wild type PTEN. The combination treatment had no effect either on cell invasion nor migration in all cell lines.

Conclusion EGFR targeted therapy using the combination of cetuximab and EGFR siRNA is effective in NSCLC cell lines harboring wild-type EGFR. Wild-type KRAS may act as a potential biomarker for response to combination treatment by the induction of apoptosis in cells with wild-type PTEN.

Key words cetuximab; EGFR siRNA; KRAS; non-small cell lung cancer

Human lung cancer is the leading cause of cancer deaths worldwide. Lung cancer is classified into small cell lung cancer (SCLC) and NSCLC, accounting for approximately 15–20% and 80–85%, respectively. In addition, NSCLC is further classified into adenocarcinoma, squamous cell carcinoma and large cell carcinoma. NSCLC is difficult to treat compared with SCLC.1 EGFR is a transmembrane protein that regulates cell proliferation, apoptosis, angiogenesis and metastasis. EGFR is activated by EGF binding, which further activates the EGFR downstream signaling pathway.2 In lung cancer, overexpression and mutation of EGFR was observed in NSCLC patients, which makes it an important therapeutic target. Studies reveal that EGFR mutations occur in 30–40% of NSCLC patients.3

Currently, two classes of EGFR targeted drugs are used to treat cancers. One of them is EGFR-tyrosine...
kinase inhibitors (TKIs) and the other is anti-EGFR monoclonal antibodies. In lung cancer, EGFR-TKIs are the predominantly used EGFR-targeted drugs, and first-generation EGFR-TKIs such as gefitinib and erlotinib are recommended for first-line treatment in NSCLC patients with EGFR mutations. However, anti-EGFR monoclonal antibodies, such as cetuximab, are not used in NSCLC patients, although they are broadly used in patients with colorectal cancer and head and neck cancers. A randomized, multicenter, phase III study [First-Line ErbituX (FLEX)] of cetuximab revealed improvements in the overall survival of NSCLC patients. However, survival upon addition of cetuximab to conventional chemotherapy was only prolonged by one month and was considered insignificant compared with the cost of treatment. Therefore, novel combination therapies with cetuximab and biomarkers to identify patients who would benefit from the therapy have been extensively sought.

RNA interference (RNAi) is a novel strategy that degrades the target mRNA by small interfering RNA (siRNA) consisting of 19–25 base pair, which leads to the down regulation of protein expression. In various fields such as medicine, biology and engineering, RNAi has been widely utilized as a tool for gene functional analysis. Recently, Food and Drug Administration (FDA) approved the first-ever therapeutic drug based on siRNA. Therefore, inhibition of specific molecules by siRNA is now a promising therapy in the cancer.

In the previous study, we found that NSCLC cell lines with EGFR mutation and lack of AKT activation were sensitive for cetuximab monotherapy. One possible mechanism of this phenomenon is that these cells might become physiologically dependent on EGFR signaling pathway for their growth, therefore they are sensitive for the inhibition of EGFR function by cetuximab. There is no report on overcoming the resistance of NSCLC cells with wild type EGFR to cetuximab.

In this study, we developed a novel combination treatment using cetuximab and EGFR siRNA to strongly suppress EGFR signaling pathways and studied its effect on NSCLC cell lines harboring wild-type EGFR. Additionally, we explored candidate gene alterations in cells showing response to treatment to find possible biomarkers for this combination treatment.

**MATERIALS AND METHODS**

**Cell lines**

Human NSCLC cell lines including the adenocarcinoma cell lines A549 and Ma10, the squamous cell carcinoma cell line Sq19, the giant cell carcinoma cell line Lu99, and the large cell carcinoma cell line 86-2 were used in this study. A549 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Sq19, Lu99, RERF-LC-AI and 86-2 were provided by the Riken Cell Bank (Riken, Tsukuba, Japan). The cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium or Dulbecco’s modified Eagle’s medium (D-MEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum(FBS), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin at 37°C in a humidified incubator containing 5% CO2. The gene status of cell lines that were not published in literature was determined using polymerase chain reaction (PCR) and direct sequencing.

**EGFR siRNA transfection**

The EGFR siRNA (sense: 5’-CUCUGAGAAGAAGAUACU-3’ and antisense: 5’-ACUUUCUUCUCCAGAG-3’) and the negative control siRNA (no information disclosure) were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Dallas, TX). The cell lines were transfected with 10 nM siRNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). Transfection was performed according to the manufacturer’s protocol.

**Western blotting**

After the cell lines were transfected with siRNA, cells were collected and washed with phosphate-buffered saline (PBS) (-), and subsequently lysed in a lysis buffer. The sample was subjected to 8–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and separated proteins were transferred to Immobilon-P PolyVinylidene DiFluoride (PVDF) membranes (Merck Millipore, Billerica, MA). The membranes were incubated with anti-EGFR, anti-PTEN, anti-Akt, anti-KRAS, anti-TP53, anti-LKB1 antibodies (1:1,000 dilution, Cell signaling technology, Beverly, MA) and β-actin antibody (1:2,000 dilution, Sigma-Aldrich, St. Louis, MO). Primary antibodies were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000 dilution, Cell signaling technology, Beverly, MA) and β-actin antibody (1:2,000 dilution, Sigma-Aldrich, St. Louis, MO). Primary antibodies were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000 dilution, anti-mouse IgG and anti-Rabbit IgG respectively; GE Healthcare Bio-Sciences Amersham, Diegem, Belgium, UK). The membranes were subjected to chemiluminescence detection assay using ECL Prime Western Blotting Detection Reagents (GE Healthcare Bio-Sciences Amersham).

**Cell proliferation assay**

Cell proliferation after treatment with 0, 0.01, 0.1 and 1.0 µM cetuximab for 6 days was detected by water-soluble tetrazolium salt (WST-8) for colorimetric cell viability assay (Dojindo Molecular Technologies, Kumamoto, Japan). The procedure was performed according to the
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<table>
<thead>
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<th>Table 1. Gene status of NSCLC cell lines</th>
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EGFR, epidermal growth factor receptor; KRAS, v-ki-ras2 Kirsten rat sarcoma viral oncogene homolog; LKB1, liver kinase B1; NSCLC, non-small cell lung cancer; PTEN, phosphatase and tensin homolog deleted from chromosome 10; TP53, tumor protein p53; WT, wild type

Active caspase-3/7 assay

Active caspase-3/7 was measured using a homogeneous luminescent method with Caspase-3/7 Glo assay kit (Promega, Madison, WI). The operating procedure was performed according to the manufacture’s instruction. After the cell lines were transfected with EGFR siRNA and treated with 0, 0.01, 0.1 and 1.0 µM cetuximab for 24 h, the luminescence was measured by using a multifunctional microplate reader, Infinite F500 (Tecan Trading AG).

Migration and invasion assay

The in vitro migration assay used an HTS Transwell-96 permeable support with 8 µm pore polyester membrane (Corning Incorporated, NY). The in vitro invasion assays were performed using HTS Transwell-96 well plate with Matrigel-coated filters (Corning Incorporated). Cell lines were seeded into the upper 96 well of the plate and exposed to 0, 0.01, 0.1 and 1.0 µM cetuximab, whereas 10% FBS medium was added into the lower well followed by incubation for 42 h. Non-migratory and non-invasive cells stayed in the upper membrane and migratory and invasive cells passed through basement membrane layer and cling to the bottom of the insert membrane. Migratory and invasive cells were dissociated from membrane by the detachment buffer and were lysed by the lysis buffer. Finally, the lysates were stained with CyQuant GR Dye (Invitrogen, Carlsbad) and the fluorescence produced were measured (excitation: 485 nm/emission: 525 nm) using multifunctional microplate reader, Infinite F500 (Tecan Trading AG).

Statistical analysis

All experiments were performed in triplicate. The values were represented as mean ± SD (standard deviation). Statistical analyses were performed by one-way ANOVA (analysis of variance) with Bonferroni’s test using SPSS Statistics 25 (IBM Japan, Tokyo, Japan). The result was considered statistically significant if $P < 0.05$.

RESULTS

Gene status of cell line

The gene status of EGFR, PTEN, KRAS, TP53 and LKB1 in all NSCLC cell lines used in this study are...
listed in Table 1. We examined KRAS gene status in 86-2, Sq19 and Ma10, PTEN gene status in Lu99, 86-2 and Sq19, TP53 gene status in Sq19, and LKB1 gene status in 86-2 and Sq19 using PCR and direct sequencing with sequence specific primers published elsewhere. The remaining gene status of the cell lines were cited from a published data as indicated in the table 1. All cell lines exhibited wild-type EGFR, whereas KRAS, PTEN, TP53 and LKB1 were mutated in A549 and Lu99; Lu99 and Sq19; Lu99, 86-2, Sq19 and Ma10; and A549, 86-2 and Sq19, respectively.

**Protein expression of NSCLC cell lines**

The expression of EGFR, PTEN, Akt, KRAS, TP53 and LKB1 proteins in all NSCLC cell lines was examined using western blotting (Fig.1). EGFR, KRAS and Akt were equally expressed in all cell lines. However, PTEN was not expressed in Sq19 cells, and the expression of Akt and KRAS were comparable with other cell lines. LKB1 was not expressed in A549 and Sq19 cells, and TP53 was not expressed in A549 and Lu99 cells.

**Effect of EGFR siRNA on EGFR expression**

Next, we tested the effect of EGFR specific siRNA on the expression of EGFR in all cell lines. As shown in Fig. 2, the expression of EGFR was almost suppressed by EGFR specific siRNA, whereas negative control siRNA that consists of non-targeting 20–25 bp nucleotide strands had no effect on the expression of EGFR. The proliferation of the cells transfected with EGFR siRNA was not different from those of the cells transfected with negative control siRNA (data not shown).

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**Fig. 2.** EGFR expression in NSCLC cell lines after EGFR siRNA treatment. Down regulation of EGFR expression was observed by western blotting. EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA.
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**Effect of cetuximab on cell proliferation in NSCLC**

We evaluated the effect of cetuximab on the cell growth in NSCLC cell lines. The number of NSCLC cells after cetuximab treatment (0, 0.01, 0.1 and 1.0 µM) was measured for 6 days using a colorimetric WST-8 assay. As shown in Fig. 3, cetuximab has no effect on the proliferation in all NSCLC cell lines. Therefore, it was confirmed that all the cell lines studied here are cetuximab-resistant cell lines.

**Effect of combination treatment of cetuximab and EGFR siRNA on cell proliferation**

Furthermore, to test the effect of EGFR downregulation on the cetuximab-resistant cells, we evaluated the effect of cetuximab on NSCLC cell proliferation in the absence or presence of EGFR specific siRNA. As shown in Fig. 4, the combination of cetuximab with EGFR siRNA showed...
significant suppression of cell proliferation in 86-2, Sq19 and Ma10 NSCLC cell lines. These cell lines have wild-type KRAS (Table 1). The cell lines that have mutated KRAS were found to be insensitive to the combination treatment. These data suggested that the KRAS status is a possible biomarker for the combination treatment of cetuximab with EGFR siRNA.

**Effect of combination treatment of cetuximab and EGFR siRNA on apoptosis**

After exploring the mechanism underlying the inhibition of cell proliferation by the combination treatment of cetuximab and EGFR siRNA, we tested these effects on apoptosis in each cell line. In this assay, we measured the production of active caspase-3/7 as a surrogate marker of apoptosis. As shown in Fig. 5, the combination treatment induced significant apoptosis in A549, 86-2 and Ma10 cell lines. These cell lines harbor wild-type PTEN, whereas the cell lines in which apoptosis was not induced by the combination treatment have mutated PTEN. As wild-type PTEN was reported to promote apoptosis, the combined effect of wild-type PTEN and...
the combination treatment might facilitate the induction of apoptosis. Moreover, induction of apoptosis by the combination treatment is a possible mechanism for the inhibition of cell proliferation in 86-2 and Ma10 cell lines.

**Effect of combination treatment of cetuximab and EGFR siRNA on migration and invasion.**

Since cell migration and invasion are the hallmarks of cancer cells, we evaluated the effect of the combination treatment of cetuximab with EGFR siRNA on cell migration and invasion. Cell migration was not affected by the combination treatment (Fig. 6). Cell invasion was suppressed by the combination treatment of 0.01 μM cetuximab and EGFR siRNA; however, it was not affected by the combination treatment of the other concentrations of cetuximab and EGFR siRNA (Fig. 7).

Therefore, we concluded that the combination treatment mostly affects EGFR pathways that relate to cell proliferation and apoptosis.

**DISCUSSION**

In this study, we explored the effect of the combination treatment of cetuximab with EGFR siRNA in NSCLC cell lines with wild-type EGFR. We found that this combination treatment suppressed the proliferation of cells that carried wild-type KRAS. Therefore, the mutational status of KRAS might be a possible biomarker of this combination treatment. In addition, we also showed that combination treatment induces apoptosis, which suppresses the proliferation of cells that carry wild-type PTEN. These observations suggest that the combination therapy of cetuximab with EGFR siRNA is promising for the treatment of NSCLC using KRAS and PTEN gene status as biomarkers.

A number of signaling molecules are involved in cancer development. These genes are classified into two categories; oncogenes that promote cancer development and tumor suppressor genes that inhibit cancer development. EGFR pathways, which play a vital role in cancer development, mainly regulate cell proliferation signals; many genes downstream of EGFR are known oncogenes and tumor suppressor genes. KRAS is the oncogene and PTEN, TP53, and LKB1 are the tumor suppressor genes involved in this pathway (Fig. 8). Dysregulation of these molecules by gene mutation or alternation of protein expression is reported to promote cancer development. For example, KRAS mutation constitutively activates the EGFR signaling pathway and promote cell proliferation. PTEN is the tumor suppressor molecule of PI3K-Akt pathway that regulates cell proliferation, cell growth, and inhibition of apoptosis. Mutation or loss of PTEN is reported to inhibit apoptosis, which leads to cell proliferation. TP53 is a multifunctional protein that is activated owing to cell-physiologic stress and prevents cancer development via the induction of apoptosis, cell cycle arrest, DNA repair, and the inhibition of angiogenesis. Indeed, TP53 mutation is reported to reduce apoptosis in cancer cells. LKB1 inhibits activation of mTOR signal functioning in the downstream of PI3K-Akt pathway, and functional loss of LKB1 leads to cell proliferation.

In this study, we examined five NSCLC cell lines. Since, these cell lines have wild-type EGFR, the aberrant proliferative activity of these cancer cells is dependent on the EGFR downstream molecules. KRAS is aberrantly activated by mutations in A549 and Lu99 cells and is the cause of the abnormal activity of the growth regulating function of these cancer cell lines. Moreover, we showed that TP53 was inactivated in all cell lines by defective expression (A549) or gene mutation (Lu99, 86-2, Sq19 and Ma10), and one or two additional tumor suppressor genes are inactivated by mutation or defective expression in each cell line. These diverse variety of inactivation of tumor suppressor genes may strongly
contribute to the cancer development.

Dysregulation of oncogenes and tumor suppressor genes are considered as the biomarkers of molecular targeted therapy in cancer. In the anti-EGFR therapy, somatic mutation of tyrosine kinase domain of EGFR that renders aberrant activation was revealed as potent predictors of response to EGFR-TKIs. In addition, increased EGFR copy number and KRAS mutation were reported as negative predictors of EGFR-TKI. For cetuximab, wild-type KRAS was reported to be a predictor of colorectal cancer responsiveness, however, it was reported that the KRAS mutation status was not useful in predicting the responsiveness for cetuximab in NSCLC patient. In the NSCLC cell lines with wild-type KRAS, it was reported that the mutation status was not a predictor of colorectal cancer responsiveness, however, it was reported that the KRAS mutation status was not useful in predicting the responsiveness for cetuximab in NSCLC patient.

In the NSCLC cell lines with wild-type KRAS, we showed that wild-type KRAS would be a common characteristic of the cells that are sensitive for the combination treatment of cetuximab with EGFR siRNA. To our knowledge, this is among the first studies that identified a biomarker of this combination treatment for NSCLC cells with wild-type EGFR.

There are several limitations in this study. First, we have presented data showing that one of the mechanisms of the susceptibility to the combination treatment of cetuximab and EGFR siRNA was the induction of apoptosis in two of three sensitive cell lines (86-2, MaI0). However, in the other sensitive cell line (Sq19), the mechanism was unclear, and a more extensive study of the other signaling molecules affected by this combination treatment is needed. Second, this was an in vitro study using NSCLC cell lines, and its results need to be validated using animal experiments and clinical testing to enable the practical application of the combination treatment of cetuximab with EGFR siRNA.

In conclusion, EGFR targeted treatment using the combination of cetuximab and EGFR siRNA is effective even in the NSCLC cell lines harboring wild-type EGFR. A possible biomarker of response to this combination treatment is wild-type KRAS, and one of the mechanisms of action is induction of apoptosis in cells with wild-type PTEN. The combination treatment of cetuximab and EGFR siRNA may be a new therapeutic potential in NSCLC patients.

The authors declare no conflict of interest.

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