Esophageal Cancer Exhibits Resistance to a Novel IGF-1R Inhibitor NVP-AEW541 with Maintained RAS-MAPK Activity

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Abstract. Aim: To assess the effects of a novel type 1 insulin-like growth factor receptor (IGF-1R) inhibitor, NVP-AEW541, on cell proliferation and signal transduction of esophageal cancer. Materials and Methods: Cell proliferation assay and western blot were conducted to assess the antitumor effects of NVP-AEW541. Genetic modification of RAS by expression vector was applied for overexpression of mutant RAS. Results: More than 2 μmol/l of NVP-AEW541 was required to effectively inhibit the proliferation of esophageal cancer. NVP-AEW541 potently blocked the activation of IGF-1R and protein kinase B (PKB, also known as AKT), but not of mitogen-activated protein kinase kinase (MEK) and extracellular-signal-regulated kinases (ERK). Active RAS was not reduced by NVP-AEW541 in esophageal cancer cells TE-1, suggesting that insensitivity of esophageal cancer to NVP-AEW541 is due to the maintained RAS-MAPK activity, which did not arise from RAS mutation. Moreover, the transduction of mutant RAS reduced the sensitivity of TE-1 cells to NVP-AEW541. Conclusion: Stimulation of RAS-MAPK pathway is associated with resistance to NVP-AEW541 in esophageal cancer. Combining NVP-AEW541 with inhibitors/antibodies against RAS-MAPK signaling molecules might be more effective for use against esophageal cancer.

The signaling system for insulin-like growth factors (IGFs) is comprised of two cognate ligands (IGF-I and IGF-II), cell surface receptors (IGF-IR, IGF-IRR, IR and hybrid receptors) and at least six IGF binding proteins (IGFBPs) (1). When the ligands bind to IGF-1R, autophosphorylation of the receptor tyrosine kinase is induced, leading to the activation of multiple downstream signaling pathways such as the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, also known as AKT), but not of mitogen-activated protein kinase (MEK) and extracellular-signal-regulated kinase (ERK). Active RAS was not reduced by NVP-AEW541 in esophageal cancer cells TE-1, suggesting that insensitivity of esophageal cancer to NVP-AEW541 is due to the maintained RAS-MAPK activity, which did not arise from RAS mutation. Moreover, the transduction of mutant RAS reduced the sensitivity of TE-1 cells to NVP-AEW541. Conclusion: Stimulation of RAS-MAPK pathway is associated with resistance to NVP-AEW541 in esophageal cancer. Combining NVP-AEW541 with inhibitors/antibodies against RAS-MAPK signaling molecules might be more effective for use against esophageal cancer.

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IGF-1R tyrosine kinase. At present, studies of the inhibitor are in preclinical stage. Several malignancies of the gastrointestinal tract, such as gastrointestinal neuroendocrine tumors (NET) (15), colorectal cancer (CRC) (16), gastrointestinal stromal tumors (2), pancreatic cancer (17) and hepatocellular carcinoma (HCC) (18), have been employed to study the effects of NVP-AEW541. However, the antitumor effect of this compound has not been thoroughly studied in ESCC.

In this study, we systematically evaluated the antiproliferative effects of NVP-AEW541 in several typical ESCC cells: TE-1, TE-4, TE-8, TE-10 and T.Tn. The potential signaling pathways that may be affected by NVP-AEW541 were assessed in detail. We also tried to explore potential mechanisms of insensitivity to this compound, such as RAS-related signaling, which will be helpful to improve the therapeutic effect of NVP-AEW541 on ESCC.

Materials and Methods

Cell lines and culture conditions. To assess the expression status of IGF-1R in human esophageal cancer cells and the antiproliferative effect of NVP-AEW541, the human ESCC cell lines TE-1, TE-4, TE-8, TE-10 and T.Tn were used, each of which was obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan). TE-1, TE-4, TE-8 and TE-10 were cultured in RPMI-1640, and T.Tn was cultured in dulbecco’s modified eagle medium nutrient mixture F-12 (DMEM/F12). All cell lines were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G sodium and 100 μg/ml streptomycin, and maintained in a monolayer culture at 37˚C in humidified air with 5% CO₂. Cellular morphology was observed through a microscope during culturing and experiments.

Reagents. NVP-AEW541, a tyrosine kinase inhibitor of IGF-1R, was synthesized and provided by Novartis Pharma AG (Basel, Switzerland) through a materials transfer agreement with Okayama University (Okayama, Japan). Stock solutions of the compound at 1 mg/ml were freshly prepared with culture medium in different concentrations (0, 0.1, 1, 10 μmol/l) of NVP-AEW541 for 48 h; another group of cells was starved for 24 h and then 50 μl of culture medium were added in different concentrations (0, 0.1, 0.5, 1, 5, 10 μmol/l) of NVP-AEW541 for 15 min, followed by adding 50 μl of culture medium with IGF-1 for a final concentration of 20 ng/ml for 48 h. After the drug treatment, 20 μl of the cell proliferation reagent WST-1 (Roche, Germany) was added to each well and incubated for 1-2 h in a humidified atmosphere (37˚C, 5% CO₂). Absorbance values were detected with a spectrophotometer set at 450/630 nm. Dose-effect plots were created to calculate the IC₅₀ of NVP-AEW541 for each cell line using Calcsyn software (Biosoft).

Western blot analysis. ESCC Cells were plated into 6-well plates at a density of 2.5x10⁵ per well and incubated for 36 h. Subconfluent cells were incubated in a serum-free medium for 24 h before drug treatment. Different concentrations (0, 0.1, 1, 10 μmol/l) of NVP-AEW541 were added to the serum-starved cells for 15 min and IGF-1 was subsequently added for a final concentration of 100 ng/ml, for 30 min. The culture medium was then carefully removed, washed once in cold PBS, and an appropriate amount of mammalian protein extraction reagent (M-PER; Thermo Scientific, Rockford, IL, USA) was added to the plate. Cell lysate was collected after shaking gently for 5 min and centrifuged at 15,000 x g at 4˚C for 20 min. The supernatant was transferred to a new tube for protein determination and western blot analysis. The concentration of protein lysates was measured with a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Equal amounts (30 μg) of protein lysate were electrophoresed under reducing conditions in 5-10% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gels. The proteins were then transferred to hybond polyvinylidene difluoride (PVDF) transfer membranes (GE Healthcare, Buckinghamshire, UK) and incubated with primary antibodies at 4˚C overnight, followed by incubation with peroxidase-linked secondary antibodies at room temperature for 1 h. Supersignal West Pico chemiluminescent substrate (Thermo Scientific) and chemiluminescence film (GE Healthcare) were used for signal detection.

The antibodies used for western blot were the following: IGF-1R (sc-713) and actin (sc-69879) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); phospho-IGF-1 receptor β (tyr1135/1136)/insulin receptor β (Tyr1150/1151) (#3024), AKT (#2922), phospho-AKT (Ser473) (#4060), ERK1/2 (Thr202/Tyr204) (#9101) and phospho-MEK 1/2 (Ser217/221) (#1504) were purchased from Cell Signaling Technology (Beverly, MA, USA); mouse anti-RAS antibody (#8985D) was purchased from Thermo Scientific. Peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were obtained from Jackson ImmunoResearch (Pennsylvania, PA, USA).

Detection of active RAS. TE-1 cells were seeded into 100-mm plate and incubated for 36 h. Subconfluent cells were incubated in a serum-free medium for 24 h before drug treatment. Different concentrations (0, 0.1, 1, 10 μmol/l) of NVP-AEW541 were added to serum-starved cells at a confluence of 80-90% for 15 min and subsequently 100 ng/ml of IGF-1 was added for 30 min. Active RAS in TE-1 cells was monitored using an Active RAS pull-down and detection kit (Thermo Scientific) following the manufacturer’s instructions. Briefly, cells were washed with ice-cold Tris-buffered saline (TBS), lysed with lysis/binding/wash buffer and scraped. After incubation on ice and centrifugation, the supernatant was transferred up to a lysis/binding/wash buffer spin cup containing glutathione resin, which binds to glutatine S-transferase (GST)-RAF1-RAS binding domain (RBD). The reaction mixture was incubated at 4˚C for 1 h and centrifuged. The resin was washed with a lysis/binding/wash buffer and reduced with a reducing sample buffer. The eluted samples were immunobloted with an anti-RAS antibody for detection.
Genomic DNAs of ESCC cell lines were released using a GenElute mammalian genomic DNA miniprep Kit (Sigma-Aldrich), following the manufacturer’s instructions. The RAS gene was amplified with RAS-specific primers (forward primer: 5’-GCTGAAAATGACTGAATATAAACTTGT-3’; reverse primer: 5’-TTGTTGGATCATATTCGTCCAC-3’) by polymerase chain reaction (PCR). To identify the k-RAS codon 12/13 mutation, the PCR product was directly sequenced on an ABI 3100-Avant DNA sequencer (Applied Biosystems, Foster City, CA). The human wild-type RAS sequence in GenBank (NG_007524.1) was a BLAST sequence.

Transfection of mutant RAS. TE-1 cells in an antibiotic-free medium were seeded in a 60-mm dish for 24 h before transfection. Plasmid PCI-mEGFP-HRASG12V (Addgene, Cambridge, MA, USA) was mixed gently with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and incubated for 20 min at room temperature. The medium was replaced by a fresh antibiotic-containing medium after adding the plasmid-Lipofectamine 2000 complexes for 6 h. The following day, mEGFP-RASG12V transfected TE-1 cells were seeded into a 96-well plate for 24 h before the drug treatment. Different concentrations of NVP-AEW541 (0, 0.5, 1, 5 μmol/ml) were added to RAS-transfected TE-1 cells for 48 h and the inhibition rate was tested by WST assay. TE-1 cells that were transfected with Lipofectamine 2000 only were used as a control group.

Statistical analyses. The comparison of categorical experimental data was conducted by Student’s t-test. Data is represented as the mean±SD. All p-values are two-sided. A value of p<0.05 was considered to be statistically significant in all experiments.

**Results**

**IGF-1R was overexpressed in ESCC cells.** To examine the role of IGF-1R in the growth and proliferation of ESCC cells, we first detected the expression of IGF-1R. Cell lysates extracted from the cultured ESCC cells (TE-1, TE-4, TE-8, TE-10 and T.Tn), from HeLa cells known to express IGF-1R at a high level (19), as a positive control, and from human foreskin fibroblast cells (HFF-1) were used as a positive and negative control, respectively. Actin was used as a loading control.

**Inhibition of IGF-1R activity by NVP-AEW541 suppressed the proliferation of ESCC cells.** We next sought to determine whether blocking IGF-1R activity using its specific inhibitor exerts an antitumor effect on ESCC. ESCC cells cultured in serum-containing medium were treated with NVP-AEW541, and cell viability was measured by a WST assay. The proliferation of ESCC cells was inhibited by NVP-AEW541 in a dose-dependent manner (Figure 2A). To assess the effect of NVP-AEW541 on IGF-1-mediated cell proliferation, ESCC cells were starved for 24 h before drug treatment and stimulated with various amounts of IGF-1 after treatment with NVP-AEW541 for 15 min. As shown in Figure 2B, NVP-AEW541 dose-dependently inhibited cell proliferation under IGF-1-stimulated conditions as well. Among the five
ESCC cell lines, the sensitivity of TE-1 and TE-8 cells to NVP-AEW541 was higher in the IGF-1-stimulated condition than in the serum-containing condition, suggesting that the proliferation of TE-1 and TE-8 cells is more IGF-1 dependent compared to the other three cell lines. In order to determine the sensitivity to NVP-AEW541 of each cell line, the half maximal inhibitory concentrations (IC50) were calculated and revealed IC50s to be more than 2 μmol/l, in both serum-containing and IGF-1-stimulated conditions, for all of the cell lines; the maximal value of 9.429 μmol/l for TE-1 cells suggests that TE-1 cells are the most insensitive to NVP-AEW541 (Table I).

NVP-AEW541 efficiently blocked PI3K/AKT signaling but not RAS/RAF/ERK signaling. The inhibitory effect of NVP-AEW541 on the PI3K/AKT signaling pathway and the RAS/RAF/ERK signaling pathway, both of which are crucial signal transduction pathways mediated by IGF-1R, were examined. Stimulation with IGF-1 after serum starvation significantly increased the phosphorylation of IGF-1R, which was dose-dependently inhibited by NVP-AEW541 in TE-1, TE-4, TE-8 and TE-10 cells (Figure 3). Although the serum was starved, AKT and ERK were constitutively activated in each cell line, revealing that both the PI3K/AKT and RAS/RAF/ERK pathways play an important role in cell growth and proliferation of these ESCC cells. The phosphorylation of AKT was enhanced by IGF-1 stimulation and markedly inhibited by NVP-AEW541. However, treatment with different concentrations of NVP-AEW541 led
to various inhibitory effects on p-ERK, which remained at 10 μmol/l (Figure 3). Therefore, a higher concentration of NVP-AEW541 was required in ESCC probably due to the small inhibitory effect of NVP-AEW541 on the RAS/RAF/ERK signaling pathway.

To find the possible signaling factor that regulates the strong activation of ERK and the activity of MEK and RAS, the upstream signaling transducers of ERK, were detected in TE-1 cells with NVP-AEW541 treatment. The activity of MEK was inhibited by 1 μmol/l of NVP-AEW541, whereas its activity was somewhat recovered at 10 μmol/l of NVP-AEW541 (Figure 4A). RAS was active under the serum-starved condition and was not potently blocked by NVP-AEW541 (Figure 4B). These results further demonstrated that RAS/RAF/ERK signaling may retard the inhibitory effect of NVP-AEW541 on the proliferation of esophageal cancer cells.

RAS in ESCC was wild-type and the exogenous transduction of G12V-mutated RAS in ESCC cells reduced cell sensitivity to NVP-AEW541. Point mutations in RAS may result in the constitutive activation of RAS. To explore whether RAS is mutated in ESCC cells and contributes to cell proliferation, we extracted the genomic DNA of ESCC for sequencing. The sequencing analysis of RAS showed that the nucleotides at codons 12 and 13 were GGT and GGC, respectively, consistent with the wild-type RAS sequence in the GenBank (NG_007524.1) (Figure 5). RAS mutations did not occur in any ESCC cells, suggesting that there may be other potential mechanisms which cause the constitutive activation of RAS.

Exogenous transduction of the G12V-mutant RAS using plasmid PCI-mEGFP-HaRASG12V was applied to determine the effect of RAS mutation on cell sensitivity to NVP-AEW541. The fusion protein mEGFP-RASG12V highly expressed in TE-1/RASG12V but not in TE-1/RASWT cells (Figure 6A). TE-1/RASG12V and TE-1/RASWT were treated with NVP-AEW541 and the inhibition rate measured by a WST assay. As shown in Figure 6B, the inhibition rate of
NVP-AEW541 was significantly reduced in TE-1/RAS\textsuperscript{G12V}, suggesting that the RAS mutation enhances cell resistance to NVP-AEW541.

In summary, NVP-AEW541 has an antiproliferative effect on cell proliferation in ESCC cells and the maintained RAS/RAF/ERK activity may be associated with the requirement for higher concentrations of NVP-AEW541 for efficacy.

**Discussion**

The disruption of IGF-1R function has been proven to be a promising therapeutic strategy in different human cancers due to the role of IGF-1R in cancer cell proliferation, survival and metastasis (6, 7). In this study, we demonstrated that IGF-1R is abnormally expressed in ESCC cells and the inhibition of IGF-1R action by the IGF-1R-specific small-molecule inhibitor NVP-AEW541 retarded cell proliferation by suppressing IGF-1-induced AKT activation. However, high doses of NVP-AEW541 were required to effectively inhibit the proliferation of ESCC cells. The reason for this may be that besides PI3K/AKT signaling, RAS/RAF/ERK signaling also contributes to cell proliferation and survival, but the stimulation with ligand IGF-1 and the treatment with NVP-AEW541 had a weak effect on the phosphorylation of ERK (Figure 3). These data are consistent with previous studies in which treatment with NVP-AEW541 failed to inhibit the activation of ERK in colon and biliary tract cancer (16, 20, 21). Genetic blockage of IGF-1R also inhibits activity of AKT but not of ERK (22, 23). The PI3K/AKT pathway might play a more important role than the RAS/RAF/ERK pathway in the downstream effectors of the IGF-1/IGF-1R axis.

ESCC cells exhibited constitutive activation of ERK. To explore the possible mechanism, we further assessed the activities of the upstream molecule of ERK upon treatment with NVP-AEW541. Our data demonstrated that the insensitivity of cancer cells to IGF-1R inhibitors may be mainly driven by the maintained activity of RAS/RAF/ERK signaling (Figure 4). One patient with advanced Ewing’s sarcoma who was resistant to a combination treatment of IGF-1R inhibitors with mammalian target of rapamycin (mTOR) inhibitor also exhibited RAS/RAF/ERK activation (24). The overexpression and constitutive activation of platelet-derived growth factor receptor α (PDGFR\textalpha) might be associated with the resistance to IGF-1R-targeted therapy because PDGFR\textalpha signaling increases proliferation signals through its downstream PI3K/AKT and RAS/RAF/ERK signaling pathway, which overlaps with IGF-1R downstream signaling (25). Indeed, PDGFR\textalpha is highly expressed in esophageal cancer (26). However, the potential mechanism needs to be further identified.

RAS oncprotein is a GTPase that is active when bound to guanosine triphosphate (GTP) and inactive when bound to guanosine diphosphate (GDP). It is an essential component of signaling pathways and regulates various cancer-driving processes, such as proliferation, survival, energy metabolism, and angiogenesis (27). The genetic mutation of RAS isoforms (H-RAS, K-RAS, and N-RAS) at residues G12, G13 and Q61 are frequently detected in human tumors. Oncogenic substitutions derived from mutations inhibit GTPase activity and thus hold the protein in an active GTP-bound state to stimulate the activation of the RAS-dependent downstream signaling pathway (28). Therefore, genetic analysis by sequencing was conducted to elucidate the constitutive active RAS status to explain the lower response to the IGF-1R inhibitor NVP-AEW541 in ESCC cells (Figure 4B). However, no mutation at codon 12 or 13 was found in ESCC (Figure 5). In addition to GTP loading by mutation, the activation of RAS is also regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The study of RASGRP1, a GEF for RAS, showed that RASGRP1 overexpression elevated the activation of endogenous wild-type RAS for tumor progression, providing an insight into RAS activation and drug resistance (29). Furthermore, recent studies have indicated that patients with colorectal cancer carrying a RAS-activating mutation were less responsive to epidermal growth factor receptor (EGFR)-targeted therapy such as cetuximab or panitumumab, two monoclonal antibodies against EGFR (30, 31). To investigate the effect of RAS mutation on IGF-1R-targeted therapy, TE-1 bearing active RAS mutation (Gly12Val) was treated with NVP-AEW541. Similar to previous studies, the RAS mutation also impaired the therapeutic effect of the IGF-1R inhibitor (Figure 6). Our result is further confirmed by a study in which the introduction of K-RAS or B-RAF mutants resulted in resistance of imatinib-sensitive gastrointestinal stromal tumors to imatinib (32). Although RAS mutations are not common in esophageal cancer (33, 34), we do not exclude the possibility that RAS mutation might be a good negative predictor in IGF-1R-targeted therapy.

Considering more effective treatment responses and dose-limiting side-effects, combinational treatment may be a more promising strategy than single-drug treatment. For IGF-1R-targeted therapy for ESCC, based on the present data, one approach may be combinational treatment with inhibitors against the effectors of the RAS/RAF/ERK signaling pathway. For example, MEK inhibitor U0126 enhanced the susceptibility to lapatinib, a dual human epidermal growth factor receptor-2 (HER2) and epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor in RAS-induced lapatinib-resistant breast cancer (SKBR3 and BT474) cells (35). Another possibly attractive approach is dual targeting of IGF-1R and other growth factor tyrosine kinase receptors [e.g. EGER, HER-2], IGF-1R cross-talks with these growth factor receptors and co-regulates tumor progression (36, 37).
Importantly, overexpression of EGFR has been associated with intrinsic and/or acquired resistance to the IGF-1R inhibitor BMS-536924 and a combination of BMS-536924 with the EGFR inhibitor gefitinib showed enhanced antitumor activity (38). More effective inhibition of the phosphorylation of AKT and ERK may be an important reason for the combined antitumor effect. In addition, gefitinib had an effective antitumor effect in ESCC cells (39). Thus, it is feasible that the combination of NVP-AEW541 and EGFR inhibitors will produce a good response in esophageal cancer.

In summary, ESCC cells with a high expression of IGF-1R required a high dose of the IGF-1R inhibitor NVP-AEW541 to achieve effects. PI3K/AKT signaling was significantly inhibited by NVP-AEW541, whereas RAS/RAF/MEK signaling was less responsive to it. RAS mutation was associated with cell resistance to NVP-AEW541. Further investigation in whether the potent antitumor effect of NVP-AEW541 observed in this study would be even more effective as part of a combinational therapy is necessary to verify the hypotheses generated by the present study.

Disclosure of Potential Conflicts of Interest

We declare that we have no conflict of interest.

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