Targeting Adenine Nucleotide Translocase-2 (ANT2) to Overcome Resistance to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor in Non-Small Cell Lung Cancer

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Abstract

EGFR tyrosine kinase inhibitor (EGFR-TKI) therapy has achieved favorable clinical outcomes in non–small cell lung cancer (NSCLC) patients with EGFR mutations. However, patients eventually develop resistance to EGFR-TKIs by several mechanisms. Adenine nucleotide translocase-2 (ANT2) is an oncogenic mitochondrial membrane–associated protein. We investigated the therapeutic potential of ANT2 inhibition to EGFR-TKI resistance in NSCLC using gefitinib-sensitive (PC9 and HCC827) and gefitinib-resistant (H1975 and HCC827/GR) NSCLC cell lines. ANT2 was inhibited by transfecting cells with an ANT2-specific shRNA. ANT2 expression was elevated in the H1975 and HCC827/GR cells compared with the PC9 and HCC827 cells. ANT2 upregulation in gefitinib-resistant cells was associated with increased SP1 binding to the ANT2 promoter. ANT2-specific shRNA decreased NSCLC cell viability. Moreover, ANT2-specific shRNA sensitized the H1975 and HCC827/GR cells to gefitinib, accompanied by HSP90 and EGFR downregulation. ANT2-specific shRNA also inactivated the PI3K/Akt signaling pathway in the H1975 and HCC827/GR cells, which was mediated by the suppression of miR-221/222 levels and by the subsequent restoration of PTEN. In EGFR-TKI–treated NSCLC patients, ANT2 expression was higher in patients exhibiting poor responses compared with patients showing excellent responses. Furthermore, ANT2 expression increased in tumor tissues biopsied after acquiring gefitinib resistance compared with tissues before gefitinib treatment. These findings suggest that ANT2 overexpression contributes to EGFR-TKI resistance in NSCLC and that ANT2 targeting may be considered a novel strategy for overcoming this resistance.

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Introduction

Lung cancer is the most common cause of cancer-related deaths worldwide, and non–small cell lung cancer (NSCLC) accounts for 85% of lung cancers (1). Adenocarcinoma is the most common NSCLC histology, and its incidence is continually increasing (2). Because more than half of the NSCLC patients present at advanced stages of the disease, systemic treatments such as chemotherapy are important for managing NSCLC (3). In recent decades, the management of patients with NSCLCs, particularly adenocarcinomas, has shifted toward personalized targeted therapy based on the genetic status of the tumor cells (4). The most common druggable genetic alterations in NSCLC are EGFR mutations, which are observed in approximately 60% of East-Asian patients with adenocarcinomas and in 30% of Western adenocarcinoma patients (5). Mutated EGFR activates the PI3K/Akt, JAK/STAT, and MAPK/ERK pathways and contributes to the carcinogenesis of NSCLC (6–8). Targeted therapies using EGFR tyrosine kinase inhibitors (TKI) have prolonged the survival of pulmonary adenocarcinomas patients with EGFR mutations (9). However, the clinical drawback of EGFR-targeted therapy is that EGFR-TKI resistance eventually develops in all patients (10). Approximately half of the acquired resistance to EGFR-TKIs is caused by a secondary gatekeeper mutation in the EGFR tyrosine kinase domain (i.e., the T790M mutation in EGFR exon 20; ref. 11). Other mechanisms that contribute to acquired EGFR-TKI resistance include MET amplification, small-cell carcinoma transformation, epithelial–mesenchymal transition, and HER2 amplification (12–15). However, the mechanisms of EGFR-TKI resistance remain unclear for 20% to 40% of patients (10), warranting the identification of EGFR-TKI resistance mechanisms to overcome acquired EGFR-TKI resistance.

Adenine nucleotide translocase (ANT) is a protein abundant in the inner mitochondrial membrane involving in mitochondrial oxidative phosphorylation and glycolytic metabolism.
(16–18). ANT also interacts with BCL2 family proteins and regulates mitochondrial membrane permeability and mitochondrial-mediated apoptosis (16–20). Four isoforms of ANT, encoded by ANT1, ANT2, ANT3, and ANT4 genes, have been identified in humans. The expressions of ANT isoforms differs depending on the tissue and cell type, developmental stage, and cellular proliferation status. Specifically, ANT3 is ubiquitously expressed in all tissues at low levels or at a degree correlating with the level of oxidative metabolism. ANT1 is abundantly expressed in terminally differentiated cells. In contrast, ANT2 is highly expressed in undifferentiated cells and tissues with proliferating and regenerating abilities including lymphocytes and the kidneys and liver (16, 17, 21). ANT2 expression is also higher than ANT1 expression in several types of human cancer cells (17, 18, 22, 23). Notably, ANT1 and ANT3 play proapoptotic roles; however, ANT2 displays an antiapoptotic function in cancer cells by inhibiting mitochondrial membrane permeabilization (17, 19, 23, 24). Furthermore, ANT2 was demonstrated to promote glycolytic metabolism in cancer cells under aerobic and anaerobic conditions (25). These observations have suggested that ANT2 functions as an oncogenic molecule. Consistently, silencing ANT2 led to an increase in mitochondrial membrane potential and to the chemosensitization of cancer cells (22). In our previous studies, ANT2 knockdown by short hairpin RNA (shRNA) showed anticancer effects via diverse mechanisms in breast and liver cancer cells (23, 26–30). Thus, we hypothesized that ANT2 might be involved in the resistance to EGFR-TKI in NSCLC cells and investigated the therapeutic potential of targeting ANT2 to overcome acquired EGFR-TKI resistance.

**Materials and Methods**

**Cell lines and reagents**

EGFR-TKI–sensitive human lung adenocarcinoma cell lines, PC9 and HCC827 (both having EGFR exon 19 deletion mutations) and EGFR-TKI–resistant human lung adenocarcinoma cell lines, H1975 (having both EGFR L858R and T790M mutations) and HCC827/GR cells, which were induced to have acquired MET amplification (31), were used. PC9 was kindly provided by Prof. Mayumi Ono (Kyushu University, Fukuoka, Japan), and HCC827 and HCC827/GR cells were kindly provided by Prof. Jae Cheol Lee of the Asan Medical Center (Seoul, South Korea). H1975, and other lung adenocarcinoma cell lines, A549, H1666, and H522 (all EGFR wild-type), were purchased from ATCC. These cell lines were obtained from R&D Systems.

**Construction of expression vector**

The ANT2-specific shRNA expression vector was constructed as reported previously (23). Briefly, an ANT2-specific siRNA (5′-GCAGAUCACUCCAGAUAAGT-3′) complementary to exon 2 of ANT2 (Genbank accession number: NM001152) was synthesized (Bioneer) to achieve the specific downregulation of ANT2. DNA vectors expressing the shRNA forms of the siRNAs were then generated using pSilencer 3.1-H1 puro plasmids with a TTCAAAGA linker sequence to form looped structures (Ambion). A scramble (sc) shRNA (Ambion) with no homology to human genes was used as a control shRNA. The HSP90 expression vector (pCMV6-entry) with TruORF Gold expression-validated cDNA clone (OriGene Technologies) was purchased. Dominant-negative PI3K (dnPI3K) was provided by Prof. Yell Cheol Kang of Yonsei University (Seoul, South Korea).

**Transfection of plasmid, siRNA, and miR inhibitor or mimics**

Cells were plated in either 6-well plates (2 × 10^5 cells/well) or 100-mm dishes (2 × 10^6 cells) and allowed to adhere for 24 hours. Then, the cells were transfected with either ANT2-specific shRNA (ANT2 shRNA) or sc shRNA vectors using Lipofectamine 2000 (Invitrogen) in Opti-MEM media (QIAGEN). The transfected cells were cultured for 6 hours, and the medium was replaced with fresh medium supplemented with 10% FBS. The cells were harvested 24 to 48 hours after transfection. In addition, Annexin V–FITC (Biosciences) was used to detect apoptosis. miR-221 inhibitor (5′-AGCUACAIUGIUGUCGUGGUUGU-3′) and miR-222 inhibitors (5′-AGCUACAIUCUGCCGUGGUGU-3′), which were chemically modified and optimized nucleic acids designed to specifically target the miRNA molecules, were purchased from GenePharma and transfected as described above.

**Protein–DNA binding assay**

Nuclear extracts were prepared from cells and subjected to protein–DNA binding assays using an EpiQuik General Protein–DNA Binding Assay Kit (Colormetric; Epigentek) to detect the binding of Sp1 to the ANT2 promoter DNA sequence. A DNA probe corresponding to the Sp1-binding sequence in the ANT2 promoter was constructed as follows: 5′-CCGGCGGCGGCGGCGGACAGC-3′.

**ChIP-qPCR assay**

ChIP-qPCR assay was performed using ChIP Ab+ Sp1 - ChIP validated antibody and primer set (Merck Millipore). In brief, chromatin from cells was cross-linked with 1% formaldehyde, sheared to an average size of approximately 500 bp, and then immunoprecipitated with anti-Sp1 antibody. Putative binding sites of Sp1 within the human ANT2 promoter region were identified by TFSEARCH. Two potential Sp1-binding sites were amplified and monitored by real-time quantitative PCR method.

**Cell viability and apoptosis assay**

Cell viability was measured using Cell Counting Kit-8 (CCK8) assays (Dojindo Molecular Technologies) according to the manufacturer’s protocol. All of the experiments were repeated at least three times in triplicate and the results are presented as the mean ± SD. Apoptosis assay was performed by Annexin V and PI staining (BD Pharmingen) followed by flow cytometry analysis (Epics XL; Beckman Coulter).
Targeting ANT2 in EGFR-TKI-Resistant NSCLC

Western blotting
Total cellular protein was extracted using lysis buffer (5 nmol/L ethylenediamine tetra-acetic acid, 300 nmol/L NaCl, 0.1% NP-40, 0.5 nmol/L NaF, 0.5 nmol/L Na₃VO₄, and 0.5 nmol/L ethylenediamine tetra-acetic acid, 300 nmol/L NaCl, 50 µg/mL each of aprotinin, pepstatin, and leupeptin; Sigma-Aldrich). In total, 50 µg of protein was subjected to 10% SDS-PAGE, and the resolved proteins were transferred to polyvinylidene difluoride membranes (Millipore), which were then incubated with antibodies directed against ANT2, phospho-Akt (P-Akt) or Akt (Cell Signaling Technology) or with antibodies directed against HSP90, EGFR, MET, PTEN, cleaved PARP, or β-actin (Santa Cruz Biotechnology). The immunoblots were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Quantitative reverse transcription PCR
Total RNA was extracted from the cells using TRIzol reagent (Life Technologies) and reverse transcribed using a PrimeScript First Strand cDNA Synthesis Kit (Takara Bio). GAPDH was used as the internal control for mRNA expression. To evaluate the miR-211/222 levels, the extracted total RNA was subjected to cDNA synthesis and quantitative reverse transcription PCR (qRT-PCR) analysis using Mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kits (Clontech Laboratories). U6-specific snRNA (catalog no. 4373381, Applied Biosystems) was used as an internal control for miRNA expression. The sequences of the primers used for qRT-PCR to analyze the miRNAs and miRNAs are as follows: for ANT2: forward, 5'-ACGTGTCTGTG-CAGGGTAT-3', and reverse, 5'-GTGTCAATGGAAGAAG-3'; for PTEN: forward, 5'-CGAAGTGCTGAATGATACT-3', and reverse 3'-CATGAACTGTGCTCCCG-3'; for GAPDH, forward 5'-CCCTCATTGACCCTAAC-3', and reverse, 5'-ACGATACCAAAAGTTGCTGAT-3'; for miR-221: forward, 5'-CGGGGGA-GCTATATTGCTGTGG-3', and, reverse, 5'-CCAGTGCCAACGT-TGGAT-3'; for miR-222: forward, 5'-CCAGTGCCAACGT-TGGAT-3', and reverse, 5'-CCAGTGCCAACGT-TGGAT-3'. The PCR reactions were performed using a Step One Plus thermal cycler (Applied Biosystems) in triplicate. The relative changes in gene expression were calculated using the following formula, and the data are represented as fold upregulation/downregulation: the fold change is indicated as 2^{-ΔΔCt}, where ΔΔCt = (Ct of gene of interest, treated − Ct of internal control, treated) − (Ct of gene of interest, control − Ct of internal control gene; control; Ct, the threshold cycle number).

Intracellular ATP assay
Intracellular ATP assays were performed using CellTiter-Glo Luminescent Cell Viability Assay Kits (Promega), which emits
light upon interactions with ATP and luciferin. In brief, lyophilized enzyme/substrate mixtures (250 μL) were transferred to opaque 96-well microplates containing cell lysates. The plates were incubated at room temperature for 10 minutes to stabilize the luminescence signals, which were then quantified using an Orion Luminometer (Berthold Detection Systems).

**Immunohistochemical analysis of human lung cancer tissues**

From the registry of lung cancer patients who underwent surgical resection for lung cancer and were treated with gefitinib for recurrent or metastatic disease at Seoul National University Hospital (SNUH; Seoul, South Korea), 84 patients who harbored EGFR mutation were collected. One medical oncologist (B. Keam), who was unaware of the ANT2 statuses of the tumors, reviewed the radiologic images by RECIST 1.0 criteria to classify the responses as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). Progression-free survival (PFS) was defined as the time from the beginning of EGFR-TKI administration to confirmed disease progression or death. The 84 patients were divided into three groups: (i) poor responders, (ii) conventional responders, and (iii) excellent responders. The poor responders were defined as PD or SD without durable (≥6 months) PFS (32). The excellent responders were defined as CR or PR with prolonged PFS (>12 months). The conventional responders were defined as neither poor nor excellent responders. Finally, 10 cases with poor responses to gefitinib and 9 cases with excellent responses to gefitinib were selected. All 19 patients had EGFR-TKI–sensitive mutations, including EGFR exon 19 deletions (n = 9), and L858R (n = 9) or T854I mutations (n = 1) in EGFR exon 21. Formalin-fixed paraffin-embedded tumor tissue blocks from these patients were subjected to immunohistochemistry (IHC) using an anti-ANT2 antibody (Creative Biogene, NY). IHC was performed using the CSA II Biotin-free Tyramide Signal Amplification System (DAKO Cytomation) and the results were interpreted by a pathologist (Y.K. Jeon) who was blinded to the treatment response. The ANT2 IHC was graded as follows: 0, negative or staining in less than 10% of tumor cells; 1, weak; 2, moderate; and 3, strong staining in ≥10% of tumor cells. This study was approved by the Institutional Review Board of SNUH (H-1401-031-548).

**Statistical analysis**

The data were analyzed using Student t tests (SPSS version 21; IBM Corp.). P < 0.05 was considered statistically significant.

**Results**

**ANT2 expression was upregulated in EGFR-TKI–resistant NSCLC cells**

First, we screened the ANT2 expression in several NSCLC cell lines with variable sensitivity to EGFR-TKI. As shown in Fig. 1A–C, ANT2 expression was lower in EGFR-TKI–sensitive cell lines (including PC9 and HCC827) and higher in EGFR-TKI–resistant cell lines (including A549, H522 H1975, and HCC827/GR) at both mRNA and protein levels.

Because Sp1 is the most important transcription factor for human ANT2 (33), we assessed the binding of Sp1 to the ANT2 promoter using protein–DNA binding assays and ChIP-qPCR assay (Fig. 1D). Sp1 binding to the ANT2 promoter was significantly elevated in H1975 and HCC827/GR cells compared with that in PC9 and HCC827 cells. Together,
these data suggest that ANT2 expression is upregulated in EGFR-TKI-resistant NSCLC cells at the transcriptional level, which may be mediated by increased binding of Sp1 to the ANT2 promoter.

Inhibition of ANT2 suppresses cell viability and restores gefitinib sensitivity in EGFR-TKI-resistant NSCLC cells

Next, it was determined whether ANT2 shRNA would suppress the viability of EGFR-TKI-resistant NSCLC cells with elevated ANT2 expression. The IC50 of gefitinib was 50 and 10 nmol/L in PC9 and HCC827 cells, respectively, but as much as 1,000-fold higher (10 μmol/L) in H1975 and HCC827/GR cells (Supplementary Fig. S1). Suppression of ANT2 by ANT2 shRNA transfection of H1975 and HCC827/GR cells (Fig. S2). As shown in Fig. 2A, ANT2 shRNA transfection of PC9 and HCC827 cells decreased cell viability by 20%, whereas ANT2 shRNA transfection of H1975 and HCC827/GR cells decreased cell viability by 40% and 50%, respectively. The combined treatment of ANT2 shRNA and gefitinib exhibited an additive effect on decrease of cell viability in PC9 and HCC827 cells as well as in H1975 and HCC827/GR cells (Fig. 2A). Furthermore, apoptosis of H1975 and HCC827/GR cells was much increased by combined gefitinib and ANT2 shRNA treatment as shown by PARP cleavage (Fig. 2B) and Annexin V/PI staining (Supplementary Fig. S3). A ura pharmacologic ANT2 inhibitor, we used lonidamine, which is a mitochondrial hexokinase inhibitor provoking a disruption of the mitochondrial transmembrane potential similar to the ANT2 shRNA-induced apoptosis. Lonidamine also compromised cell survival in lung cancer cells along with gefitinib (Supplementary Fig. S4). Taken together, these data suggested that ANT2 knockdown by ANT2 shRNA might effectively suppress the viability of gefitinib-resistant NSCLC cells and overcome gefitinib resistance mediated by T790M mutations and by MET amplification.

EGFR-TKI sensitization by ANT2 inhibition is mediated by the suppression of HSP90 and EGFR expression

To address the mechanism by which ANT2 shRNA sensitizes H1975 and HCC827/GR cells to gefitinib, we focused on

![Intracellular ATP assay](image1)

**Figure 3.**

ANT2 inhibition sensitizes NSCLC cells to gefitinib by suppressing the expression of HSP90 and EGFR. A, cells were transfected with ANT2 shRNA or sc shRNA for 24 hours and then lysed to quantify total intracellular ATP levels. The results are tabulated as relative ATP production by normalizing luminescence units to total protein levels. B, at 24 hours after transfection with ANT2 shRNA or sc shRNA, Western blotting was performed to determine the levels of HSP90, EGFR, P-Akt, Akt, and MET. C, H1975 and HCC827/GR cells were transfected with ANT2 siRNA, HSP90 siRNA, or sc siRNA for 24 hours, and then treated with gefitinib. After an additional 24-hour incubation, cell viability was measured using CCK8 assays and presented as a relative value compared with that of sc siRNA-treated cells. D, H1975 and HCC827/GR cells were transfected with ANT2 shRNA or sc shRNA with/without HSP90 expression vector. At 24 hours after transfection, the cells were treated with gefitinib (50 nmol/L) or DMSO as a control and subsequently incubated for an additional 24 hours. Then, cell viability was measured using CCK8 assays. The values presented in all of the histograms are the mean values ± SD. *P < 0.05; **P < 0.005, statistically significant differences as determined by unpaired Student t tests; n.s., not significant.
HSP90 expression. As shown in Fig. 3A, ANT2 shRNA decreased the intracellular ATP level in NSCLC cells in a degree proportionate to the ANT2 expression level (i.e., greater in H1975 and HCC827/GR cells compared with PC9 and HCC827 cells). ANT2 shRNA also suppressed HSP90 expression in these NSCLC cells, which was accompanied by the downregulation of EGFR expression (Fig. 3B). However, the levels of Akt and MET, which are other HSP90 client proteins, were not affected by ANT2 shRNA (Fig. 3B).

To determine whether ANT2 shRNA-mediated HSP90 downregulation could be involved in the sensitivity of H1975 and HCC827/GR cells to gefitinib, we knocked down HSP90 using an HSP90 siRNA and evaluated cell viability after combined treatment with gefitinib. The transfection of ANT2 siRNA and HSP90 siRNA reduced the expression of ANT2 and HSP90 by approximately 30% in H1975 and HCC827/GR cells (Supplementary Fig. S5). ANT2 siRNA and HSP90 siRNA transfection led to approximately 30% and 10% decreases in cell viability in H1975 and HCC827/GR cells, respectively (Fig. 3C). In addition, combined treatment of gefitinib and ANT2 siRNA or HSP90 siRNA transfection further suppressed cell viabilities in these cells (Fig. 3C). Moreover, rescue of HSP90 expression in ANT2 shRNA-transfected cells restored the cell viability after gefitinib treatment (Fig. 3D). These data suggested that the ANT2 shRNA-mediated downregulation of HSP90 might be involved in the restoration of gefitinib sensitivity in EGFR-TKI–resistant NSCLC cells. Meanwhile, the degree of suppression of cell viability by combined HSP90 siRNA and gefitinib was much lower compared with that observed by combined ANT2 siRNA and gefitinib treatment (Fig. 3C). Thus, we hypothesized that mechanisms other than HSP90 suppression might contribute to the restoration of gefitinib sensitivity by ANT2 shRNA in EGFR-TKI–resistant NSCLC cells.

Figure 4. ANT2 inhibition sensitizes NSCLC cells to gefitinib by downregulating the levels of miR-221/222, consequently restoring PTEN expression and inactivating the PI3K/Akt pathway. A and B, the expression levels of miR-221 and miR-222 and PTEN were assessed by qRT-PCR in EGFR-TKI–sensitive (PC9 and HCC827) and EGFR-TKI–resistant (H975 and HCC827/GR) cells. The relative expression levels (2^ΔΔt) of these molecules were compared with those of PC9 cells. Western blotting was performed to determine the levels of PTEN. C and D, H975 and HCC827/GR cells were transfected with miR-221 or miR-222 inhibitors or negative inhibitors, and the changes in PTEN levels were measured by qRT-PCR and Western blotting. The relative expression levels (2^ΔΔt) of PTEN were compared with those of H975 cells transfected with negative inhibitors. E–G, At 24 hours after transfection with ANT2 shRNA or sc shRNA, the expression levels of miR-221 and miR-222 (E) and PTEN (F) were measured by qRT-PCR. The relative expression levels (2^ΔΔt) of these molecules were compared with those of cells transfected with sc shRNA. PTEN expression was also assessed by Western blotting (G). H, H1975 and HCC827/GR cells were transfected with ANT2 shRNA, dominant-negative PI3K (dnPI3K) vector, or sc shRNA for 24 hours, and then treated with gefitinib. After an additional 24-hour incubation, cell viability was measured via CCK8 assays and presented as a relative value compared with that of sc shRNA-transfected cells. The values presented in all of the histograms are the mean values ± SD. *, P < 0.05; **, P < 0.005; statistically significant differences as determined by unpaired Student t tests; n.s., not significant.
EGFR-TKI sensitization by ANT2 inhibition is mediated by the downregulation of miR-221/222, and the restoration of PTEN and the subsequent inactivation of the PI3K/Akt pathway.

We focused on the PI3K/Akt pathway as another mechanism underlying the restoration of gefitinib sensitivity mediated by ANT2 shRNA because P-Akt was much decreased by ANT2 shRNA in NSCLC cells (Fig. 3B). Because the Akt level was not altered by ANT2 shRNA (Fig. 3B), we hypothesized that ANT2 shRNA might affect the regulatory molecules in the PI3K/Akt pathway, which are representative by PTEN. An examination of the miRNA profile after ANT2 shRNA transfection in A549 cells revealed that miR-221 and 222 were most markedly downregulated (Supplementary Fig. S6), and a database search indicated that miR-221 and 222 were most markedly downregulated in H1975 and HCC827/GR cells compared with those in PC9 and HCC827 cells (Fig. 4A and B). Furthermore, transfection with miR-221 or 222 inhibitors in H1975 and HCC827/GR cells resulted in the upregulation of PTEN (Fig. 4C and D).

Moreover, ANT2 shRNA transfection significantly downregulated miR-221 and 222 levels in both H1975 and HCC827/GR cells (Fig. 4E), which was accompanied by PTEN upregulation at both the mRNA and protein levels (Fig. 4F and G). Furthermore, the transfection of H1975 and HCC827/GR cells with a dominant-negative PI3K (dnPI3K) construct suppressed cell viability, suggesting the PI3K/Akt pathway might be important for the survival of these cells (Fig. 4H). Together, these data suggested that ANT2 inhibition downregulated miR-221 and 222 expressions, subsequently restoring PTEN expression and inactivating the PI3K/Akt signaling pathway, which might contribute to the sensitization of H1975 and HCC827/GR cells to gefitinib, along with other mechanisms.

**ANT2 overexpression in NSCLC tissues from patients showing resistance to EGFR-TKI therapy**

Finally, we evaluated ANT2 expression in human NSCLC tissues by IHC to determine whether ANT2 overexpression is related to the responsiveness of NSCLC patients to gefitinib. As shown in Fig. 5A, ANT2 overexpression was more frequently observed in patients who showed poor responses to gefitinib therapy than in patients who showed excellent responses. Furthermore, ANT2 expression was frequently (4 of 7, 57%) increased in tumor tissues biopsied from patients with acquired resistance to gefitinib compared with paired tumor biopsies obtained before gefitinib treatment. However, ANT2 expression was maintained in two samples and decreased in 1 of 7 patients (Fig. 5B).

**Discussion**

This study demonstrated that ANT2 expression was upregulated in EGFR-TKI–resistant NSCLC cells and that ANT2 knockdown by ANT2 shRNA could suppress cell viability in ANT2-overexpressing NSCLC cells and restore the EGFR-TKI sensitivity of NSCLC cells with acquired resistance to EGFR-TKIs. Moreover, ANT2 shRNA was revealed to overcome EGFR-TKI resistance by

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**Figure 5.**

Expression levels of ANT2 in tumor tissues from NSCLC patients treated with gefitinib. A, ANT2 expression was determined using IHC in tumor tissues from NSCLC patients who showed good primary responses (n = 9) or poor responses to gefitinib (n = 10). The level of ANT2 IHC was presented as the IHC grade. The statistical significance in difference in the ANT2 IHC grade between good responder and poor responder was determined using unpaired Student’s t test, which was nonsignificant (ns). B, ANT2 expression was compared in paired tumor tissues obtained before gefitinib treatment (“before gefitinib”) and after acquiring resistance to gefitinib (“after gefitinib”) using IHC. Changes in IHC grade are depicted in each patient (n = 7), and representative IHC images from patient 5 are shown below. The statistical significance in the ANT2 IHC grade before gefitinib treatment and after acquiring resistance to gefitinib was determined using paired Student’s t test, which was nonsignificant (ns).

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www.aacrjournals.org Mol Cancer Ther; 15(6) June 2016 1393
various mechanisms, as illustrated in Fig. 6, including the depletion of intracellular ATP, the degradation of HSP90, the down-regulation of EGFR, and the suppression of the PI3K/Akt signaling pathway through PTEN restoration.

ANT2 mRNA expression was consistently upregulated in several cancer cells, but not in non-neoplastic cells (17, 22, 23, 29). Moreover, ANT2 mRNA was overexpressed in primary cancer tissues, including lung tumors, but not in normal tissues (22). We previously screened the ANT2 mRNA levels in various cancer cell lines, and found that lung cancer cells also over-expressed ANT2 (23). In this study, we demonstrated that ANT2 mRNA and protein expression is upregulated in EGFR-TKI–resistant NSCLC cells compared with EGFR-TKI–sensitive cells. Of note, ANT2 overexpression was observed in NSCLC cells that achieved resistance to EGFR-TKIs by variable mechanisms, including the T790M mutation and MET amplification. Furthermore, ANT2 expression was also higher in NSCLC tissues from patients who showed primary or acquired resistance to EGFR-TKIs. In this study, ANT2 upregulation in EGFR-TKI–resistant NSCLC cells was mediated by Sp1 at a transcriptional level and the activated EGFR signaling in human glioma cells was previously shown to enhance the activity of the Sp1 transcription factor (34). Thus, we speculated that persistent EGFR activation in EGFR-TKI–resistant NSCLC cells might contribute to the activation of Sp1 and its binding to the promoter region of and subsequent transcription of ANT2.

ANT2 is important for glycolytic metabolism, which is a characteristic feature of energy metabolism in many tumors (25). In addition, ANT2 plays an antiapoptotic role by maintaining the mitochondrial membrane potential (16, 17). The above-mentioned unique expression pattern and the biologic function of ANT2 suggested a protumoral role of ANT2; therefore, ANT2 was considered as a promising therapeutic target for cancer treatment. Previously, ANT2 silencing by ANT2 siRNA resulted in chemosensitization to lonidamine in HeLa cells (22). We also previously reported that ANT2 knockdown by ANT2 shRNA suppressed cell growth, proliferation, and invasion and induced apoptotic cell death in breast and liver cancer cells. Of note, this antitumor effect of ANT2 shRNA was mediated by variable mechanisms including the depletion of ATP, the repression of HSP90, the regulation of miRNA targeting signaling suppressive molecules, and the regulation of epigenetic pathways (23, 26–28, 30). ANT2 shRNA also enhanced chemosensitivity in stem cell-like breast cancer cells (29). In this study, ANT2 shRNA compromised the viability of NSCLC cells, and the degree of cytotoxicity was proportionate to the level of ANT2 expression, which suggested that a specific cytotoxic effect was elicited by ANT2 shRNA. Moreover, using gefitinib-resistant NSCLC cell lines having two most mechanism underlying acquired resistance to EGFR-TKI, that is, T790M (H1975) or MET amplification (HCC827/GR), we demonstrated that ANT2-specific shRNA overcome EGFR-TKI resistance in NSCLC.
Recently, many strategies to overcome EGFR-TKI resistance have been developed in vitro and in vivo, and several agents are undergoing clinical trials (10). Among these agents, HSP90 inhibitors have shown favorable results for sensitization to EGFR-TKIs in NSCLC; the effects of these drugs are mediated by downregulation of HSP90 client proteins including EGFR, MET, Akt, and ErbB3 (35, 36). Previously, we demonstrated that ANT2 shRNA downregulated HER2 expression in breast cancer cells, thereby exerting an antitumor effect. This effect was mediated by the ANT2 shRNA–induced depletion of intracellular ATP and the subsequent suppression of HSP90 function (27). In this study, the transfection of H1975 and HCC827/GR cells with an ANT2-specific shRNA led to ATP depletion and HSP90 downregulation, which was accompanied by EGFR suppression. Although HSP90 inhibitors such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) are known to be functional inhibitors of HSP90, reduction in HSP90 levels by 17-AAG have been reported previously (37). However, the mechanism by which ANT2 shRNA led to the suppression of HSP90 levels must be elucidated by further study. Unexpectedly, the levels of other HSP90 client proteins (Akt and MET) were not affected by ANT2 shRNA, even in MET-amplified HCC827/GR cells. However, the P-Akt level was markedly downregulated. These observations suggest that ANT2 shRNA–mediated sensitization to gefitinib cannot be fully explained by HSP90 suppression.

Meanwhile, it was observed that ANT2 shRNA markedly downregulated P-Akt expression in both gefitinib-resistant and gefitinib-sensitive cell lines. In addition, P-Akt expression was elevated in gefitinib-resistant H1975 and HCC827/GR cells compared with gefitinib-sensitive PC9 and HCC827 cells, whereas PTEN levels were lower in gefitinib-resistant cells but higher in gefitinib-sensitive cells. Furthermore, ANT2 shRNA led to the restoration of PTEN expression in H1975 and HCC827/GR cells. Thus, to identify the regulatory mechanism of PTEN by ANT2 shRNA, we performed screening for miRNAs whose expression was changed by ANT2 shRNA. By doing this, we demonstrated that ANT2 shRNA suppressed miR-221 and miR-222 with the concomitant upregulation of PTEN and the inhibition of P38/Akt signaling in the EGFR-TKI–resistant H1975 and HCC827/GR cell lines, which might underlie a mechanism associated with ANT2 shRNA–induced cell death. Consistently, P13K/Akt pathway inhibition has been suggested as one approach to overcome EGFR-TKI resistance in NSCLC (38, 39), and this inhibition might provide a rationale for ANT2 shRNA as a strategy to restore the responsiveness of EGFR-TKI-resistant cells to EGFR-TKI.

In conclusion, this study demonstrated that ANT2 overexpression might contribute to resistance to EGFR-TKIs in NSCLC and that ANT2 knockdown using ANT2 shRNA can suppress cell viability and overcome EGFR-TKI resistance in NSCLC cells by suppressing HSP90 and downregulating the P13K/Akt pathway. Thus, targeting ANT2 may be considered a novel therapeutic strategy to overcome primary or acquired resistance to EGFR-TKIs in NSCLC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: J.-Y. Jang, C.W. Kim
Development of methodology: Y.K. Jeon
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References


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Targeting Adenine Nucleotide Translocase-2 (ANT2) to Overcome Resistance to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor in Non–Small Cell Lung Cancer

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