Abstract. Background/Aim: We have reported that embryonic stem cell-expressed Ras (ERas) is expressed in human gastric cancer and is associated with its tumorigenicity. Here, we asked whether ERas plays a role in resistance to chemotherapy in gastric cancer. Materials and Methods: To assess the cytotoxicity of chemotherapeutic agents, ERas-overexpressing human gastric cancer GCIY cells were exposed to anticancer agents, including CPT-11 and inhibitor of mammalian target of rapamycin (mTOR). We also investigated the mechanisms by which ERas induces chemoresistance. Results: ERas-overexpressing clones were significantly more resistant to CPT-11 than were the control (p<0.001). Administration of rapamycin was significantly cytotoxic to the ERas-overexpressing clones compared with the control (p<0.01). Electrophoresis mobility shift assay revealed that ERas enhanced nuclear factor (NF)-κB activity. PCR array demonstrated that ERas up-regulated several multidrug efflux transporter genes, including ABCG2. Conclusion: ERas induces chemoresistance to CPT-11 via activation of phosphatidylinositol-3 kinase-protein kinase β (mTOR) pathway and NF-κB, and consequently results in up-regulation of ABCG2.

Several chemotherapy regimens have been clinically demonstrated to prolong survival of gastric cancer patients and improve patients’ quality of life (1-3). However, it remains difficult to achieve sufficient chemotherapeutic responses in the majority of patients with gastric cancer. Resistance to chemotherapy is one of the primary factors accounting for this. With a few exceptions, gastric tumors exhibit marked resistance to currently available pharmacological approaches. Moreover, most of them develop chemoresistance during treatment. Although many mechanisms, such as drug efflux, cell growth signaling and cell cycle control, have been suggested to play important roles in the resistance of gastric cancer cells to chemotherapeutic agents (4-6), the detailed molecular mechanisms involved have not been fully elucidated. Therefore, it is crucial to identify novel molecular mechanisms underlying the chemoresistance of gastric cancer which will help in the development of more effective therapeutic strategies.

ERas, a novel member of the Ras protein family, is expressed in embryonic stem cells (ESCs) and plays a crucial role in their tumor-like growth properties (7). Previous research has shown that ERas mRNA is expressed not only in ESCs, but also in several cancer cell lines, including colorectal cancer, pancreatic cancer, and breast cancer, revealing that ERas gene transcription is regulated by epigenetic silencing (8). Recently, we reported that ERas is expressed in gastric cancer and promotes activation of the phosphoinositide 3-kinase (PI3K)/Protein kinase β (AKT) pathway, anchorage-independent growth, and epithelial mesenchymal transition (EMT) (9). These features of ERas imply its relation with chemoresistance because activation of the PI3K/AKT pathway and the acquisition of EMT have been reported to be related to chemoresistance (10-13).

In this study, we examined the role of ERas in chemoresistance to anticancer agents used for clinical treatment of gastric cancer patients and the mechanisms of ERas-related resistance to Camptothecin-11 (CPT-11). In addition, we investigated the efficacy of inhibiting mammalian target of rapamycin (mTOR), a downstream effector of the PI3K/AKT pathway in gastric cancer cells expressing ERas.
Materials and Methods

Cell culture and transfection. The gastric cancer cell line, GCIY (RIKEN, Saitama, Japan) was cultured in Eagle’s minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 15% fetal bovine serum (FBS) under an atmosphere of 5% CO2 at 37°C. We established stable transfectants of GCIY using ERas expression plasmid for ERas, pCAG-hERas, or empty pCAG-IP plasmid as previously described (14).

Total RNA isolation and real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted with the Aurum total RNA fatty and fibrous tissue kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. Reverse transcription was carried out using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA) according to the manufacturer’s instructions. The synthesized cDNA from each sample was subjected to real-time quantitative RT-PCR. TaqMan® Gene Expression Assays for ATP-binding cassette sub-family G member 2 (ABCG2) (Hs00184979_g1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) were purchased from Applied Biosystems and real-time quantitative RT-PCR analyses were performed in triplicate using Applied Biosystems ABI Prism 7500 according to the supplier’s recommendations. The housekeeping gene GAPDH was chosen as an endogenous control to normalize the expression data for each gene.

Gene array analysis. Target genes of chemoresistance were identified by comparing mRNA expression between GCIY cells transfected with empty vector and GCIY cells overexpressing ERas using RT2 Profile PCR Array System containing drug resistance-related genes (PAHS-004; SABiosciences, Frederick, MD, USA). Row data were normalized using PCR Array analyzed software (SABiosciences).

Immunoblots. Cells were lysed in a lysis buffer (Cell Signaling, Beverly, MA, USA) on ice for 15 min followed by sonication. An equal volume of 2× sample buffer [0.1 M Tris/HCl, 4% w/v sodium dodecyl sulfate (SDS), 20% glycerol, and 100 mM dithiothreitol, pH 6.8] was added. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% polyacrylamide gels. PAGE-separated proteins were electrophoretically transferred onto nitrocellulose membranes (GE Healthcare Bio-sciences, Piscataway, NJ, USA). The membrane filters were blocked with 5% powdered milk in 0.1% Tween 20, 20 mM Tris-HCl, 137 mM NaCl pH 7.6 (TBS-T) for 1 h and then incubated in rabbit anti-ERas antibody (gift from Dr. Yamanaka, Department of Stem Cell Biology, Kyoto University, Institute for Frontier Medical Sciences) diluted 1:1000, rabbit anti-AKT antibody diluted 1:3000, rabbit anti-phospho-AKT antibody diluted 1:1000, rabbit anti-phospho-p70S6k antibody (Cell Signaling, Danvers, MA, USA) diluted 1:1000, rabbit anti-phospho-mTOR antibody (Cell Signaling) diluted 1:1000, anti-Topo I antibody (Topogen, Inc., USA) diluted 1:2000, or mouse anti-p-actin antibody (Sigma-Aldrich) diluted 1:2000 in 0.1% powdered milk in TBS-T at 4°C overnight, and then with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:3000; Cell Signaling) for 1 h at room temperature. Antigens on the membrane were detected with enhanced chemiluminescence detection reagents (GE Healthcare Bio-sciences).

Growth inhibition assay. Cells were seeded into 96-well plates at a density of 5×10³ cells/well in medium overnight and then treated with cis-diaminedichloroplatinum(II) (CDDP) (30 μM), CPT-11 (100 μM), paclitaxel (100 μM), 5-Fluorouracil (5-FU) (7700 μM), or etoposide (100 μM) for 48 h. Cell viability after treatment with anticancer agents was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfoophenyl)-2H-tetrazolium (MTS) assays (Promega Corp., Madison, WI, USA) according to the manufacturer’s instructions. The IC50 values for CDDP, paclitaxel, and etoposide were defined as the drug concentration at which half of the maximum growth inhibition was achieved and was determined using GraphPad PRISM4 software (La Jolla, CA, USA).

Casparase-Glo™ 3/7 assay. For estimation of apoptosis induced by CPT-11, we measured activation of caspase-3 using the substrate Asparagine – Glutamic acid – Valine - Asparagine (DEVD)-aminoluciferin from Caspase-Glo™ 3/7 assay kit (Promega Corp., Madison, WI, USA) according to the manufacturer’s recommendations.

Electrophoretic mobility gel shift assay (EMSA). Nuclear protein were extracted from cells using NE-PER nuclear and cytoplasmic extraction regents (Pierce, France) according to the manufacturer’s recommendation. Nuclear extract was used for EMSA of nuclear factor (NF)-κB activation. EMSA was performed with the Light Shift™ Chemiluminescent EMSA kit (Pierce). Briefly, 5′-end labeled NF-κB probes with biotin (20 fmol), 10 μg nuclear extracts of cells, and 20 μl binding reaction system consisting of 10× binding buffer 2 μl, and 1 μl each of 50% glycerol, 100 mM MgCl2, 1 μg/μl poly (dI:dC) and 1% NP-40 were incubated for 20 min at 4°C. Products of binding reactions were separated in 5% polyacrylamide gel with 0.5× Tris-borate EDTA (TBE) and then transferred to nylon membranes with positive charge. After the transferred DNA was cross-linked to the nylon membranes, the subsequent steps were performed using the Light Shift™ Chemiluminescent EMSA kit according to the manufacturer’s protocol. The specificity of NF-κB binding was determined in competition reactions in which 200-fold molar excess of unlabeled NF-κB DNA probe was added to the assay.

Statistical analysis. Unpaired Student’s t-test and Fisher’s exact test were used to analyze the data. The level of significance was set at 5%, using two-sided analysis.

Results

ERas enhances chemoresistance to CPT-11. We examined the effect of ERas on chemoresistance to five anti-cancer agents usually used for treatment of gastric cancer patients including CDDP, CPT-11, paclitaxel, 5-FU, and etoposide. GCIY cells overexpressing ERas cells showed significant resistance to CPT-11 compared to control cells (Figure 1A). However, ERas did not enhance resistance to CDDP, paclitaxel, 5-FU, and etoposide. Table I shows the IC50 values for CPT-11, CDDP and etoposide. Expression of ERas resulted in about 0.5-fold decrease in potency of CPT-11. Next, we estimated apoptosis induced by CPT-11 by Caspase-Glo™ 3/7 assay. Caspase-3 activity of cancer cells after 48 h treatment with CPT-11 was significantly higher in control cells than in gastric cancer cells expressing ERas.
Rapamycin inhibits growth of gastric cancer cells expressing ERas. We previously reported that ERas activates the PI3K/AKT pathway but does not enhance the mitogen-activated protein kinase (MAPK) pathway in gastric cancer (14). Here, first we examined whether ERas activates mTOR and p60S6K, a downstream effector of mTOR, by Western blot. ERas enhanced the phosphorylation of AKT, mTOR and p60S6K in gastric cancer (Figure 2A). Next, we investigated the effects of mTOR inhibition by rapamycin. Rapamycin significantly inhibited growth of gastric cancer cells expressing ERas compared with control cells (Figure 2B). In contrast, there was no significant differences in the effect of U0126, an inhibitor of MEK 1/2, between gastric cancer cells expressing ERas and control cells (Figure 2C).

Discussion

Our study demonstrated that gastric cancer cells expressing ERas are resistant to CPT-11, a commonly used chemotherapeutic agent for gastric cancer, and are susceptible to rapamycin, an inhibitor of mTOR.

It is well known that aberrant Ras promotes not only malignant transformation and cell proliferation but also resistance to chemotherapeutic agents. Constitutive expression

**Table 1. Anticancer drug concentrations for 50% cell survival (IC50) of GCIY cells with ERas construct and control cells (empty vector).**

<table>
<thead>
<tr>
<th>Anti cancer agent</th>
<th>Mean±S.D. (μM)</th>
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<tbody>
<tr>
<td></td>
<td>Empty vector</td>
</tr>
<tr>
<td>CPT-11</td>
<td>83.5±8.6</td>
</tr>
<tr>
<td>CDDP</td>
<td>13.9±3.4</td>
</tr>
<tr>
<td>Etoposide</td>
<td>19.4±2.4</td>
</tr>
</tbody>
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*p<0.05 (vs. control).
of the c-H-Ras oncogene suppressed doxorubicin-induced apoptosis in a rat rhabdomyosarcoma cell line (15), and activated N-Ras significantly contributes to chemoresistance of a human melanoma cell line (19). We have previously reported that ERas promotes tumorigenicity of gastric cancer through activation of the PI3K/AKT pathway and its expression is associated with gastric cancer metastasis (14). In our recent study, we also demonstrated that the ERas/P13K/AKT pathway may provide resistance to chemotherapy and promote transforming activity in neuroblastoma (14). However, it is not known whether ERas plays a role in chemoresistance of gastric cancer. Ras-
Figure 3. ERas enhances NF-κB DNA-binding activity and expression of ABCG2. A: Nuclear extracts were prepared from GCIY cells transfected with empty vector and ERas-overexpressing GCIY clones treated with CPT-11 for 1, 3, and 6 h and NF-κB DNA-binding activity was evaluated by EMSA. *Positive control with unlabelled NF-κB probe. **Positive control. B: Real-time quantitative RT-PCR of ABCG2 mRNA levels in ERas-overexpressing GCIY clones relative to GCIY cells transfected with empty vector. Columns represent the means of three independent experiments; bars, SD. *p<0.01 vs. control. C: Western blotting of total lysates of GCIY cells transfected with empty vector and ERas-overexpressing GCIY clones with anti-topoisomerase I. The cell lysates were probed for β-actin to control for equal protein loading.

Table II. Expression profiles of genes related to drug resistance that were changed by ERas expression.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>GenbankID</th>
<th>Fold change</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCC3</td>
<td>NM_003786</td>
<td>2.59</td>
<td>ATP-binding cassette, sub-family C (CFTR/MRP), member</td>
</tr>
<tr>
<td>ABCC6</td>
<td>NM_001171</td>
<td>2.59</td>
<td>ATP-binding cassette, sub-family C (CFTR/MRP), member 6</td>
</tr>
<tr>
<td>ABCG2</td>
<td>NM_004827</td>
<td>2.58</td>
<td>ATP-binding cassette, sub-family G (WHITE), member 2</td>
</tr>
<tr>
<td>BRCA2</td>
<td>NM_000059</td>
<td>–3.62</td>
<td>Breast cancer 2, early onset</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>NM_000777</td>
<td>4.02</td>
<td>Cytochrome P450, family 3, subfamily A, polypeptide 5</td>
</tr>
<tr>
<td>ERBB3</td>
<td>NM_001982</td>
<td>–8.44</td>
<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)</td>
</tr>
<tr>
<td>RARB</td>
<td>NM_000965</td>
<td>3.49</td>
<td>Retinoic acid receptor, beta</td>
</tr>
<tr>
<td>SULT1E1</td>
<td>NM_005420</td>
<td>4.74</td>
<td>Sulfortransferase family 1E, estrogen-preferring, member 1</td>
</tr>
<tr>
<td>TNFRSF11A</td>
<td>NM_003839</td>
<td>–4.52</td>
<td>Tumor necrosis factor receptor superfamily, member 11a, NFkB activator</td>
</tr>
</tbody>
</table>
mediated chemoresistance might be associated with two of the major downstream pathways, including the mitogen-activated protein kinase (MAPK) pathway and the PI3K/AKT pathway. In this study, we demonstrated that ERαs expression in gastric cancer cells results in the promotion of resistance to cell death from CPT-11. Selective activation of the PI3K/AKT pathway is a unique characteristic of ERαs (7). PI3K/AKT pathway is considered a crucial intracellular signal transduction pathway involved in chemoresistance of gastric cancer (12, 17), and inhibition of this pathway enhances cytotoxicity of CPT-11 (18, 19). Therefore, we speculate the activation of the PI3K/AKT pathway may be one mechanism that accounts for resistance to CPT-11 induced by ERαs.

mTOR, which is located downstream of AKT, has been reported to be frequently activated in gastric cancer and its expression plays an important role in gastric cancer development and progression (20, 21). There are several reports about the possibility of mTOR as a new molecular target for cancer therapy (22-25), and a recent phase II clinical trial in Japan provided evidence that treatment with inhibitor of mTOR, everolimus, shows efficacy and safety in pretreated patients with advanced gastric cancer (26). In our current study, we showed that ERαs activates the AKT/mTOR/p70S6K signal transduction pathway, and that rapamycin significantly inhibits proliferation of GCIY cells expressing ERαs compared with control cells. In addition, we demonstrated the significant effect of rapamycin in combination with CPT-11 for gastric cancer expressing ERαs. Although rapamycin did not completely inhibit chemoresistance to CPT-11 induced by ERαs, these results indicate that ERαs expression might be a molecular marker predicting sensitivity to rapamycin in gastric cancer.

The transcription factor NF-κB is a protein complex that controls the transcription of DNA and is involved in the regulation of cell survival. NF-κB is activated in many types of malignant tumor and plays a role in the resistance to chemotherapeutic agents (27). Previously, others have reported inhibition of NF-κB enhances chemosensitization to CPT-11 (28-30). In our study, we revealed that ERαs expression in gastric cancer resulted in canonical NF-κB DNA-binding activity. Furthermore, NF-κB was more activated in gastric cancer cells expressing ERαs compared to control cells at all time points when cells were exposed to CPT-11. This indicates that activation of NF-κB induced by ERαs is associated with resistance to CPT-11.

To investigate other mechanisms whereby ERαs enhances resistance to CPT-11, we sought ERαs target genes contributing to chemoresistance by PCR array. PCR array showed that ERαs up-regulates expression of multidrug-resistance associated genes, including ABCG2 (Table II). ABCG2 is a member of the ATP-binding cassette transporter family of transmembrane, substrate-effluxing proteins. Expression of ABCG2 in cancer cells causes chemoresistance by actively effluxing anticancer drugs including CPT-11 (31-33). Cancer cells expressing ABCG2 identified using fluorescence-activated cell sorter are called side population (SP), which have some intrinsic stem cell properties (34, 35). SP cells are also recognized as so-called cancer stem cells (CSCs) in some tumors (24, 36-38). CSCs have been identified in a wide variety of human solid cancers, including gastric cancer, as a fraction of cells, which are capable of reproducing the human cancer in mouse models and which are resistance to tumor therapies, including radiotherapy and chemotherapy (39-42). Yashiro et al. showed the SP fraction was significantly higher in ERαs-positive gastric cancer cells than in ERαs-negative cells, and concluded that ERαs might be a putative molecule responsible for CSC-like characteristics in gastric cancer (43). Our finding that ERαs up-regulates ABGC2 expression and enhances chemoresistance supports their hypothesis.

In conclusion, we demonstrated that ERαs expression is associated with resistance to CPT-11, and rapamycin enhances the therapeutic efficacy of CPT-11 for gastric cancer expressing ERαs (Figure 4). Although further work is need to clarify the mechanisms whereby ERαs enhances chemoresistance to CPT-11, ERαs might be a potential maker to predict efficacy of CPT-11, and the combination therapy of CPT-11 and rapamycin may improve outcomes of gastric cancer patients.

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