Targeting Src in Mucinous Ovarian Carcinoma

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Abstract

Purpose: Mucinous ovarian carcinomas have a distinct clinical pattern compared with other subtypes of ovarian carcinoma. Here, we evaluated (i) stage-specific clinical significance of mucinous ovarian carcinomas in a large cohort and (ii) the functional role of Src kinase in preclinical models of mucinous ovarian carcinoma.

Experimental Design: A total of 1,302 ovarian cancer patients including 122 (9.4%) cases of mucinous carcinoma were evaluated for survival analyses. Biological effects of Src kinase inhibition were tested using dasatinib-based therapy in a novel orthotopic mucinous ovarian cancer model (RMUG-S-ip2).

Results: Patients with advanced-stage mucinous ovarian cancer had significantly worse survival than those with serous histology: median overall survival, 1.67 versus 3.41 years, $P = 0.002$; median survival time after recurrence of 0.53 versus 1.66 years, $P < 0.0001$. Among multiple ovarian cancer cell lines, RMUG-S-ip2 mucinous ovarian cancer cells showed the highest Src kinase activity. Moreover, oxaliplatin treatment induced phosphorylation of Src kinase. This induced activity by oxaliplatin therapy was inhibited by concurrent administration of dasatinib. Targeting Src with dasatinib in vitro showed significant antitumor effects in the RMUG-S-ip2 model but not in the serous ovarian carcinoma (SKOV3-TR) model. Combination therapy of oxaliplatin with dasatinib further showed significant effects on reducing cell viability, increasing apoptosis, and in vivo antitumor effects in the RMUG-S-ip2 model.

Conclusions: Our results suggest that poor survival of women with mucinous ovarian carcinoma is associated with resistance to cytotoxic therapy. Targeting Src kinase with a combination of dasatinib and oxaliplatin may be an attractive approach for this disease. Clin Cancer Res; 17(16); 5367–78. ©2011 AACR.

Introduction

In 2010, more than 21,000 women in the United States were diagnosed with ovarian carcinoma and almost 14,000 died from this disease, which ranks as the most common cause of death among gynecologic malignancies (1). The majority of ovarian carcinomas are of serous histology. Mucinous histology is relatively rare, accounting for 2% to 10% of all subtypes of epithelial ovarian carcinomas (2, 3). Because of its relatively rare incidence, mucinous ovarian carcinomas are understudied but are thought to have poorer response to taxane and platinum chemotherapy, resulting in poor survival outcomes compared with serous ovarian carcinomas (4–7). Therefore, understanding the mechanisms contributing to mucinous ovarian cancer development and progression as well as novel therapeutic approaches are urgently needed.

Recent evidence suggests that mucinous ovarian carcinoma is histologically and molecularly similar to colorectal carcinomas and has a distinct clinical pattern compared
Translational Relevance

Recent studies have shown that mucinous ovarian carcinomas have a distinct clinical pattern compared with other subtypes of ovarian carcinomas. The first part of our study has shown poorer survival outcome in mucinous ovarian carcinoma than in serous ovarian cancer, the most common subtype of ovarian carcinoma, in a large sample size. Genomic approaches using mucinous ovarian cancer cell lines pointed to the Src kinase being involved in many of the activated pathways. In validation studies, Src was found to be highly activated in mucinous ovarian cancer. The mucinous ovarian cancer cells were resistant to most chemotherapy drugs. An Src inhibitor dasatinib, inhibited oxaliplatin-induced Src activation and enhanced antitumor effects of oxaliplatin in both in vitro and in vivo models of mucinous ovarian carcinoma. These findings implicate Src as a critical therapeutic target in mucinous ovarian carcinoma.

with other subtypes of ovarian carcinomas (8, 9). The majority of colorectal carcinomas are of mucinous histology, and patients with advanced-stage colorectal carcinoma are treated with chemotherapy containing oxaliplatin, a third-generation platinum compound (10). Despite proven efficacy, resistance to oxaliplatin is a rising issue (11). Among proposed mechanisms, Src kinase has been shown to play an important role in oxaliplatin resistance in colorectal and pancreatic carcinomas (12, 13). Src kinase is a nonreceptor tyrosine kinase that regulates various aspects of tumor progression via multiple signaling pathways, including cell survival (AKT), growth (Ras/MEK/ERK), metastasis (FAK/paxillin/c-Jun), and angiogenesis (STAT3/VEGF; ref. 14). Src kinase is known to be overexpressed in colorectal, pancreatic, lung, breast, and prostatic carcinomas (15) and is thought to contribute to chemotherapy resistance (13). In serous ovarian carcinoma, Src kinase was reported to be overexpressed in advanced-stage disease (16). However, the role of Src kinase in mucinous ovarian carcinoma is not known and was examined in this study.

Here, we show that (i) advanced-stage mucinous ovarian carcinoma was associated with shorter survival time after progression or recurrence of disease than with serous histology in a large cohort study, (ii) Src kinase is highly activated among mucinous ovarian carcinomas, and (iii) targeting Src kinase with a combination of oxaliplatin and dasatinib showed synergistic antitumor effects in mucinous ovarian cancer models.

Materials and Methods

Clinical data

A total of 1,302 ovarian cancer patients from cancer centers in the United States and Japan were evaluated; 122 mucinous carcinoma patients (fourth common, 9.4 ± 0.8%) were compared with 698 serous carcinoma patients (most common, 53.4 ± 1.4%) for survival. Patient age, preoperative cancer antigen 125 (CA-125) value, histologic subtypes, International Federation of Gynecology and Obstetrics (FIGO) stage, grade, and cytoreduction were evaluated to determine potential impact on survival. Early- and advanced-stage diseases were defined as FIGO stages I/II and III/IV, respectively. Appendectomy is generally carried out as a standard surgical procedure for mucinous ovarian carcinoma in the participating institutions. Gynecologic pathologists at each institution reviewed all of the specimens for assessing histology. Institutional Review Board approval was obtained at each institution.

Cell lines and cultures

RMUG-S and RMUG-L were cultured in RPMI-1640 media, supplemented with 10% FBS and 0.1% gentamicin at 37°C in 5% CO2 with 95% air. These cell lines were originally isolated from women with mucinous ovarian carcinoma (17). Ovarian serous carcinoma cell lines (HeyA8, HeyA8-MDR, SKOV3-ip1, and SKOV3-TR), clear cell carcinoma cell lines (ES-2 and RMG-2), and undifferentiated carcinoma cell lines (A2780 and A2780-CP20) were cultured in RPMI-1640 media supplemented with 15% FBS and 0.1% gentamicin at 37°C in 5% CO2 with 95% air. In vitro experiments were conducted with 80% cell confluence. All the cell lines were purchased from American Type Culture Collection or Japanese Collection of Research Bioreresources.

Drugs and reagents

Anti-Src (#2108) and anti-phospho-Src (Tyr419; #2101) antibodies for Western blotting were purchased from Cell Signaling Technology. Anti-phospho-Src antibody (Tyr419; AF2685) for immunohistochemical staining was purchased from R&D Systems. Anti-CD31 (#53370) and anti-Ki67 (CP2498) antibodies were purchased from BD Pharmingen and BioCare Medical, respectively. Anti-vinculin (V9131) and anti-β-actin (A5316) antibodies were purchased from Sigma-Aldrich. Oxaliplatin (Weyth) was purchased from the institutional pharmacy. Dasatinib (Bristol-Myers Squibb) was prepared as a 20 mmol/L stock solution in dimethyl sulfoxide (DMSO). Paclitaxel, cisplatin, carboplatin, doxorubicin, and topotecan were purchased from Sigma-Aldrich. Etoposide was purchased from EMD4 Biosciences.

Microarray analysis

Gene expression was compared between mucinous (RMUG-S and RMUG-L) and serous (SKOV3, OVCA5, 420, 429, 432, and 433) ovarian cancer cell lines. Pathway analysis was used to assess significant genes in mucinous compared with serous ovarian carcinoma (Ingenuity Pathway Analysis, version 7.5; ref. 18; GeneChip Human Genome U133 Plus 2.0 Array). Significant gene network was plotted (both direct and indirect relationships) using the cutoff value of ±1.5-fold change. Src kinase–specific pathway analysis was carried out and was...
shown as functional gene network ontology. Extraction of networks of molecular interactions for each data set was conducted using NetWalk, a random walk-based network retrieval algorithm (19), and NetWalk-mediated analyses and visualizations were carried out using NetWalker, an integrated platform for network-based data analyses and visualization.

**Apoptosis assay**

For evaluation of apoptosis, PE Annexin V Apoptosis Detection Kit I (BD Pharmingen) was used as described previously (20). Briefly, 5 × 10^5 RMUG-S-ip2 cells with serum-containing medium were plated in 10-cm plates and incubated for 24 hours. Then, the media was replaced with fresh serum-free medium. Treatment was started with control, oxaliplatin, dasatinib, and oxaliplatin with dasatinib for time periods ranging from 24 to 96 hours. Cell morphology was assessed by phase-contrast microscopy. Then, cells were removed from plate by trypsin-EDTA, washed twice with PBS, and resuspended with binding buffer at 10^6 cells/mL. FITC Annexin V and propidium iodide were added (each at 5 μL/10^6 cells). Cells were incubated for 15 minutes at room temperature in the dark. Percentage of apoptosis was analyzed with an EPICS XL flow cytometer (Beckman-Coulter). Each sample was analyzed in triplicate.

**Cytotoxicity assays**

Cytotoxicity of oxaliplatin, dasatinib, and oxaliplatin with dasatinib treatment in RMUG-S-ip2 cells was assessed with MTT uptake assay (Sigma-Aldrich) as described previously (20). Briefly, 2 × 10^5 RMUG-S-ip2 or SKOV3-TR cells with serum-containing medium were plated in each well of a 96-well plate and incubated for 24 hours. Then, the media was replaced with fresh serum-free medium. Treatment in each condition was stopped at 48-, 72-, and 96-hour time points, and cells were removed from the plate by trypsin-EDTA, washed twice with PBS, and fixed with 70% ethanol (4°C overnight). Ethanol was removed and incubated with propidium iodide for 10 minutes. Cell-cycle analysis was carried out with an EPICS XL flow cytometer (Beckman-Coulter). Each sample was analyzed in triplicate.

**Western blotting**

Preparation of lysates from cultured cells was carried out as previously described (23, 24). Briefly, cells with 80% confluence were harvested and lysed in modified radioimmunoprecipitation (RIPA) assay buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 0.5% deoxycholate, 25 μg/mL leupeptin, 10 μg/mL aprotinin, 2 mmol/L EDTA, and 1 mmol/L sodium orthovanadate) as described previously (24). Protein concentrations were measured with BCA Protein Assay Reagent Kit (Pierce Biotechnology), and 50 μg of lysate protein was mixed with 10% SDS-PAGE gels transferred electrophoretically onto a nitrocellulose membrane. Nonfat milk powder (5%) in TBS-T [10 mmol/L Tris (pH 8), 150 mmol/L NaCl, and 0.05% Tween-20] was used for protein block for 1 hour. The blots were incubated with anti-Src (band at 60 kDa) and anti-phospho-Src (60 kDa) antibodies at dilutions of 1:1,000 for 4°C overnight and washed with TBS-T. Antibody binding was probed by incubating the blots with horseradish peroxidase–conjugated goat anti-rabbit antibodies (GE Healthcare) in 5% nonfat milk powder diluted with TBS-T. Antibody binding was probed by incubating the blots with horseradish peroxidase–conjugated goat anti-rabbit antibodies (GE Healthcare) in 5% milk diluted with TBS-T for 1 hour at room temperature. Reactivity was visualized with an enhanced chemiluminescence detection kit (Pierce Biotechnology). Anti-vinculin (120 kDa) or anti-β-actin (42 kDa) was used to evaluate an equal protein loading. Densitometry (Image); NIH) was used to interpret the difference in the results of Western blot.

**Immunohistochemistry**

Immunohistochemical analysis for phospho-Src (Tyr419) and Src kinase was evaluated for human tissue samples embedded in paraffin blocks. For mouse tissues, phospho-Src, Src kinase, and Ki67 were evaluated using formalin-fixed paraffin-embedded tumors. Optimal cutting temperature (OCT) compound-fixed tumor samples were used for CD31 staining. Briefly, the paraffin-embedded block was sectioned to 5-μm thickness and deparaffinized (60°C overnight) and rehydrated. Antigen retrieval was done using the pretreatment reagent Borg Decloaker (BioCare Medical) with pressure cooker for anti-phospho-Src and anti-Src antibodies or Diva (BioCare Medical) with steamer for anti-Ki67 antibody, respectively. For CD31 staining, sections were done on freshly cut frozen slides. These were fixed in cold acetone, and no antigen retrieval was necessary. Endogenous peroxidase and non-specific epitopes were blocked with 3% H2O2 (Fisher Scientific) in PBS for 12 minutes at room temperature, nonspecific protein binding was blocked with 5% normal

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**Cell-cycle analysis**

Proportions of cells in G1, S, and G2 phases were evaluated following treatment with either oxaliplatin, dasatinib, or oxaliplatin with dasatinib and compared with untreated controls. Briefly, 5 × 10^5 RMUG-S-ip2 cells with serum-containing medium were plated in a 10-cm dish and incubated for 24 hours. Then, the media was replaced with fresh serum-free medium. Treatment in each condition was stopped at 24-, 48-, 72-, and 96-hour time points, and cells were removed from the plate by trypsin-EDTA, washed twice with PBS, and fixed with 70% ethanol (4°C overnight). Ethanol was removed and incubated with propidium iodide for 10 minutes. Cell-cycle analysis was carried out with an EPICS XL flow cytometer (Beckman-Coulter). Each sample was analyzed in triplicate.
horse serum and 1% normal goat serum for anti-Ki67 and anti-CD31 antibodies or 4% cold water fish skin gelatin (Electron Microscopy Science) for anti-phospho-Src or anti-Src antibodies for 20 minutes at room temperature, respectively. Sections were incubated with primary antibodies at 4°C overnight. For negative control, sections were incubated without primary antibody and with human IgG antibody (Jackson ImmunoResearch Laboratories). Goat anti-rabbit horseradish peroxidase–conjugated antibody (Jackson ImmunoResearch Laboratories) for anti-phospho-Src and anti-Src antibodies (20 minutes, room temperature) were used for secondary antibody, respectively. Signal was visualized after incubating with 3,3′-diaminobenzidine (Phoenix Biotechnologies) and counterstaining with Gill’s no. 3 hematoxylin (Sigma-Aldrich).

**Animal care**

Nude mice (8–12 weeks old) were purchased from the National Cancer Institute (NCI)/Frederick Cancer Research and Development Center (athymic female, Ncr-nu). The mice were quarantined, housed, and maintained under specific pathogen-free environment in the animal facility that is approved by the American Association for Accreditation of Laboratory Animal Care in agreement with the current regulations and standards of United States Department of Agriculture, Department of Health and Human Service (DHHS), and NIH. Approval of the study protocols was obtained and supervised by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center.

**In vivo therapeutic experiment**

In *in vivo* model of mucinous ovarian carcinoma (RMUG-S-ip2) was created for the experiment (Supplementary Methods) and the characteristics are shown in Supplementary Figure S1. RMUG-S-ip2 cells were injected into the peritoneal cavity of 40 orthotopic nude mice (4 x 10^6 cells/mouse). After randomization into 4 groups of 10 mice (control, oxaliplatin alone, dasatinib alone, and oxaliplatin with dasatinib), treatment was initiated at 4 weeks following injection. Oxaliplatin (5 mg/kg/mouse) was given intraperitoneally (i.p.) twice weekly after being dissolved in 5% dextrose and diluted with HBSS (13). Dasatinib (15 mg/kg/d/mouse) was given orally every day after being solubilized in citrate/citric acid buffer. Control mice received HBSS i.p. twice a week and citrate buffer orally every day. Mice were monitored on a daily basis and weighted weekly. After 8 weeks of treatment, the mice were sacrificed and total body weight of mouse, tumor locations and weight, and the number of tumor nodules were recorded. Tumor samples were fixed with 10% formalin and embedded in paraffin or with OCT compound in liquid nitrogen. A similar experiment was conducted with 25% drug dose reduction for both oxaliplatin and dasatinib in the RMUG-S-ip2 model. In addition, an experiment with the SKOV3-TR model (1.25 x 10^6 cells/mouse) was carried out (mice were randomized into 4 groups as noted earlier and treatment was started 1 week after tumor cell injection and continued for 5 weeks).

**Statistical analysis**

Continuous variables were assessed for normal distribution (Kolmogorov–Smirnov test) and expressed as appropriate (mean ± SD (or SE) or median with range). Student’s *t* test or the Mann–Whitney *U* test was conducted to determine the statistical significance. Categorical variables were evaluated with Fisher’s exact test (OR and 95% CI). For clinical data analysis, to determine the significance of variables for the survival outcomes, such as progression-free survival (PFS), overall survival (OS), and survival time after progression of disease or recurrence, univariate (log-rank test) and multivariate (Cox regression proportional hazard test) analyses were carried out as appropriate. Survival curves were estimated with the Kaplan–Meier method. *P* values of less than 0.05 were considered as statistically significant (all, 2-tailed). The Statistical Package for Social Scientists software (SPSS; version 18.0) was used for all analyses.

**Results**

**Mucinous ovarian carcinomas have distinct clinical characteristics**

We first examined the clinical features of mucinous ovarian carcinoma compared with serous subtypes. The average age of women with a diagnosis of mucinous carcinoma was significantly less than those with serous carcinoma (mean age, 54.1 ± 14.8 vs. 59.9 ± 11.3, *P* = 0.005). Distribution of FIGO stages among women with mucinous carcinomas was also significantly different from those with serous histology; majority of mucinous cases were early-stage carcinomas, whereas majority of serous cases were advanced-stage carcinomas (*P* < 0.0001; Fig. 1A). CA-125 is a common biomarker for ovarian cancer used for diagnosis or follow-up after therapy. In women with mucinous ovarian carcinomas, CA-125 was infrequently elevated compared with those with serous carcinoma (proportion of elevated CA-125 >35 IU/L in all stage cases, 66.7% vs. 96.0%, OR 0.56, 95% CI 0.38–0.83, *P* < 0.0001; Fig. 1B). Same results were noted in advanced-stage carcinomas (78.6% vs. 97.4%, OR 0.1, 95% CI 0.02–0.43, *P* = 0.009). Among cases with elevated CA-125 (>35 IU/L), mucinous carcinoma patients showed lower CA-125 values than those with serous carcinoma (median 229 vs. 475 IU/L, *P* < 0.0001; Fig. 1B). In multivariate analysis adjusted for other significant variables, such as age, cytoreduction, and stage, mucinous histology remained an independent risk factor for survival of ovarian cancer patients (OS, *P* = 0.045; survival time after recurrence, *P* < 0.0001). In early-stage disease, mucinous carcinoma patients showed better survival outcomes than those with serous carcinoma (10-year PFS, 78.7% vs. 66.6%; *P* = 0.039; 10-year OS, 89.5% vs. 53.6%, *P* = 0.086; Fig. 1C). This difference likely represents the lower frequency of recurrence in women with...
early-stage mucinous ovarian carcinoma than among those with serous histology (12.5% vs. 30%, OR 0.33, 95% CI 0.15–0.76, \( P = 0.009 \)). In advanced-stage disease, representing 36.9% of mucinous ovarian carcinoma cases, although there was no statistically significant difference in PFS between mucinous and serous carcinoma patients (\( P = 0.92 \)), OS of mucinous carcinoma patients was significantly shorter than those with serous carcinoma (median OS, 1.67 vs. 3.41 years, \( P = 0.002 \), multivariate analysis). Similarly, survival time after progression of disease or recurrence after primary cytoreductive surgery was significantly shorter for women with mucinous carcinoma than for those with serous histology (median survival time, 0.53 vs. 1.66 years, \( P < 0.0001 \), multivariate analysis).

**Gene expression and protein activity of Src kinase in mucinous ovarian carcinomas**

Given the poor clinical outcome of women with mucinous ovarian cancer, new therapeutic approaches are needed. In search of potentially useful targets, we next evaluated gene expression in mucinous ovarian cancer cell lines (heat map shown in Supplementary Fig. S2). In pathway analysis, 41,329 genes were identified and 12,568 genes were functional and eligible for analysis. The top 5 functional networks and 10 significant genes in mucinous ovarian carcinomas, when compared with serous ovarian carcinomas, are shown in Supplementary Table S1 and Figure S3A–E, respectively. Src kinase was included in the top 3 networks for amino acid, molecular transport, and small molecular biochemistry, although Src

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**Figure 1.** Clinical significance of mucinous ovarian carcinomas. A, the proportion of FIGO stage in 122 mucinous (black bar) and 698 serous (white bar) carcinoma patients is shown. B, the proportion of patients with elevated CA-125 (>35 IU/L) and median CA-125 value among patients with mucinous or serous carcinomas is shown. C, Kaplan–Meier survival curves for PFS and OS in early-stage disease are shown. D, Kaplan–Meier survival curves for OS and survival time after progression or recurrence of disease are shown.
mRNA was not differentially expressed between mucinous and serous cancer cell lines (Supplementary Fig. S3C). These data suggest that regulation of Src would be at the protein level. Thus, we examined the phosphorylation status of Src in 12 ovarian cancer lines and 10 clinical mucinous ovarian cancer samples. Because Src kinase activity is proposed to be an important mechanism of resistance to chemotherapy (13), Src kinase–specific pathway analysis was carried out for mucinous compared with serous ovarian carcinoma cell lines (Fig. 2A). Among upregulated genes associated with Src kinase, 3 of 12 genes were cadherin and catenin family members (CDH1, CDH5, and JUP; Supplementary Table S2). Because increased expression of E-cadherin is a characteristic molecular feature of mucinous ovarian carcinoma (25), the increased activity of cadherin/catenin pathway in mucinous ovarian carcinoma may be associated with decreased Src expression in mucinous ovarian carcinoma. There were some downregulated genes associated with cell growth, adhesion, and motility in the mucinous ovarian cancer cells (ASAP1, ITGB3, RAC2, DNM1, and MYLK; Supplementary Table S2).

Next, protein expression of Src kinase was evaluated in 12 ovarian cancer cell lines and 2 nontransformed cell lines. The results were similar to the microarray analysis, and the level of total Src expression in mucinous ovarian carcinoma cell line (RMUG-S-ip2) was the lowest among tested cell lines (Fig. 2B). However, RMUG-S-ip2 showed the highest activity of Src kinase, as determined by the ratio between phospho-Src kinase and Src kinase. Expression of Src kinase [total Src and phospho-Src (Tyr419)] was also evaluated in 10 mucinous ovarian cancer samples (Fig. 2C). p-SrcY419 was localized in the cytoplasm of cancer cells, and all samples showed overexpression of Src kinase.

**Mucinous ovarian carcinomas are drug resistant**

On the basis of clinical findings suggesting relative drug resistance, we next examined the sensitivity of mucinous (RMUG-S and RMUG-L) and serous (SKOV3) ovarian cancer cell lines to various chemotherapeutic agents (Fig. 2D). Compared with serous cancer cell line, mucinous carcinoma cell lines had significantly higher IC_{50} values for all tested chemotherapeutic agents. IC_{50} doses for oxaliplatin in RMUG-S-ip2 and RMUG-L-ip2 cells were 15.6 and 16.4 μg/mL, respectively (Supplementary Fig. S4). These values are comparable with colorectal cancer cell lines reported previously (2.8–29 μg/mL; refs. 26, 27). IC_{50} level of oxaliplatin in drug resistant–cell line SKOV3-TR cells was 5.2 μg/mL (Supplementary Fig. S4).

**Oxaliplatin-induced Src kinase activity is inhibited by dasatinib**

Next, we examined the effects of oxaliplatin on Src expression and activation in the RMUG-S-ip2 and SKOV3-TR cells. Oxaliplatin induced Src phosphorylation but had no effect on total Src levels in the RMUG-S-ip2 cells (Fig. 3A). Oxaliplatin did not affect Src phosphorylation in the SKOV3-TR cells (Supplementary Fig. S5). Treatment with dasatinib downregulated the phosphorylation of Src kinase in the RMUG-S-ip2 cells (Fig. 3B). We then examined the effect of the combination of oxaliplatin and dasatinib on Src activation in RMUG-S-ip2 cells. In combination treatment, dasatinib blocked the oxaliplatin-induced increase in Src activity (Fig. 3B).

We next examined the effect of dasatinib on cell viability in the RMUG-S-ip2 and SKOV3-TR cells. RMUG-S-ip2 cells showed a dose-dependent decrease in cell viability with dasatinib (Fig. 4A). Conversely, SKOV3-TR cells did not show changes in viability with dasatinib (Fig. 4B). RMUG-S-ip2 cells were then treated with various concentrations of oxaliplatin and dasatinib (Fig. 4C). Oxaliplatin treatment alone affected cell viability of RMUG-S-ip2 cells (IC_{50}, 15.6 μg/mL), and the addition of dasatinib enhanced cytotoxic effects of oxaliplatin treatment. Isobologram analysis was carried out, and the interaction index was less than 1 at all examined points, confirming the synergistic effects of oxaliplatin and dasatinib in cell viability (Fig. 4D). Dasatinib concentration of 182 nmol/L showed a 50% reduction of oxaliplatin IC_{50}. In the SKOV3-TR cells, there was no difference in cell viability between oxaliplatin treatment alone and oxaliplatin with dasatinib (data not shown).

**Effects of combination therapy with oxaliplatin and dasatinib**

Effects of combination therapy with oxaliplatin and dasatinib were evaluated using apoptosis assays in RMUG-S-ip2 cells (Fig. 4E and Supplementary Fig. S6A). Monotherapy with either oxaliplatin or dasatinib significantly increased apoptosis compared with controls (proportion of apoptosis, control vs. dasatinib vs. oxaliplatin, 4.1 ± 0.8 vs. 8.5 ± 0.3 vs. 9.5 ± 0.5%; for both oxaliplatin and dasatinib, P < 0.01). Combination therapy with oxaliplatin and dasatinib further enhanced the extent of apoptosis compared with monotherapy either with dasatinib or with oxaliplatin (proportion of apoptosis, combination therapy vs. dasatinib vs. oxaliplatin, 26.8 ± 0.3 vs. 8.5 ± 0.3 vs. 9.5 ± 0.5%; for both oxaliplatin and dasatinib, P < 0.01). These significant effects were observed at all time points examined (24–96 hours), and the effects were maximal at the 96-hour time point. Effects of therapy on cell-cycle were also analyzed in the RMUG-S-ip2 cells (Fig. 4F and Supplementary Fig. S6B). Oxaliplatin significantly increased the proportion of cells in S-phase and decreased in both G_{1} and G_{2} phases compared with control (P < 0.001). Dasatinib treatment did not affect cell-cycle distribution. The results of the combination of oxaliplatin and dasatinib remained similar to the results of oxaliplatin treatment (P < 0.001 compared with control).

**In vivo antitumor effects of oxaliplatin and dasatinib in ovarian carcinoma**

Next, an in vivo experiment was carried out to evaluate the antitumor effects of combination therapy with oxaliplatin and dasatinib. Compared with the control group, monotherapy with oxaliplatin or dasatinib resulted in significantly smaller tumor weight (tumor weight reduction, control vs. oxaliplatin, 58.6%, P < 0.01; and control vs. dasatinib 65.8%, P < 0.01) and number of tumor metastasis.
Figure 2. Expression and activity of Src kinase in mucinous ovarian carcinomas. A, Src pathway–specific analysis with gene network ontology is shown. Expression in mucinous ovarian carcinoma cell lines was compared with serous ovarian carcinoma cell lines. B, Western blot analysis for phospho-Src and Src kinase in 14 cell lines is shown. C, immunohistochemical staining for Src kinase and phospho-Src in human mucinous ovarian carcinomas is shown (magnification 200×). H&E, hematoxylin–eosin. D, cell viability assay for 3 cell lines and 6 chemotherapeutic agents is shown.
nodules (tumor nodule reduction, control vs. oxaliplatin, 45.9%, \(P < 0.05\); and control vs. dasatinib 53.6%, \(P < 0.05\); Fig. 5A). Combination therapy with oxaliplatin and dasatinib further showed significant antitumor effects compared with either monotherapy: reduction of tumor weight, oxaliplatin versus combination, 79.1%, \(P < 0.01\); and dasatinib versus combination, 74.7%, \(P < 0.01\), respectively (Fig. 5A). Compared with controls, combination therapy resulted in 91.3% tumor weight reduction and 77.4% decrease in tumor nodule number, respectively (both \(P < 0.01\)). There were 4 of 10 mice with metastasis to liver parenchyma in the control group, but none in the treatment groups. Mice treated with dasatinib did not have any differences in body weight compared with the control group (data not shown). To evaluate whether there may be synergistic antitumor activity with oxaliplatin and dasatinib, we conducted an in vivo experiment using RMLIG-S-ip2 with 25% dose reduction of oxaliplatin and dasatinib (Supplementary Fig. S7). The results of antitumor effects in low-dose oxaliplatin and dasatinib were similar to the results of original experiment: antitumor effects of oxaliplatin alone, dasatinib alone, and combination of oxaliplatin and dasatinib were 65.4%, 60.3%, and 88.8%, respectively, when compared with the control group (all \(P < 0.05\)). These results suggest possible synergistic antitumor effects of oxaliplatin and dasatinib in mucinous ovarian carcinoma.

In contrast to mucinous ovarian carcinoma, serous ovarian carcinoma showed different antitumor effects with dasatinib treatment (Fig. 5A). Although RMLIG-S-ip2 showed significant antitumor effects with dasatinib monotherapy, there were no significant antitumor effects in the SKOV3-TR model (mean tumor weight, dasatinib vs. control, 1.09 ± 0.29 vs. 1.42 ± 0.29, \(P = 0.42\)). These in vivo effects with dasatinib monotherapy corresponded well with the effects noted in the in vitro experiments with the mucinous and serous cancer cell lines (Fig. 4A and B). Oxaliplatin alone showed significant antitumor effects in the SKOV3-TR model when compared with control (mean tumor weight, oxaliplatin vs. control, 0.48 ± 0.12 vs. 1.42 ± 0.29, \(P = 0.01\)), and the extent of tumor reduction rate with oxaliplatin therapy was similar to the RMLIG-S-ip2 model (58.6% and 65.9%, respectively).

Immunohistochemical staining was carried out on the RMLIG-S-ip2 tumor tissues obtained from the in vivo experiments to assess the effects on proliferation and microvessel density (MVD; Fig. 5B). Tumors obtained from the dasatinib treatment group showed substantially decreased p-SrcY419 expression compared with the control group. In tumors obtained from the oxaliplatin treatment group, there were areas that showed focally increased expression of p-SrcY419. This was not seen in tumors treated with oxaliplatin and dasatinib. This induction of activated Src following oxaliplatin treatment supports the in vitro findings. Numbers of positive-Ki67 cells as well as MVD were significantly decreased in the combination therapy group compared with monotherapy group (all \(P < 0.05\); Fig. 5B).

Discussion

Our clinical, in vitro, and in vivo results highlight important features that contribute to the distinct mechanisms of mucinous ovarian carcinoma pathogenesis. Targeting Src kinase with dasatinib inhibited oxaliplatin-induced Src kinase activity and showed synergistic antitumor effects in a mucinous ovarian carcinoma model. Several key areas in this notable observation deserve special mention. Mucinous ovarian carcinoma is known to be associated with poorer patient outcome than with other subtypes of ovarian carcinoma (4, 7, 8). Our results not only support previous studies but also imply that mucinous ovarian...
carcinomas are associated with slow progression and chemoresistance that may partly be explained by the hypothesis that mucinous ovarian carcinomas are genetically stable (28). To explain the distinct clinical characteristics of mucinous ovarian carcinomas, there are several molecular characteristics that can distinguish these tumors from serous carcinomas (8). Well-studied biomarkers of mucinous ovarian carcinomas include cadherin, matrix metalloproteinases, WT-1, CA-125, and carcinoembryonic antigen. In gene expression analyses with microarrays, the profile patterns among these 2 cell lines were distinctly different (8). Although mucinous ovarian carcinomas are less likely to have BRCA or p53 mutations, Kras mutations are seen with greater frequency than with serous carcinomas (8). Kras mutation, reported in nearly 40% of carcinomas, is also common in colorectal carcinoma (29). These molecular characteristics further support the premise that mucinous ovarian carcinoma is distinct not only from serous ovarian carcinomas but also from histologically and molecularly mimics colorectal carcinoma (8).

Oxaliplatin has been used for the treatment of ovarian carcinomas in various clinical trials and has shown a wide range of antitumor activity (20%–75%; refs. 12, 30). Induction of Src kinase activity via reactive oxygen species (ROS) produced during the process of oxaliplatin–DNA adduct formation has been proposed as one of the mechanisms associated with oxaliplatin resistance in colorectal carcinoma (13). Thus, dasatinib holds potential

Figure 4. In vitro effects of combination therapy with oxaliplatin and dasatinib in mucinous ovarian carcinoma. A and B, cell viability assays for dasatinib treatment in RMUG-S-ip2 (A) and SKOV3-TR (B) cells are shown. IC50 dose of dasatinib in RMUG-S-ip2 was 3.1 μmol/L. DMSO was used as the drug vehicle. C, cell viability assay for the combination of oxaliplatin and dasatinib in RMUG-S-ip2 cells is shown following treatment for 72 hours. D, isobologram analysis for oxaliplatin and dasatinib in RMUG-S-ip2 cells is shown. Interaction index was less than 1 at all examined points. Dasatinib concentration that reduced 50% of oxaliplatin IC50 dose was 182 nmol/L. E, apoptosis assays for oxaliplatin and dasatinib are shown at the 96-hour time point. Chemotherapeutic doses used for the assay were oxaliplatin 12.4 μg/mL and dasatinib 100 nmol/L, respectively. F, cell-cycle analysis of RMUG-S-ip2 cells treated with oxaliplatin and dasatinib is shown. A–D, dots represent mean ± SE. E and F, bars represent mean ± SE.
for enhancing the efficacy of oxaliplatin chemotherapy (31). In our study, similar findings were observed in mucinous ovarian carcinoma and treatment with oxaliplatin induced activation of Src kinase in RMUG-S-ip2 cells. Similar induction was not observed in serous ovarian cancer cells. Induction of Src kinase activity may be
associated with drug resistance via AKT and Ras pathways in mucinous ovarian carcinoma (13, 32, 33). However, it is not completely clear how Src kinase activity is induced in mucinous ovarian carcinoma but not in serous cancer cells. To date, the mechanism of ROS-induced Src activation is not fully understood (13). As shown in colorectal carcinoma models, pretreatment with an antioxidant agent, such as a vitamin E analogue, inhibited phosphorylation of Src kinase (13). Thus, it is possible that cell innate functions of antioxidative enzymes such as superoxide dismutase in mucinous ovarian carcinoma may be different from that of serous carcinoma (13).

Mucinous ovarian carcinomas showed not only induction of Src kinase activity with oxaliplatin treatment but also increased activity of Src kinase in nonstress conditions. However, expression of Src kinase at mRNA and protein levels in RMUG-S-ip2 cells was lower than in serous cancer cell lines. This suggests that there is a mechanism that contributes to the activation of Src kinase at the protein level but not necessarily at the gene expression level. Theoretically, increased upstream signaling, phosphatase activity, and protein–protein interactions downstream of Src could be the mechanisms that explain increased activity of Src kinase. To date, there is little evidence about the mutation of Src kinase (14, 15). Phosphorylation of Y419 tyrosine residue activates Src kinase, whereas that of Y530 tyrosine residue inactivates Src kinase (15). It is possible that the RMUG-S-ip2 cells may have increased phosphatase activity that dephosphorylates Y530. For protein–protein interaction as the mechanism of Src kinase activation, there are several binding partners of Src kinase that can regulate its catalytic activity including the p130cas/paxillin complex (34). Role of p130cas/paxillin in mucinous ovarian carcinomas is yet to be elucidated and needs further investigation. Because RMUG-S-ip2 cells showed highest Src activity whereas SKOV3-TR showed minimum Src activity, baseline activity of Src kinase may be a predictor of response to dasatinib therapy.

In summary, mucinous ovarian carcinoma has distinct characteristics from serous carcinoma. In light of results presented here, combination therapy with oxaliplatin and dasatinib is an attractive approach for further clinical development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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