Abstract. As few prognostic markers and symptoms have been identified, ovarian cancer is typically diagnosed at an advanced stage, and a majority of patients will relapse and develop resistance to anticancer drugs such as paclitaxel. Musashi-2 (MSI2) is a regulator of gene translation and functions as an oncogenic protein and a marker of poor prognosis in various types of cancer. However, the biological and clinical significance of MSI2 in ovarian cancer remains unclear. Using a tissue microarray-based assay, we demonstrated that MSI2 was highly expressed in advanced, serous ovarian cancer tissues. In addition, MSI2-overexpressing ovarian cancer cells exhibited increased viability, proliferation and growth. We found that MSI2 was overexpressed in paclitaxel-resistant ovarian cancer SKOV3-TR cells but not in paclitaxel-sensitive cell lines. The loss of MSI2 expression in lentivirus-mediated stable MSI2 knockdown SKOV3-TR cells impaired paclitaxel resistance as determined using cell viability and apoptosis assays. In contrast, lentivirus-mediated MSI2 overexpression promoted the development of paclitaxel resistance in paclitaxel-sensitive ovarian cancer cells. The results of the present study are the first to demonstrate that MSI2 is a valuable marker of advanced, serous ovarian cancer and that MSI2 plays an important role in paclitaxel resistance.

Introduction

The Musashi family of proteins comprises two evolutionarily conserved RNA-binding proteins: Musashi-1 (MSI1) and Musashi-2 (MSI2). MSI1 plays a pivotal role in stem cell maintenance (1,2), whereas MSI2 is a recently identified oncogenic protein (3). MSI2 is frequently overexpressed in various types of human cancers and is associated with disease progression. It is overexpressed in patients with acute myeloid leukemia (AML) and chronic myeloid leukemia (CML), and MSI2 expression in AML and CML is directly correlated with a poor clinical prognosis (4,5). Consistent with these findings, another report has demonstrated that elevated levels of MSI2 expression correlate with poor prognosis in patients with B-cell acute lymphoblastic leukemia (6). In addition, MSI2 is highly expressed in hepatocellular carcinoma (HCC) tissues but not in its corresponding non-tumorous tissues and that MSI2 overexpression correlates with highly invasive HCC (7). A recent study has confirmed that colorectal adenocarcinomas express high levels of MSI2 and that MSI2 functions as an oncogenic protein by promoting intestinal cell transformation (3). A subsequent study has demonstrated that the oncogenic function of MSI2 depends on its own structural feature, i.e., the presence of an RNA-recognition motif (8). This motif binds to specific cancer-associated target mRNAs, including the tumor suppressors p21 and NUMB (3,9-11), and inhibits their translation, subsequently resulting in the development of various types of cancer (3,11,12). Together, these reports indicate that MSI2 plays an important role in the development and progression of various cancers and that MSI2 is a potential target of a novel anticancer therapeutic approach.

Musashi-2 is one of the most fatal gynecological malignancies worldwide. The International Agency for Research on Cancer reported that in 2008, 225,000 new cases of ovarian cancer were diagnosed and that 140,000 patients died from the disease worldwide (13,14). In Asian countries, there were an estimated 110,526 new cases of ovarian cancer diagnosed and 65,668 deaths from ovarian cancer in 2012 (14). As there are few distinguishing symptoms and etiological
factors associated with the early stages of ovarian cancer (15), over 70% of cases are diagnosed at advanced metastatic stages (stage III or IV), a stage at which the 5-year survival rate is only 30% (16). Surgical debulking of tumors is the preferred initial treatment; however, approximately 80-90% of patients who undergo this procedure relapse in a relatively short period (17). The current standard of care consists of surgery followed by paclitaxel-based chemotherapy (18), despite the observation that patients who frequently relapse develop chemoresistance (19).

It has been reported that chemoresistance is responsible for 90% of deaths in patients with advanced ovarian cancer (20), indicating that chemoresistance is the primary cause of ovarian cancer relapse. Although there have been considerable efforts to elucidate the mechanism of chemoresistance, molecular mechanisms underlying ovarian cancer relapse remain largely unknown. Therefore, we investigated the function of MSI2 in tumor growth and paclitaxel-resistance in ovarian cancer.

Materials and methods

Cell culture and reagents. The A2780 human ovarian cancer cell line was purchased from the European Collection of Cell Culture (Salisbury, UK). HeyA8, SKOV3, paclitaxel-resistant SKOV3-TR and immortalized ovarian surface epithelial (IOSE) cells were kindly provided by Dr Anil K. Sood (The University of Texas MD Anderson Cancer Center, Houston, TX, USA) and maintained in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 95% air and 5% CO

Water-soluble tetrazolium salt (WST-1) assay. Paclitaxel-induced cytotoxicity was assessed using a WST-1 assay (EZ-Cytox cell viability assay kit; ITSBio, Seoul, Korea). The cells were seeded in a 96-well culture plate and incubated overnight. Then, the cells were treated with the indicated doses of paclitaxel for 48 h; WST-1 solution was subsequently added to each well and the cells were incubated for 0.5 h. Cell viability was determined by measuring absorbance at 450 nm using an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

BrdU incorporation assay. The rate of cell proliferation was evaluated using the BrdU Cell Proliferation Assay kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. Briefly, the cells were transfected with control or MSI2-expressing plasmids and incubated overnight. The transfected cells were subsequently treated with 0.5% trypsin-EDTA (Gibco-Thermo Fisher Scientific). Then, 0.5x10^5 cells were added to each well of the 96-well plate; the cells were then incubated for 24 h and subsequently incubated in BrdU solution (1:10; Cell Signaling Technology) for 2 h. Following the incubation, the cells were washed, treated with a fixing/denaturing solution (Cell Signaling Technology), and incubated with an antibody detection solution (Cell Signaling Technology) at room temperature for 1 h. Then, the cells were washed, incubated in horseradish peroxidase (HRP)-conjugated second antibody solution (Cell Signaling Technology) for 30 min, and incubated in TMB substrate solution (Cell Signaling Technology) for 30 min. At the end of the incubation, cell proliferation was determined by measuring absorbance at 450 nm using an iMark microplate reader (Bio-Rad Laboratories).

Immunoblot assay. Changes in protein levels were measured using immunoblotting assays. The cells were lysed in a radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The proteins were separated using SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked with 5% skim milk for 1 h at room temperature and incubated overnight with the indicated antibody at 4˚C. HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technology) were used, followed by enhanced chemiluminescence (ECL; Pierce, Thermo Fisher Scientific) and autoradiography, to detect the proteins. The primary antibody against MSI2 was purchased from Abcam (Cambridge, UK). Anti-MDR1 and anti-Myc tag antibodies were purchased from Cell Signaling Technology. β-actin was used as a loading control and was detected using an antibody against it (Sigma-Aldrich).

Quantitative real-time PCR (qRT-PCR). Changes in mRNA levels were measured using qRT-PCR. The cells were lysed with TRIzol reagent (Invitrogen-Thermo Fisher Scientific), and total RNA was purified according to the manufacturer's instructions. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen-Thermo Fisher Scientific). PCR was performed using HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) with a StepOnePlus Real-Time PCR System (Applied Biosystems-Thermo Fisher Scientific). The primers used to amplify human MSI2 were 5'-GGATTCCTATGTGGGCGACGA-3' (forward) and 5'-CTCTGTGCCCTGACATTTATTCAAAGTTAAA-3' (reverse). The primers used to amplify human MDR1 were 5'-TGACATTTATTCCAAGATTAAA GCA-3' (forward) and 5'-TAGACACTTTATGCAAACATT TCA-3' (reverse). The primers used to amplify human β-actin were 5'-GGATTCCTATGTGGGCGACGA-3' (forward) and 5'-CGCTCGGTGAGGATCTTCATG-3' (reverse). The C

Transfection and lentiviral stable RNA interference. The human MSI2 open reading frame (ORF) was cloned into the pCMV-Myc/His vector (Invitrogen-Thermo Fisher Scientific), and the cells were transfected into the plasmids using Lipofectamine® (Invitrogen-Thermo Fisher Scientific). The MSI2 and scramble siRNAs were purchased from Bioneer Corp. (Daejeon, Korea), and the cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen-Thermo Fisher Scientific). To generate the MSI2 lentiviral plasmid, ORF of MSI2 was cloned into the pLentiHT vector. The lentiviral vectors expressing shRNA against MSI2 were purchased from Sigma-Aldrich. To generate MSI2 ORF and MSI2...
shRNA lentiviral particles, the 293T cells were co-transfected with the pCMV-VSV-G plasmid (Addgene, Cambridge, MA, USA) and pCMV-dR 8.2 (Addgene), and the viral particles were harvested after 72 h. The cells were infected in the presence of polybrene (8 µg/ml). The cells were subsequently transduced and selected using 1 µg/ml puromycin (Amresco, Solon, OH, USA).

**Annexin V/PI apoptosis assays.** To analyze apoptosis, the cells were washed with phosphate-buffered saline (PBS; Thermo Fisher Scientific) and resuspended in 1X binding buffer (100 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM CaCl$_2$). The cells were incubated with Annexin V-FITC and PI staining solution (FITC Annexin V apoptosis detection kit; BD Biosciences, Franklin Lakes, NJ, USA) for 15 min at RT in the dark. The quantity of apoptotic cells was determined using a BD FACSCalibur (BD Biosciences), and the results were analyzed using the CellQuest program (BD Biosciences).

**Tissue immunohistochemistry (IHC) analysis.** The expression level of MSI2 in ovarian cancer tissues was analyzed using ovarian cancer tissue microarrays (58 cases) (Super BioChips, Seoul, Korea). The tissue array was deparaffinized using xylene (Thermo Fisher Scientific) and rehydrated using graded ethanol (100, 95, 90, 80 and 70%). After the slide was washed with distilled water, H&E staining was performed using a Mayer’s hemalum solution (Merck-Millipore, Billerica, MA, USA) and an Eosin Y-solution 0.5%, aqueous (Merck-Millipore). The tissue array was then dehydrated using graded ethanol (70, 90, 95 and 100%) and xylene (Thermo Fisher Scientific), and the staining was visualized under a microscope. For IHC analysis, the ovarian cancer tissues were first incubated with a normal goat serum blocking solution (2% goat serum) and then stained for MSI2 by incubating with the MSI2-antibody (Abcam) diluted 1:100 overnight at 4°C. The slide was washed with PBS supplemented with Tween-20 (0.05% PBST) and subsequently incubated with HRP-conjugated secondary antibody diluted 1:5,000 for 90 min at room temperature. After the slides were washed in PBST, staining was visualized using a DAB substrate (Thermo Fisher Scientific). MSI2-positive cells were counted by a pathologist (Dr Yi Kyeong Chun, Cheil General Hospital and Women’s Healthcare Center, Dankook University, College of Medicine, Seoul, Korea). Staining intensity was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining.

**Statistical analysis.** The results of three experiments per group were statistically analyzed. The data are presented as the mean ± standard deviation (SD). Statistical analyses were conducted using a two-tailed Student’s t-test analysis, chi-square test, or Fisher’s exact test and Spearman’s rank correlation coefficient analysis. P<0.05 was considered statistically significant.

**Results**

**MSI2 expression in ovarian cancer tissues.** To establish the clinical significance of MSI2, we examined MSI2 expression in ovarian cancer tissues. Using a tissue microarray comprising 58 human ovarian cancer tissues, we conducted an IHC analysis of MSI2 expression and analyzed the correlation between MSI2 expression and various clinicopathological parameters such as tumor stage and subtype. In addition to being the most common histological subtype, serous ovarian cancer is the most lethal type of ovarian cancer, followed by...
mucinous, endometrioid and clear cell carcinoma (21). Our IHC results indicated that MSI2 was expressed at significantly higher levels in serous ovarian carcinoma tissues (P<0.001) than in other types of ovarian carcinoma tissues (Fig. 1A). In addition, MSI2 expression levels in advanced ovarian cancer tissues (grade III and IV) were significantly greater than in grade I and II tissues (P<0.05; Fig. 1B and C). Together, these data suggested that MSI2 plays an important role in ovarian cancer growth and progression.

**MSI2 overexpression accelerates the rate of ovarian cancer cell growth.** Previous studies have shown that MSI2 regulates cell growth in several types of cancers including colorectal and brain cancers (3,22). Therefore, we hypothesized that MSI2 affects the growth and proliferation in ovarian cancer cells. To address this hypothesis, we transfected MSI2 plasmids into ovarian cancer SKOV3 and A2780 cells and conducted a WST-1-based cell viability assay. As shown in Fig. 2A, cell viability significantly increased in cells overexpressing MSI2 than in the control cells. We further analyzed cell proliferation in the control and MSI2-transfected SKOV3 and A2780 cells using the BrdU incorporation assay. Consistent with the results of the cell viability assay, MSI2 overexpression significantly increased cell proliferation (Fig. 2B). We confirmed the results of the cell proliferation and viability assays using a cell growth assay. The control and MSI2-transfected cells were cultured for 5 days, and the relative cell numbers were evaluated daily using trypan blue staining. As expected, MSI2-overexpressing SKOV3 and A2780 cells exhibited a significant increase in growth rate compared with the control cells (Fig. 2C). Together, these results suggested that inhibiting MSI2 expression is a promising strategy for regulating ovarian cancer growth.

**MSI2 is a novel mediator of paclitaxel sensitivity in ovarian cancer cells.** Approximately 70% of patients with ovarian cancer are diagnosed at an advanced stage, and a majority of patients who initially respond to treatment ultimately relapse (23), which is commonly due to the development of chemoresistance (24). We found that MSI2 expression levels were significantly greater in stage III and IV ovarian cancer tissues than in stage I and II tissues (Fig. 1) and that MSI2 overexpression enhanced ovarian cancer cell growth (Fig. 2). Therefore, we sought to determine if MSI2 also plays a role in paclitaxel resistance. To this end, we evaluated the expression levels of MSI2 in IOSE ovarian epithelial cells and various paclitaxel-sensitive and -resistant ovarian cancer cell lines.
To evaluate MSI2 protein levels, the cultured cells were lysed and analyzed using SDS-PAGE and immunoblotting with an MSI2-specific antibody. As shown in Fig. 3A, MSI2 protein levels were the highest in the paclitaxel-resistant SKOV3-TR cell line compared to the other cell lines. Moderate MSI2 levels were observed in A2780 cells, and relatively lower levels were observed in the IOSE, HeyA8 and SKOV3 cells. Next, we investigated MSI2 mRNA levels using qRT-PCR and found that MSI2 mRNA was highly expressed in the SKOV3-TR cells (Fig. 3B). However, the overall pattern of MSI2 mRNA expression was not identical to that of MSI2 protein expression, suggesting that post-transcriptional regulation influences the pattern of MSI2 expression in ovarian cancer cells. To further analyze the relationship between MSI2 protein levels and paclitaxel sensitivity in ovarian cancer cells, we conducted a WST-1-based cytotoxicity assay in the paclitaxel-treated cells. As shown in Fig. 1C, the SKOV3-TR cells, which exhibited elevated MSI2 protein levels, exhibited the strongest paclitaxel resistance at all doses examined. The A2780 cells, which exhibited only moderate MSI2 protein levels, exhibited moderate paclitaxel resistance at low doses (<20 ng/ml); however, higher doses of paclitaxel were significantly cytotoxic. Ovarian cancer cells with low MSI2 protein levels (IOSE, HeyA8 and SKOV3) exhibited paclitaxel sensitivity even at low doses (Fig. 3C). These data suggested that paclitaxel sensitivity and resistance are regulated by MSI2 protein levels in ovarian cancer cells.

MSI2 depletion increases paclitaxel sensitivity in paclitaxel-resistant SKOV3-TR cells. Based on the results described above, we hypothesized that MSI2 regulates paclitaxel cytotoxicity in ovarian cancer cells. To investigate this hypothesis, we generated stable MSI2 knockdown cells using an shRNA-expressing lentivirus. As shown in Fig. 4A, we observed a significant reduction in MSI2 mRNA and protein levels in shMSI2-expressing SKOV3-TR cells. In addition, paclitaxel exerted a significantly greater toxicity in the shMSI2-expressing SKOV3-TR cells than in the corresponding control cells (SKOV3-TR shScramble) (Fig. 4B), indicating MSI2 knockdown abolished paclitaxel resistance in the SKOV3-TR cells. To confirm these data, we evaluated apoptosis in the control and MSI2-knockdown SKOV3-TR cells treated with paclitaxel. Consistent with the results of the cytotoxicity assays, paclitaxel-induced apoptosis (both early and late apoptosis) increased in the MSI2-knockdown SKOV3-TR cells compared with that in the control SKOV3-TR cells (Fig. 4C). Previous reports have demonstrated that paclitaxel resistance is correlated with multidrug resistant 1 (MDR1) expression.
levels (25-27). To determine if shMSI2-induced paclitaxel sensitivity was associated with changes in MDR1 levels, we evaluated MDR1 levels in the cells transfected with MSI2 plasmids, and MDR1 mRNA and protein levels were analyzed using qRT-PCR and immunoblotting assays. As shown in Fig. 4D, MDR1 mRNA and protein levels significantly decreased in the SKOV3-TR cells transfected with MSI2 siRNA in a dose-dependent manner. Together, these data suggested that MSI2 plays an important role in paclitaxel resistance in ovarian cancer cells.

MSI2 overexpression induces paclitaxel resistance in ovarian cancer cells. Next, we examined if MSI2 overexpression can induce paclitaxel resistance in paclitaxel-sensitive SKOV3 cells. To this end, we generated stable MSI2-overexpressing SKOV3 (SKOV3-MSI2) cells using an MSI2-expressing lentivirus (Fig. 5A, right panel) and analyzed their cell viability after paclitaxel treatment. As shown in Fig. 5A, the paclitaxel-mediated inhibition of cell viability significantly decreased in the SKOV3-TR cells transfected with MSI2 siRNA in a dose-dependent manner. Together, these data suggested that MSI2 plays an important role in paclitaxel resistance in ovarian cancer cells.

Discussion

The results of the present study describe two novel functions of MSI2 in ovarian cancer and provide the first evidence...
suggesting that MSI2 plays an important role in the development of ovarian cancer. Tissue microarray experiments revealed that MSI2 expression was significantly correlated with advanced (III-IV), metastatic ovarian cancer and that elevated MSI2 expression levels are correlated with serous ovarian carcinoma tissues. We also demonstrated that transient MSI2 overexpression strongly increased cell viability, proliferation, and growth in the SKOV3 and A2780 cells, which are two established models of serous ovarian carcinoma. The most common type of ovarian cancer is epithelial ovarian cancer, of which serous ovarian carcinoma is the most commonly diagnosed subtype (16). Indeed, approximately 80-85% of cases of ovarian carcinomas in the Western world are of the serous subtype, and up to 95% of patients with advanced ovarian cancer are diagnosed with serous carcinomas (28). Because serous carcinoma is the most prevalent subtype, it is likely that previous clinical and molecular studies on ovarian cancer highly represent cases of serous carcinomas (16). In other types of cancers, MSI2 has been considered a promising therapeutic target due to its anti-apoptotic and oncogenic functions and its correlation with poor prognosis (4,7,11). Therefore, our finding that MSI2 expression is elevated in advanced, serous ovarian cancer tissues might provide novel insights into the study of ovarian cancer. Although the molecular mechanisms that regulate MSI2 in ovarian cancer and the association of MSI2 expression with other clinicopathological features of ovarian cancer remain unknown, the findings of this study merit further investigation.

Paclitaxel, a derivative of the chemotherapeutic drug taxane, plays a key role in the treatment of ovarian cancer (16). Although up to 42% of patients with ovarian cancer initially respond to paclitaxel treatment, most patients ultimately experience disease relapse and become chemoresistant (29). Currently, ovarian cancer is primarily diagnosed at advanced stages; therefore, overcoming the challenge of paclitaxel resistance is essential in prolonging overall survival. Our data further suggest that MSI2 induces paclitaxel resistance in ovarian cancer cells. This hypothesis is supported by the observation that MSI2 is expressed at significantly higher levels in the paclitaxel-resistant SKOV3 cells than in the paclitaxel-sensitive cells. In addition, we demonstrated that the incidence of paclitaxel-induced cell death increased in the MSI2-depleted SKOV3-TR cells compared with that in the control SKOV3-TR cells. In addition, MSI2 overexpression induced paclitaxel resistance in the paclitaxel-sensitive ovarian cancer cells. These data suggest that MSI2 plays an essential role in paclitaxel resistance and that high levels of MSI2 expression might correlate with paclitaxel resistance in ovarian cancer. Consistent with our results, recent reports have shown that MSI2 is associated with the development of chemoresistance in other types of cancer. One report has demonstrated that MSI2-expressing cells are more tumorigenic than non-MSI2-expressing cells and that MSI2 promotes resistance to anticancer drugs in pancreatic cancer cells (30). Another report has demonstrated that the loss of MSI2 inhibits tumor cell proliferation and sensitizes AML cells to anticancer drugs (12). Finally, we found that MSI2 knockdown decreased MDR1 expression levels in the SKOV3-TR cells, whereas transient MSI2 overexpression increased MDR1 expression levels. Together, these findings suggest that MSI2 is a novel regulator of MDR1. MDR1, also referred to as P-glycoprotein (ABCBI), is an important regulator of chemoresistance that is overexpressed in various drug-resistant cancer cells such as paclitaxel-resistant cells (31). siRNA-induced MDR1 knockdown significantly sensitized SKOV3-TR and OVCAR8-TR cells to paclitaxel (26). These results indicate that the molecular functions of MSI2 and MDR1 chemoresistance in ovarian cancer cells are related. Further investigation into the mechanism underlying the interplay between MSI2 and MDR1 might provide new insights into the resistance of cancer cells to anticancer drugs.

Acknowledgements

The present study was supported by the KU Research Professor Program of Konkuk University (to S.B.) and the National Research Foundation of Korea (NRF) grants from the Ministry of Science, ICT and Future Planning (grant 2013R1A1A1012205 to S.B., and grant 20110028846 to S.A.).

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