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ORIGINAL ARTICLE

Downregulation of miRNA-31 induces taxane resistance in ovarian cancer cells through increase of receptor tyrosine kinase MET

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Ovarian cancer is one of the most aggressive female reproductive tract tumors. Paclitaxel (PTX) is widely used for the treatment of ovarian cancer. However, ovarian cancers often acquire chemotherapeutic resistance to this agent. We investigated the mechanism of chemoresistance by analysis of microRNAs using the ovarian cancer cell line KFr13 and its PTX-resistant derivative (KFr13Tx). We found that miR-31 was downregulated in KFr13Tx cells, and that re-introduction of miR31 re-sensitized them to PTX both *in vitro* and *in vivo*. miR-31 was found to bind to the 3′-UTR of mRNA of MET, and the decrease in MET correlated to higher sensitivity to PTX. Furthermore, co-treatment of KFr13Tx cells with MET inhibitors sensitized the tumor cells to PTX both *in vitro* and *in vivo*. In addition, lower levels of miR31 and higher expression of MET in human ovarian cancer specimens were significantly correlated with PTX chemoresistance and poor prognosis. This study demonstrated miR31-dependent regulation of MET for chemoresistance of ovarian cancer, raising the possibility that combination therapy with a MET inhibitor and PTX will increase PTX efficacy.

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INTRODUCTION

Ovarian cancer is the leading cause of death among malignancies of the female reproductive system, resulting in ~125 000 deaths annually.[1] Because of the wide variety of symptoms of ovarian cancer, most patients are in advanced stages (International Federation of Gynecology and Obstetrics (FIGO) stage III and IV) at the time of initial diagnosis,[2] and the 5-year overall survival rates are only 30–40%.[3,4] The current therapy for ovarian cancer is debulking surgery followed by chemotherapy using carboplatin and paclitaxel (PTX).[5] Although ovarian cancer in advanced stages initially appears to be chemotherapy sensitive as response rates to platinum-based therapy exceed 80%, long-term survival remains poor as a result of recurrence and emergence of drug resistance.

MicroRNAs (miRs) are endogenous non-coding RNAs of ~23-mer, which have important roles in regulation of gene expression. Mature form of miRs silence gene expression by binding to the 3′-UTR of target mRNAs and initiate translational repression or cleavage of cognate mRNAs.[6] Following the initial demonstration of the important role for miR in human cancer, such as downregulation of miR-15a-miR-16-1 in chronic lymphocytic leukemia,[7] a number of cancers have been shown to exhibit distinct miR expression patterns related to various phenotypes with remarkable cytogenetic abnormalities.[8,9] Implication of miR in chemoresistance was reported in several cancers other than ovarian origin.[10,11] Although there was a report for correlation of miR, such as let7i, for chemoresistance of ovarian cancer,[12] the precise mechanism underlying the association is unknown.

In this study, we aimed to investigate the correlation between miR and sensitivity to PTX, and demonstrated that miR-31 was decreased in chemoresistant ovarian cancer. In addition, one of the direct targets of miR-31 was found to be receptor tyrosine kinase MET, and upregulation of MET caused the chemoresistance to PTX. These results suggest that MET inhibitors administered concurrently with PTX could prevent the development of resistance to PTX.

RESULTS

miR-31 was downregulated in ovarian cancer cells that acquired PTX resistance

To identify miRNAs that potentially cause the resistance to taxanes, such as PTX, we performed microarray analysis of miRNAs in KFr13 and PTX-resistant KFr13Tx cells. Fifty-five miRNAs were found to be downregulated below the half amount, while two miRNAs were upregulated more than twofold in KFr13Tx cells compared with wild-type KFr13. miRNAs that exhibited remarkable alterations are shown in Supplementary Table 1. Among these miRNAs, we focused on miRNA-31 (miR-31) because miR-31 was the only miR that has been reported to regulate tumor phenotype, an anti-metastatic effect in breast cancer.[13] In fact,

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miR-31 was negatively correlated with IC50 values of PTX between six cell lines including KFr13, RMG-1, SK-OV-3, OVCAR-3, KF and TU-OM-1 (Figure 1a).

Overexpression of miR-31 re-sensitized PTX-resistant ovarian cancer cells to PTX *in vitro*

To confirm the results of array analysis in which levels of miR-31 was decreased in PTX-resistant cells, quantitative real-time PCR analysis was performed, and as expected, the levels of miR-31 was significantly suppressed in KFr13Tx cells (Figure 1b). To assess the biological role of miR-31, we next tried to establish ovarian cancer cells overexpressing miR-31 by introducing pre-miR31 using lentivirus vectors. We used KFr13Tx cells because miR-31 was substantially decreased in KF13rTx when it acquired PTX resistance among several cell lines mentioned above and lentivirus vector was efficiently transfected in this cell line. Finally, we established KFr13Tx cells overexpressing three different levels of miR-31 designated as KFr13Tx miR-31(1), miR-31(2) and miR-31(3) (Figure 1c), and performed MTT assays to evaluate the sensitivity to PTX. After incubation with 500 nM of PTX for 72 h, we found that the ovarian cancer cells with higher amounts of miR-31 exhibited lower cell viability (Figure 1d). Conversely, inhibition of miR-31 expression in KFr13 cells by oligonucleotides increased cell viability after incubation with 500 nM PTX for 48 h (Figure 1e). It is notable that miR-31 introduction did not change the sensitivity to other agents such as carboplatin, irinotecan, doxorubicin and gemcitabine in KFr13Tx (Supplementary Table 2). These results suggested that the decrease of miR-31 caused PTX-specific resistance in KFr13.

miR-31 reduced protein levels of MET through the inhibition of translation

To elucidate the mechanism by which miR-31 regulates PTX sensitivity, *in silico* prediction models were employed to identify the target mRNA of miR-31 for chemosensitivity in ovarian cancer[6] (Supplementary Table 3). Among these targets, we focused on receptor tyrosine kinase MET because one potential binding site for miR-31 was found in 3′-UTR of MET mRNA (Supplementary Figure S1a). To ensure that MET is a *bona fide* target of miR-31, RNA fragments that bind to Ago2 were analyzed, and the 3′-UTR region of MET was found to be isolated from the Ago2-dependent immunoprecipitated RNA fraction of KFr13Tx miR-31 cells (Figure 2a). For further confirmation, the protein levels of MET was analyzed in KFr13 cells overexpressing miR-31, and we found that MET was downregulated in miR31-overexpressing cells in a dose-dependent manner (Figure 2b). As expected, the expression level of MET was increased in KFr13Tx cells compared with that in KFr13 cells (Figure 2b). Conversely, an increase of MET protein levels was observed after introduction of anti-miR-31 oligonucleotides into KFr13 cells (Supplementary Figure S1b). The same tendency that ovarian cancer cells with higher miR-31 showed lower MET expression was also observed in other cell lines used in the PTX sensitivity experiment mentioned above. Expression of MET was extremely low in RMG-1 and relatively low in SK-OV-3, OVCAR-3 and KFr13, and high in KF and TU-OM-1, both of which were resistant to PTX and expressed low miR-31 (Figure 2c). Subsequently, we analyzed the mechanism by which miR-31 regulates endogeneous protein levels of MET, focusing on transcriptional or translational regulation. As no significant difference of MET mRNA was observed between KFr13 and KFr13Tx cells (Figure 2d), miR-31 did not seem to inhibit transcription. On the other hand, when translation was inhibited by CHX, levels of MET were decreased, suggesting translational regulation of MET by miR-31, although a decrease in MET levels in the presence of CHX does not necessarily demonstrate a direct translational regulation of MET by miR-31, as the suppressive effect by CHX on translation is nonspecific and may inhibit expression of various proteins including those affecting the regulation of MET levels. The levels of MET were low in spite of the presence or absence of CHX in case of miR31-overexpressing cells (Supplementary Figure S1c).

To ensure that MET mRNA is a target of miR-31, we utilized a luciferase reporter assay. Normalized luciferase activity revealed that miR-31 significantly suppressed the activity of luciferase combined with wild-type MET 3′-UTR in KFr13Tx miR-31 cells, whereas no difference was observed with the control luciferase vector (Figure 2e). Furthermore, miR-31 did not affect luciferase with MET 3′-UTR possessing a mutation in the putative miR-31-binding site in KFr13Tx miR-31. These results suggest that miR-31 directly suppressed the protein expression of MET *via* sequence-specific interactions with 3′-UTR of MET mRNA.

As MET was reported to be degraded by the ubiquitin-proteasome pathway,[14] we utilized a proteasome inhibitor, MG132, to exclude the possibility that miR-31 indirectly regulates protein levels of MET through a ubiqutin-dependent protein degradation mechanism. After treatment with MG132 for 4 h, MET expression in KFr13Tx miR-31 was not altered by inhibition of proteasome function, while the amount of p53 was increased as positive control for ubiquitin-dependent protein degradation[15] (Supplementary Figure S1d). These results suggest that miR-31 directly binds to MET mRNA and regulates MET expression by translational inhibition.

MET contributes to PTX resistance of ovarian cancer cells

To investigate whether MET is responsible for the resistance of KFr13Tx cells to PTX, expression of MET was suppressed by three different small interfering RNAs (siRNAs) (Figure 3a, bottom) and the treated cells were evaluated for chemosensitivity by the MTT assay. After incubation with 500 nM of PTX for 72 h, significantly lower viability was observed in cells with MET suppression, whereas nonspecific siRNA did not affect the viability of KFr13Tx cells (Figure 3a, top). These results suggest that MET contributes to chemoresistance to PTX in ovarian cancer cells.

Based on our results suggesting the presence of MET-dependent chemoresistance, we explored molecular-targeting treatments to determine whether MET inhibitors could re-sensitize resistant tumors to PTX. Two different MET kinase inhibitors, SU11274 and PHA665752, were utilized at the concentration with minimum effect on viability of KFr13Tx mock cells (Figure 3b, lanes 1 and 2). At this concentration, the protein levels of phospho-MET and phospho-Akt were significantly decreased (Figure 3b bottom). After incubation with MET inhibitors together with PTX, the KFr13Tx cells were sensitive to PTX. Furthermore, even in the presence of HGF stimulation, MET inhibitors reversed the chemoresistance to PTX in KFr13Tx cells (Supplementary Figure S2). Based on these results, we concluded that the downregulation of miR-31 induced chemoresistance of ovarian cancer cells to PTX through the upregulation of MET *in vitro*.

Overexpression of miR-31 re-sensitized PTX-resistant ovarian cancer cells to PTX *in vivo*

To investigate the effects of miR-31 on PTX sensitivity *in vivo*, we examined a murine xenograft tumor model. KFr13Tx mock or miR-31 were injected subcutaneously into the flank of mice, and chemosensitivity of the tumors was evaluated. Without PTX treatment, the weight of the tumors was similar in mock and miR31-overexpressing KFr13 cells. In contrast, under treatment with PTX, miR31-overexpressing tumors were significantly smaller than that of mock expressing tumors (Figure 4a, top). Consistent with the smaller tumor size, mice harboring miR31-overexpressing tumor survived longer during the treatment with PTX (Figure 4a, bottom). The weights of subcutaneous tumor nodules derived from drug-resistant KFr13Tx mock cells were reduced by the combination of MET inhibitor SU11274 with PTX (Figure 4b, top).

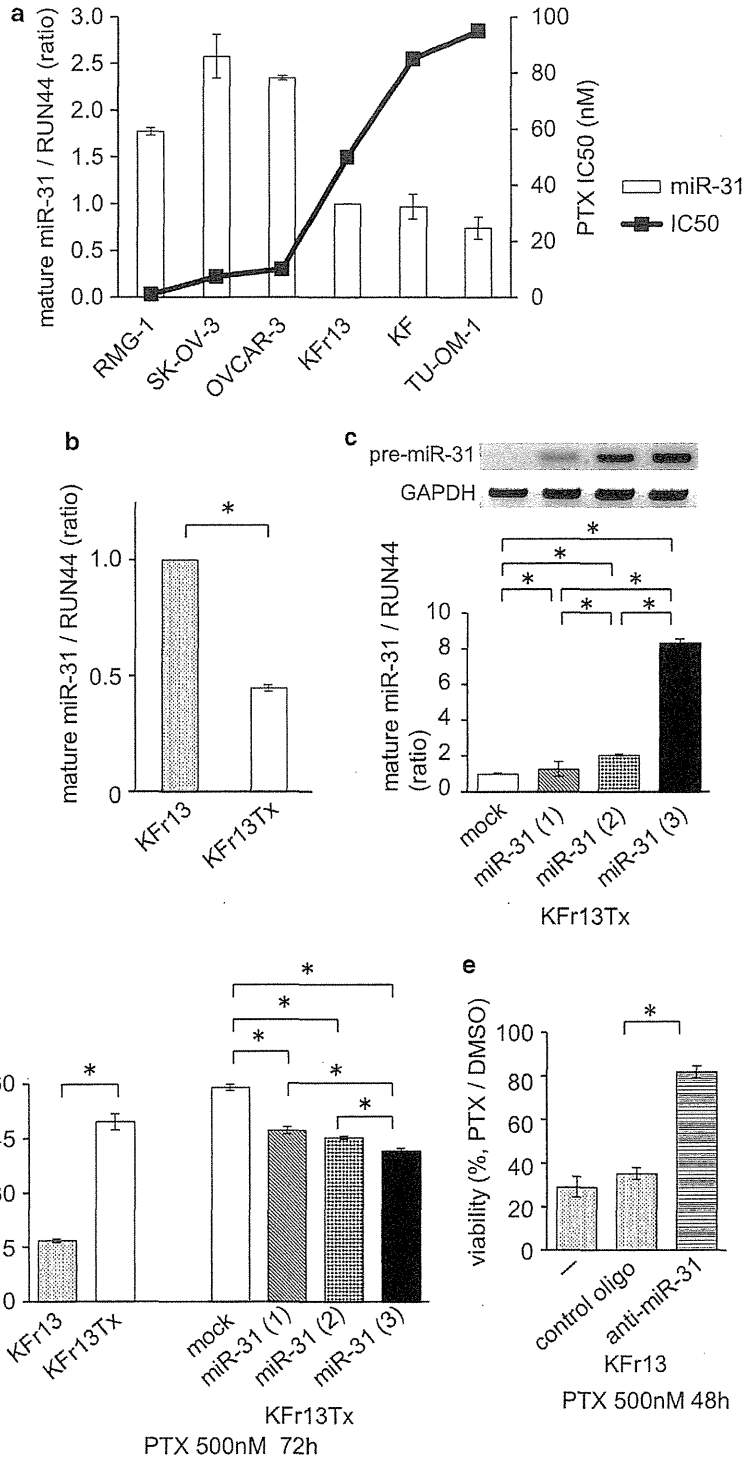
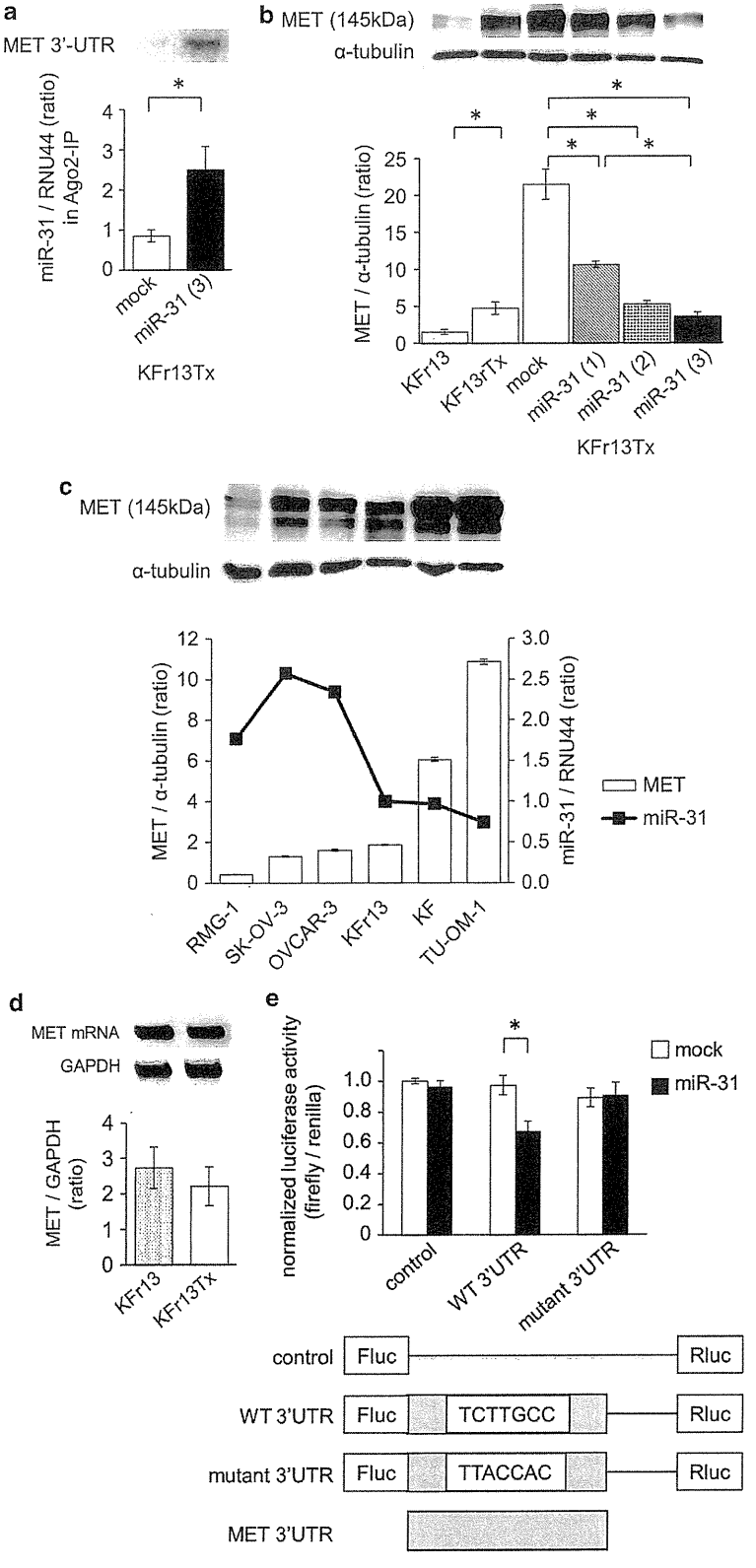


Figure 1. Overexpression of miR-31 re-sensitized PTX-resistant ovarian cancer cell to PTX *in vitro*. (**a**) Real-time PCR analysis of expression levels of miR-31 and IC_{50} of PTX in six human ovarian cancer cell lines. (**b**) Real-time PCR analysis of expression levels of miR-31 in parental (KFr13) and PTX-resistant (KFr13Tx) cells. $^*P<0.05$. (**c**) Establishment of three cell lines of KFr13Tx expressing miR-31. Expression levels of miR-31 in cells named as miR-31 (1), miR-31 (2) and miR-31 (3) are low, middle and high, respectively. Representative RT–PCR bands are shown as top panel. Real-time PCR analysis of expression levels of miR-31 in miR-31 (1), miR31 (2) and miR (3) are shown in bottom bar graph. Original gel is presented in Supplementary Figure S3a, $^*P<0.05$. (**d**) Chemosensitivity to PTX is shown by MTT assay, $^*P<0.05$. (**e**) Effect of anti-miR-31 on chemosensitivity to PTX analyzed by MTT assay, $^*P<0.05$.



Furthermore, overall survival of the mice with the KFr13Tx mock tumors was found to be improved by the combination treatment of MET inhibitor with PTX (Figure 4b, bottom). These results suggest that the combination of PTX and SU11274 may be an option for the treatment of ovarian cancer refractory to PTX.

Lower expression levels of miR-31 and higher levels of MET in human ovarian cancer specimens are associated with chemoresistance

To explore the relevance of our findings to the clinic, we analyzed the expression levels of miR-31 in surgical specimens from human ovarian cancer patients (Table 1). We needed to investigate the patients with measurable target lesions in advanced-stage cancer, namely with suboptimal resection, to examine the correlation between chemosensitivity and miR-31 expression and selected the patients with serous adenocarcinomas that usually show advanced-stage disease.[16] All 12 cases were women with serous adenocarcinomas with FIGO Stage IIIc or IV,[16] who underwent surgery as their initial treatment. As the initial surgery was suboptimal in all patients, the subjects were stratified by response to subsequent chemotherapy (more than three courses in all cases) according to the Response Evaluation Criteria in Solid Tumors (RECIST version1.1).[17]

The 12 tumors were divided into two groups, sensitive and resistant, based on the response to chemotherapy. We found that the expression levels of miR-31 were lower in resistant tumors compared with sensitive ones (Figure 5a). Moreover, the lower expression of miR-31 strongly correlated with reduced overall survival in Stage IIIc patients, who were treated with PTX and carboplatin (Figure 5b). In agreement with our *in vitro* data, the higher protein levels of MET were correlated with lower levels of miR-31 in the cohort analysis of the tumors (Figure 5c). These data

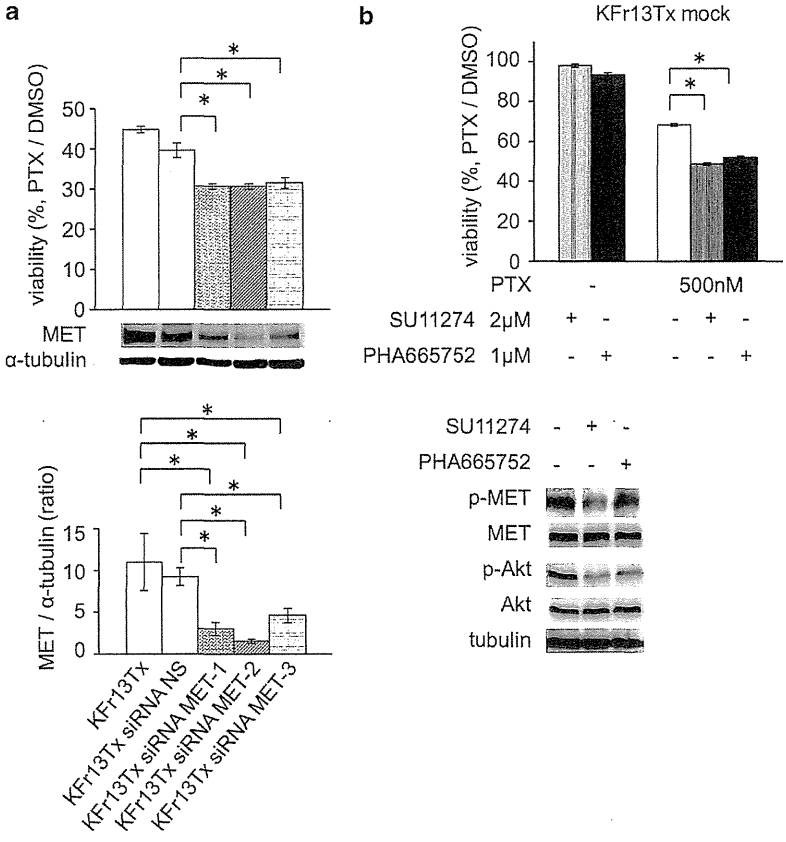


Figure 3. MET regulates PTX sensitivity in ovarian cancer cell *in vitro*. (**a**) Chemosensitivity to PTX of KFr13Tx analyzed by MTT assay are shown as bar graph (top). Immunoblotting of MET and α-tubulin when siRNAs were transfected (middle panel). The ratio of MET/α-tubulin is shown as bar graph (lower). Original blots are presented in Supplementary Figure S3e, $^*P<0.05$. (**b**) Effect of MET inhibitor, SU11274 and PHA665752 on chemosensitivity to PTX analyzed by MTT assay. Effect of MET inhibitor was validated by immunoblotting for phosphorylated form of MET (pMET) and Akt (pAkt). Original blots are presented in Supplementary Figure S3f, $^*P<0.05$.

Figure 2. miR-31 regulates MET expression by translation inhibition. (**a**) Detection of MET mRNA by RT–PCR (top panel) and miR-31 by real-time PCR in Ago2-mediated immunoprecipitated RNA fraction in KFr13Tx. Original gel is presented in Supplementary Figure S3b, $^*P<0.05$. (**b**) Expression levels of MET in wild-type and miR31-overexpressing cells. Results of immunoblotting of MET and α-tubulin are shown as top panel and the ratio of MET/α-tubulin are shown as bar graph. Original blots are presented in Supplementary Figure S3c, $^*P<0.05$. (**c**) Expression levels of MET and miR-31 in six human ovarian cancer cell lines. Results of immunoblotting of MET and α-tubulin are shown as top panel. The ratio of MET/α-tubulin and miR-31 expression is shown in bottom bar and line graph, respectively. (**d**) mRNA levels of MET in KFr13 and KFr13Tx analyzed by RT–PCR are shown as top panel. Ratio of MET/GAPDH is shown as bar graph. Original gel is presented in Supplementary Figure S3d. (**e**) Luciferase activity after transfection of the indicated 3′-UTR-driven reporter constructs. Reporter plasmid containing 3′-UTR region of MET as WT3′-UTR and its mutant as mutant 3′-UTR, $^*P<0.05$.

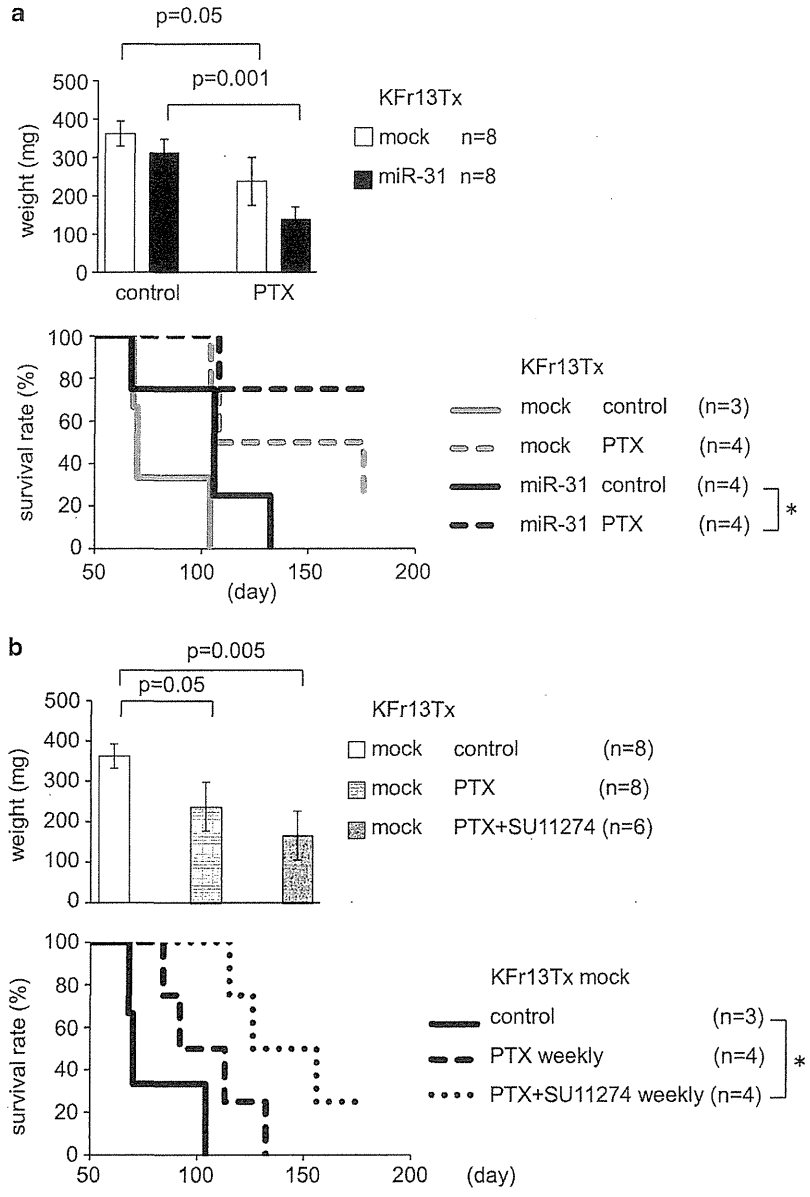


Figure 4. (**a**, top graph) Overexpression of miR-31 re-sensitized PTX-resistant ovarian cancer cell to PTX *in vivo*. Average of the weight of tumor in flank of mice. Solid bar indicates miR-31-expressing KFr13Tx cells. Open bar indicates KFr13Tx cells. Unpaired one-tailed *t*-test. (**a**, bottom graph) Survival of mice with intraperitoneal injection of KFr13Tx or KFr13Tx expressing miR-31 treated with PTX. KFr13Tx with PTX (gray broken line), KFr13Tx without PTX (gray solid line), KFr13Tx expressing miR-13 with PTX (black broken line), KFr13Tx expressing miR-13 without PTX (black solid line). $^*P<0.05$, log-rank test. (**b**, top graph) Effect of combination treatment of MET inhibitor and PTX on KFr13Tx cells *in vivo*. Average of the weight of tumor in flank of mice. Open bar indicates no treatment, closed bar as PTX alone, hatched bar as combination of both PTX and MET inhibitor SU11274. $^*P<0.05$, unpaired one-tailed *t*-test. (**b**, bottom graph) Survival of mice with intraperitoneal injection of KFr13Tx with no treatment, PTX alone, and both PTX and SU11274. Kaplan–Meier curves, $^*P<0.05$, log-rank test.

suggest the levels of miR-31 may predict the response to standard chemotherapy in ovarian cancer and serve as a prognostic factor.

DISCUSSION

The survival rate of early ovarian cancer is good and the outcome of early-stage disease might largely depend on the surgical factor, that is, the complete resection of tumors. On the other hand, the prognosis of the patients with optimally resected stage III–IV ovarian cancer is also known to be relatively good. However, when there are residual tumors of a size more than the greatest dimension of 1 cm, the prognosis of those patients largely depends on chemosensitivity, because complete resection of the residual tumors will be possible when chemotherapy is effective.[18] As ovarian cancer is usually undetected until it reaches an advanced stage, ~50–60% of patients gain no improvement in their prognosis from cytoreductive surgery.[19] However, there is a certain population of women who have disease that is highly responsive to chemotherapy with PTX after suboptimal surgical ablation and who achieve a long survival.

In this study, we aimed to identify markers that identify chemotherapy responder patients by the comparing characteristics of

Table 1. Chemosensitivity and clincal features of human ovarian cancer

Case	*Age*	*Histology*	*FIGO stage*	*Primary debulking surgery*	*Response to chemotherapy*	*Regimen*
sensitive						
1	42	serous	IIIc	suboptimal	PR	TC
2	47	serous	IIIc	suboptimal	PR	TC
3	47	serous	IIIc	suboptimal	PR	TC
4	52	serous	IIIc	suboptimal	PR	TC
5	51	serous	IV	suboptimal	CR	TC
6	66	serous	IV	suboptimal	PR	TC
resistant						
7	38	serous	IIIc	suboptimal	SD	TC
8	53	serous	IIIc	suboptimal	SD	DC
9	54	serous	IIIc	suboptimal	SD	TC
10	67	serous	IIIc	suboptimal	PD	TC
11	52	serous	IV	suboptimal	SD	TC
12	51	serous	IV	suboptimal	PD	DC

Abbreviations: CR, complete response; DC, docetaxel + carboplatin; PD, progressive disease; PR, partial response; SD, stable disease; TC, paclitaxel + carboplatin. Suboptimal, >1 cm of residual disease.

chemosensitive and chemoresistant ovarian cancer cells. We discovered that tumor miR-31 expression levels might be a useful marker of chemotherapy response. In addition, we also demonstrated that MET is one of the targets of miR-31, whose overexpression is responsible for chemoresistance. Moreover, we provide evidence that poor responders might be rescued by a combination of standard chemotherapy with inhibitors for MET.

PTX is thought to dysregulate tubulin polymerization and microtubule formation,[20] inhibiting cell cycle and mitosis resulting in apoptosis of tumor cells.[21] Previously, several mechanisms for development of PTX resistance have been reported.[22–26] Amplification and increased expression of MDR1-encoded phosphoglycoprotein (PGP), which belongs to the superfamily of ATP-binding cassette (ABC) transporters, promotes efflux of anticancer drugs such as PTX.[27] It is noteworthy that one of these transporters, ABCB9, was a candidate target of miR-31, but protein expression levels of ABCB9 were similar between KFr13Tx mock and KFr13Tx expressing miR-31 (data not shown). The detailed molecular mechanisms that may contribute to drug resistance in ovarian cancers are still not fully understood, and new therapeutic strategies to overcome drug resistance need to be established.[5]

Recently, versatile roles for miRs have been identified in various human cancers[9] and there are several reports about the correlation between miRs and chemoresistance. Upregulation of miR-125b and repression of its direct target Bak1 inhibited PTX-induced apoptosis in breast cancer.[10] miR-199a-3p increased doxorubicin sensitivity of human hepatocarcinoma cells by repressing mTOR.[11] miR-21 induced resistance to 5-fluorouracil by downregulating human DNA MutS homolog2 (hMSH2) in colorectal cancer cells, and reducing G2/M arrest and apoptosis following exposure to 5-fluorouracil.[28] These reports suggest that miRs are potential targets for the development of novel approaches for cancer treatment. In this study, we compared the profiles of miRs between PTX-resistant and their chemosensitive parental cells, and demonstrated a role for miR-31 in ovarian cancer chemoresistance. Thus, miR profiling and the identification of miR targets is a fruitful approach to identify the molecular basis of cancer cell phenotypes.

It should be noted that, beside miR-31, the levels of several cancer-related miRs[29,30] were also significantly altered in the acquisition of chemoresistance (Supplementary Table S1). Thus, miR-31 may not be the only miR regulating the malignant potential of ovarian cancer. Based on our findings, we can conclude that miR-31 is involved at least in PTX resistance of ovarian cancers. Other studies have suggested that miR-221, miR-181b and miR-181d induce tamoxifen resistance in breast cancer, S-1 resistance in colon cancer and docetaxel-induced multidrug resistance in head and neck squamous cell cancer, respectively.[30–32] Therefore, there may be tissue specificity for miRs in terms of chemoresistance in various different cancers.

In this present study, we have focused on miR-31 and our data represent the first demonstration of miR-31-mediated chemoresistance. However, it should be noted that miR-31 might have other roles associated with human cancers. Overexpression of miR-31 inhibits cancer cell proliferation by p53-dependent mechanisms in ovarian cancer cells.[33] miR-31 was also reported to inhibit metastasis, while it enhanced primary tumor growth of breast cancer.[13] Furthermore, there is a report suggesting that miR-31 inhibits cell proliferation, migration and invasion in malignant mesothelioma.[34] Contributions of miR-31 to the activation of hypoxia-inducible factor for development of head and neck squamous cell cancer were also described.[35] Considering these reports, the roles of miR-31 in tumor cell proliferation and metastasis are complex.

The HGF receptor tyrosine kinase MET was originally isolated as oncogene product and is well known to possess versatile roles in cells including cancer cell invasion, motility and growth.[36] Recent studies have highlightened the effects of MET inhibitors on lung cancer with mutations of EGFR and amplification of MET.[37,38] MET has been shown to mediate apoptotic resistance to therapeutic drugs in case of ovarian cancer through the activation of a PI-3 kinase-/AKT-dependent mechanism.[39,40] Multikinase inhibitors suppressing MET and VEGFR activity have been reported to inhibit proliferation and metastasis of ovarian cancer.[41] Together with the report that MET was frequently expressed in ovarian cancers, and elevated levels of MET correlate with lower overall survival,[42] our results suggest that concurrent use of MET inhibitor with conventional anticancer drugs may improve outcomes for ovarian cancer patients.

In conclusion, the present study suggests that downregulation of miR-31 induces resistance of ovarian cancer to taxanes like PTX through upregulation of MET. Our findings provide a basis for clinical studies to determine if miR-31 expression levels are a marker of chemosensitivity in ovarian cancer patients, and if MET kinase inhibitors can rescue the PTX response in women with chemoresistant ovarian cancers.

MATERIALS AND METHODS

Cell lines

Human KF ovarian cancer cells and KFr13 cisplatin-resistant KF ovarian cancer cells were kindly provided by Prof Yoshihiro Kikuchi (National Defense Medical College, Saitama, Japan).[43] SK-OV-3 and OVCAR-3 were obtained from the ATCC (Manassas, VA, USA). RMG-1 was obtained from Health Science Research Resources Bank (Osaka, Japan). TU-OM-1[44] was kindly provided by Dr Junzo Kigawa (Tottori University School of Medicine). KFr13, KF were maintained in RPMI 1640 with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin and streptomycin. To establish PTX-resistant KFr13 (KFr13Tx), cells were cultured with 2 nM of PTX, then the PTX concentration was gradually increased to 30 nM. SK-OV-3 was maintained in McCoy's 5a Medium Modified with 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin and streptomycin. OVCAR-3 was maintained in RPMI 1640 with 20% FBS, 0.01 mg/ml bovine insulin, 2 mM L-glutamine and 100 U/ml penicillin and streptomycin. TU-OM-1 was maintained in Dulbecco's modified eagle's medium with 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin and streptomycin.

Reagents and measurement of drug sensitivity by MTT assay

PTX, irinotecan (Sigma, St Louis, MO, USA), MET kinase inhibitors, such as SU11274 (Merckmillipore, Darmstadt, Germany), PHA665752 (Santa Cruz Biotechnology, Dallas, TX, USA), epidermal growth factor receptor inhibitor

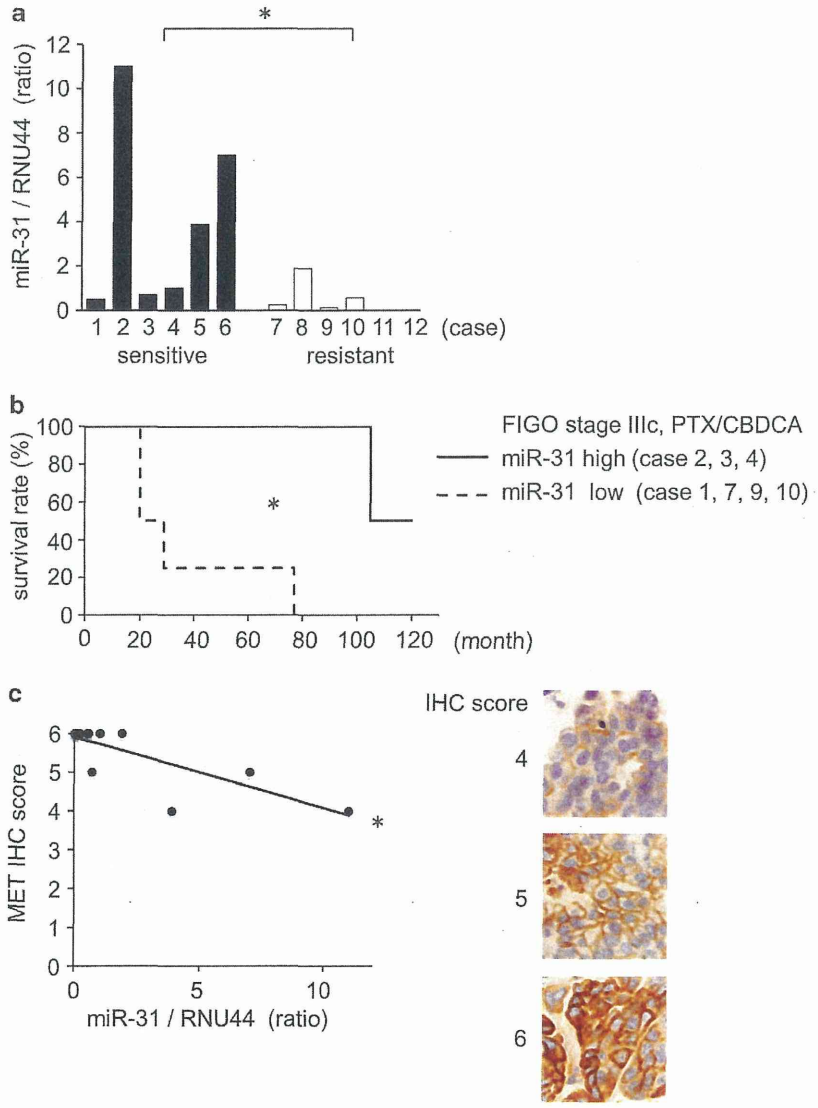


Figure 5. miR-31 expression decreased with chemosensitivity to PTX in human ovarian cancers. (**a**) Expression levels of miR-31 in human ovarian cancers were analyzed by real-time PCR. Cases 1–6 are chemosensitive and cases 7–12 are chemoresistant to taxane/platinum reagents. $^*P<0.05$, unpaired one-tailed *t*-test. (**b**) Prognosis of patients of ovarian cancers classified as miR31-high (solid line) and miR31-low (broken line) group. Kaplan–Meier curves for seven FIGO stage IIIc human primary ovarian cancers depicting overall survival, stratified based on miR-31 level. $^*P<0.05$, log-rank test. CBDCA, carboplatin; PTX, paclitaxel. Median follow-up = 67 months. (**c**) Correlation of expression levels of miR-31 and immunohistochemical reactivity of MET. IHC score, immunohistochemistry score. Representative results of IHC with scores 4, 5 and 6 are shown as photographs, × 400; $^*P<0.05$, single regression analysis.

AG490 (Calbiochem) and proteasome inhibitor MG132 (Sigma), were dissolved in dimethyl sulfoxide (DMSO). Recombinant human hepatocyte growth factor (HGF) (Peprotech, Rocky Hill, NJ, USA) was dissolved in double distilled water (DDW) with 0.9% NaCl. Carboplatin, doxorubicin and gemcitabine (Sigma) were dissolved in DDW. Cycloheximide (CHX) (Sigma) was dissolved in ethanol (EtOH).

The MTT assay was performed for drug sensitivity assays using Cell Proliferation Kit I (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 1.0×10^4 cells were seeded onto 96-well plates in 100 μl culture medium with reagents. An equal volume of DMSO was used as control. The wavelength to measure absorbance of the formazan product was 570 nm, and the reference wavelength was 690 nm.

MicroRNA microarray

Microarray analyses were performed as previously described.[45] Total RNA was isolated from KFr13 and KFr13Tx by using RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacture's protocol. In brief, 50 μg of total RNA was enriched for the specimens of small amount of RNA, tailed by using the *mirVana miRNA Labeling kit* (Ambion, Austin, TX, USA), and fluorescently labeled by using amine-reactive Cy5 dyes (Amersham Pharmacia, Piscataway, NJ, USA). The fluorescence-labeled RNAs were hybridized for 16 h with miRNA array slides. Microarrays were performed using mirVanaTM miRNA Bioarray V2 (Ambion) and scanned by GenePix 4000B (Molecular Devices Inc., Sunnyvale, CA, USA). This microarray carries genes for a total 633 kinds of miRNAs containing 328 human genes, 238 rat genes and 266 mice, with each gene spotted in quadruplicate. Raw data were normalized and analyzed using Array ProTM Analyzer Ver4.5 (Media Cybernetics Inc., Rockville, MD, USA). Analyzed data were selected by using MicroArray Data Analysis Tool (Filgen Inc., Nagoya, Japan).

Overexpression of miR-31

Pre-miR-31 was transfected into KFr13Tx using BLOCK-iT Lentiviral miR RNAi Expression System (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The sequences for miR-31 precursor (pre-miR-31)

were as follows: forward 5′-TGCTGGGAGAGGAGGCAAGATGCTGGCATAGCT GTTGAACTGGGAACCTGCTATGCCAACATATTGCCATCTTTCC-3′ and reverse 5′-CCTGGGAAAGATGGCAATATGTTGGCATAGCAGGTTCCCAGTTCAACAGCT ATGCCAGCATCTTGCCTCCTCTCCC-3′. *pcDNATM6.2-GW/EmGFP-miR plasmid* (Invitrogen) was used as a negative control (mock). We repeated blasticidin selection at a concentration of 15–90 μg/ml and obtained three clones with different miR-31 expression. Total RNA was extracted by using TRIzol Reagent (Invitrogen). miR-31 was quantified by quantitative real-time PCR using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and TaqMan MicroRNA Assays (Applied Biosystems) according to manufacturer's instructions. We assessed miRNA expression by relative quantification using the 2-ΔΔCt method[46] to determine fold changes in expression. The small nucleolar RNA RNU44 served as an endogenous control.

Inhibition of translation and of proteasome activity

Cells with ~80% confluence in 6-well plate were pretreated with 10 μg/ml of CHX to prevent *de novo* synthesis of MET for 4 h, or 0, 10 and 15 μM of MG132 to inhibit proteasome for 4 h. Equal volume of EtOH or DMSO were used as controls.

Luciferase reporter assay

Luciferase vectors (Genecopoeia, Rockville, MD, USA) were employed. The wild-type (NM_000245.2) or mutant MET 3′-UTR sequence was inserted into downstream of the firefly luciferase reporter gene, which was controlled by the SV40 enhancer for expression in mammalian cells (Genecopoeia, HmiT011181-MT01, HmiT011181-MT01-02), whereas no oligonucleotides were inserted in control vector (Genecopoeia, CmiT000001-MT01). Renilla luciferase was used as a tracking indicator for successful transfection. KFr13Tx mock and miR-31 cells with 80% confluence in 6-well plate were transfected with 2 μg of each vector using Lipofectamine 2000 (Invitrogen). Cells were harvested after 48 h and lysed with passive lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured by a dual luciferase reporter assay system (Promega). Firefly luciferase activities were normalized by the Renilla luciferase activity.

Cloning of target mRNA in miRNA–mRNA–Argonaute2 (Ago2) complex

miRNA–mRNA–Ago2 complex was immunoprecipitated from 7.0×10^6 cells of KFr13Tx mock and KFr13Tx miR-31 using microRNA Isolation Kit, Human Ago2 (Wako, Osaka, Japan), then cDNA amplification from mRNA in miRNA–mRNA–Ago2 complex was performed using Target mRNA Cloning Kit (Wako). PCR amplification of MET 3′-UTR was performed using GoTaq Green Master Mix (Promega). miR-31 in miRNA–mRNA–Ago2 complexes of both cell lines were also quantitatively measured by real-time PCR.

RNA interference for MET

Three siRNAs against MET (Thermo Fisher Scientific, Waltham, MA, USA) and non-targeting siRNA (Thermo Scientific Dharmacon) were transfected into KFr13Tx cells by using HiPerFect transfection reagent (Qiagen) in six-well plate. Cells were harvested and lysed after 48 h.

miRNA inhibitor

Two hundred and fifty nanomolar miRIDIAN microRNA hairpin inhibitor and its negative control (Thermo Scientific Dharmacon) were employed to transiently inhibit miR-31 and transfected 48 h before seeding with Oligofectamine (Invitrogen).

Immunoblotting

Cells were lysed with lysis buffer (10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4). Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride filter (Millipore, Billerica, MA, USA) by standard method. Filters were incubated with rabbit polyclonal antibody against MET (Santa Cruz Biotechnology), and mouse monoclonal antibodies against p53 (Dako, Glostrup, Denmark), actin (Millipore) or α-tubulin (Santa Cruz Biotechnology) were incubated overnight at 4 °C, and then with peroxidase-labeled secondary antibodies for 1 h. Proteins were visualized by Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen) and quantified using a Lumino Image Analyzer (LAS1000, Fuji Film, Tokyo, Japan).

Reverse transcription PCR

Total RNA was extracted by using TRIzol Reagent (Invitrogen), and reverse transcription was performed using SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's instruction. The PCR amplification was performed using GoTaq Green Master Mix (Promega). Primers used for expression analysis were as following: pre-miR31—sense, 5′-GGAGAGGAGGCAAGATGCTG-3′; pre-miR31—antisense, 5′-GGAAAGATG GCAATATGTTG-3′: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) —sense, 5′-CTCATGACCACAGTCCATGC-3′: GAPDH—antisense, 5′-TTACTC CTTGGAGGCCATGT-3′: MET—sense, 5′-GGTGAAGTGTTAAAAGTTGGA-3′: MET—antisense, 5′-ATGAGGAGTGTGTACTCTTG-3′: MET 3′-UTR—sense, 5′-TTGAGTTTGGCTGTTGTTGC-3′; MET 3′-UTR—antisense, 5′-CCTGTTGAT GGGATGTTTCC-3′.

Human ovarian tumors

Tumor specimens from patients with ovarian cancer were obtained from Hokkaido University Hospital under institutional review board-approval. Informed consent was obtained from each patient. Patients treated at Hokkaido University Hospital between 1999 and 2010 were eligible. All samples were obtained at the initial surgery. miRNA was extracted by RecoverAllTM Total nucleic Acid Isolation Kit (Ambion) from formalin-fixed, paraffin-embedded tissues, of which epithelial tumors were confirmed by microscopical examination, and miR-31 was detected by quantitative real-time PCR described above.

Immunohistochemistry

The formalin-fixed, paraffin-embedded tissues were used for detection of MET expression. The sections were incubated with anti-MET rabbit monoclonal antibody (EP1454Y) (Abcam, Cambridge, UK) with 1:250 dilution. All of the slides were reviewed by three full-boarded pathologists without knowledge of the clinical data. Immunohistochemical positivities were evaluated by proportion and intensity. For analysis of proportion, four tired evaluation was applied as 0 to 3; as no staining (0), 1–10% (1), 11–50% (2) and 51–100% of tumor cells (3). For evaluation of intensity, there are following four criteria: as negative (0), weak (1), intermediate (2) and strong (3). MET immunohistochemistry score was shown as sum of proportional and intensity scores (0 to 6).

Analysis of tumor-forming potential *in vivo*

All experiments were conducted in accordance with guidelines authorized by the Animal Research Committee Hokkaido University. Six-week-old BALB/c nude mice (Clea, Tokyo, Japan) were injected subcutaneously into their flanks with 2×10^7 KFr13Tx mock or KFr13Tx miR-31 cells in 200 μl of matrigel (BD Biosciences, San Jose, CA, USA) and 50 μl of normal culture medium. PTX and/or SU11274 was administered intraperitonealy at 10 mg/kg, respectively, in 300 μl of normal culture medium on day 1. All mice were killed on day 28 and tumor weight was measured. In another experiment, six-week-old BALB/c nude mice were intraperitonealy injected with 2×10^7 KFr13Tx mock or miR-31 cells. PTX was administered intraperitonealy at 10 mg/kg with or without 3 μM of SU11274, respectively, in 300 μl of normal culture medium on day 1 or weekly (days 7, 14 and 21).

Statistical analysis

Data were presented as mean ± s.e.m., and unpaired two-tailed Student's *t*-test was used for comparisons, with $P<0.05$ considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Tumor volume successively reflects the state of disease progression in endometrial cancer

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HIGHLIGHTS

- Tumor volume successively reflects the state of progression of endometrial cancer.
- Tumor volume is an independent prognostic factor of endometrial cancer.

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ABSTRACT

Objective. This study aimed to clarify the clinical significance of tumor volume in endometrial cancer.

Methods. A total of 667 patients with endometrial cancer who underwent preoperative MRI and surgical treatment including lymphadenectomy were enrolled. As the surrogate marker of actual tumor volume, the volume index was defined as the product of the maximum longitudinal diameter along the uterine axis, the maximum intersecting anteroposterior diameter of the sagittal section image, and the maximum horizontal diameter of the horizontal section image from the MRI data. The volume index was divided into five categories: Group 1 (<8), Group 2 (8 to <27), Group 3 (27 to <64), Group 4 (64 to <125), and Group 5 (125 or more). The relationships between various clinicopathologic factors and volume index were investigated, and Cox regression analysis was conducted to assess the significance of volume index with respect to prognosis.

Results. High-risk clinicopathologic findings increased with tumor volume. The lymph node metastasis rate was 3% in Group 1, 9% in Group 2, 17% in Group 3, 25% in Group 4, and 53% in Group 5. Cox regression analysis showed that the volume index (≥36) was a prognostic factor (hazard ratio: 2.0, 95% confidence interval: 1.3–3.1) independent of older age (≥58 years), high-risk histological grade/subtype, deep myoinvasion, lymph node metastasis, and type of surgery.

Conclusion. Tumor volume successively reflects the state of disease progression in endometrial cancer. The volume index can give information on both the staged prognosis and surgical management.

Introduction

There has been controversy between the systemic theory and the spectrum theory in terms of metastatic progression, especially in the field of breast cancer. Fisher stated that breast cancer is a systemic disease and that variations in local regional treatment are unlikely to substantially affect survival [1]. In the systemic theory, lymph node involvement is not an orderly, contiguous extension, but rather a marker of distant disease. On the other hand, Hellman stated that breast cancer is a heterogeneous disease that can be thought of as a clinical spectrum extending from a disease that remains local throughout its course to one that is systemic when first detectable [2]. Lymph node involvement can be regarded as the source of distant disease, and there can be clinical situations in which lymph nodes are involved but distant disease has not yet developed. In contrast to the systemic theory, locoregional therapy is important in the spectrum theory. Many physicians regard breast cancer as a systemic disease. Therefore, lymphadenectomy for patients with breast cancer is currently performed less commonly than in the past.

Which theory accounts for metastatic progression of endometrial cancer? In 2000, Mariani et al. showed that the 5-year overall survival (OS) for patients who had undergone para-aortic lymphadenectomy was 77% compared with 42% for patients who had not undergone para-aortic lymphadenectomy according to data of patients with lymph node metastasis [3]. In 2007, Fujimoto et al. reported that the 5-year disease-related survival for patients who had undergone para-aortic

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lymphadenectomy was 69% compared with 54% for patients who had not undergone para-aortic lymphadenectomy according to data of patients with lymph node metastasis [4]. In 2011, the author and colleagues reported that age (≥58 years), number of metastatic lymph nodes (≥4), type of lymphadenectomy (excluding para-aortic lymphadenectomy), and type of adjuvant therapy (excluding chemotherapy) were significantly and independently related to poor survival according to data of patients with FIGO stage IIIc endometrial cancer [5]. These studies suggest that lymph node metastasis is associated with a wide spectrum of prognoses for endometrial cancer and that a significant proportion of patients with high-risk endometrial cancer can be cured by appropriate removal of regional lymph nodes even if some lymph nodes are already affected. It seems that the spectrum theory gives a more reasonable explanation for metastatic progression of endometrial cancer than does the systemic theory. Hellman insisted on validity of the spectrum theory using two concepts: metastagenicity and virulence [2]. Metastagenicity refers to the ultimate likelihood of distant metastasis, and virulence refers to the pace or rate of metastatic dissemination. Hellman stated that tumor size is the most important parameter indicating both metastagenicity and virulence. In this article, we discuss the clinical significance of tumor volume in endometrial cancer.

Materials and methods

Patients

This study was carried out using data of 667 patients with endometrial carcinoma for whom tumor volume was preoperatively confirmed and extensive surgical staging was performed from 1993 to 2005 at 13 hospitals. All patients underwent lymphadenectomy in addition to hysterectomy and bilateral salpingo-oophorectomy. The common iliac, external iliac, internal iliac, obturator, circumflex iliac, parametrial, and sacral lymph node groups in the pelvic area were all dissected. Although para-aortic lymph nodes that were inferior to the level of the inferior mesenteric artery and para-aortic lymph nodes that were superior to the inferior mesenteric artery up to the renal vessels were dissected, para-aortic lymphadenectomy was performed at the discretion of the surgeon. Approval by each institutional review board was separately obtained from the hospitals' ethics boards.

Postoperative risk assessment

Risk of recurrence was assessed by the International Federation of Gynecology and Obstetrics (FIGO) (2008) staging, grade/histology, peritoneal cytology, and lymphovascular space invasion. Patients with FIGO stage III or IV disease were classified as high risk; those with FIGO stage IA disease, grade 1 to 2 tumors, negative peritoneal cytology, and no lymphovascular space invasion were classified as low risk; and those with all other tumors were classified as intermediate risk. Intermediate- and high-risk patients were offered adjuvant therapy with either radiotherapy or chemotherapy. Radiotherapy was performed using whole-pelvis external beam radiation (50 Gy/25 Fr), and chemotherapy comprised a platinum-based regimen for four to six cycles.

Assessment of tumor volume

Tumor volume was substituted by volume index, which was obtained from MRI data in a preoperative setting. The index was defined as the product of the maximum longitudinal diameter (cm) along the uterine axis; the maximum anteroposterior diameter (cm), namely the thickness, on a sagittal section image; and the maximum horizontal diameter (cm) on a horizontal section image (Fig. 1) [6,7]. Because measurement of the volume index is in cubic centimeters, the authors divided the index into five categories based on the third power of 2, 3, 4, and 5: Group 1 (<8), Group 2 (8 to <27), Group 3 (27 to <64), Group 4 (64 to <125), and Group 5 (125 or more).

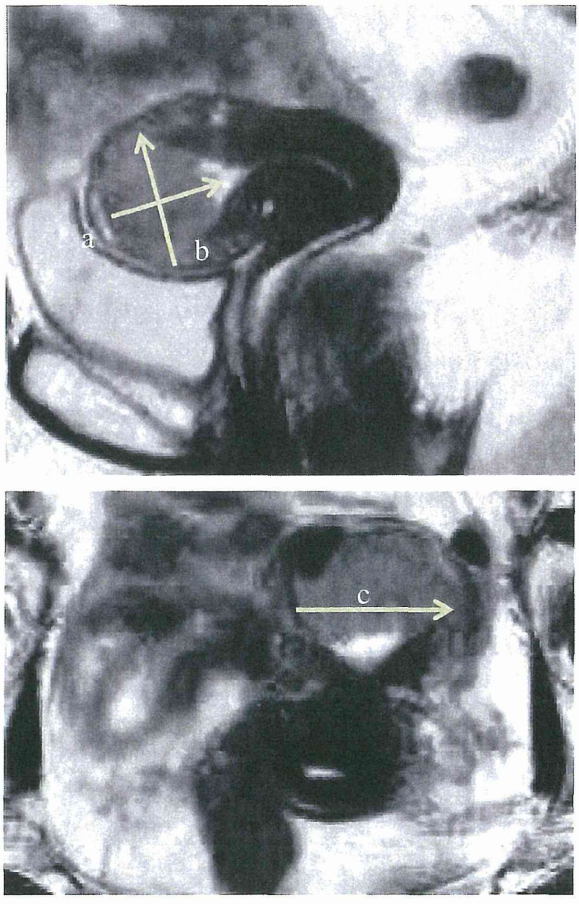


Fig. 1. Volume index, defined as the product of the maximum longitudinal diameter (a) along the uterine axis, the maximum anteroposterior diameter (b) of the sagittal section image, and the maximum horizontal diameter (c) of the horizontal section image. The volume index of the upper image was 34.

Clinicopathologic factors for survival analysis

The clinicopathologic factors examined were patient's age (less than the median vs. the median or higher), histology (G1/G2 vs. G3/others), myometrial invasion (<50% vs. ≥50%), lymph node metastasis (negative vs. positive), volume index (<36 vs. ≥36), type of lymphadenectomy (pelvic lymphadenectomy vs. pelvic lymphadenectomy + para-aortic lymphadenectomy), and type of adjuvant therapy (none/radiation therapy vs. chemotherapy). The volume index cut-off value was determined based on the results of a previous paper [7]. In the literature, measurement of the volume index was used to obtain a receiver operating characteristic curve for lymph node metastasis. Based on this curve, the cut-off value for lymph node metastasis was 36.

Statistical analysis

Correlations of variables were evaluated with the chi-square test. Survival outcome measure was overall survival (OS), defined as the time from initial treatment to death of any cause. Patients known to still be alive or lost to follow-up at the time of analysis were censored at their last follow-up. Survival rates were estimated by the Kaplan–Meier method. The log-rank test was used to compare survival curves. Logistic regression analysis was used to demonstrate the relationship between the measured volume index and the incidence of recurrence with an odds

ratio. Cox regression analysis was used to demonstrate the relationship between the measured volume index and the censored OS with a hazard ratio. Cox regression analysis was also used to select the risk factors for prognosis with hazard ratios. We regarded p values of <0.05 to be statistically significant. For multiple comparisons of the percentages of several clinicopathologic factors or survival curves among the five groups of patients, we applied Bonferroni's correction. During the analyses, we regarded p values of <0.005 to be statistically significant. Statistical analyses were performed with StatView-J 5.0 (SAS Institute, Cary, NC).

Results

The clinical and pathological characteristics of the patients are shown in Table 1. A total of 509 patients (76.3%) were in stage I (FIGO 2008), 40 (6.0%) were in stage II, 106 (15.9%) were in stage III, and 12 (1.8%) were in stage IV. A total of 380 patients (57.0%) had grade 1 endometrioid adenocarcinoma, 156 (23.4%) had grade 2 endometrioid adenocarcinoma, 86 (12.9%) had grade 3 endometrioid adenocarcinoma, 10 had clear cell carcinoma, 23 had serous adenocarcinoma, 3 had other types of carcinoma, and 9 had carcinosarcoma. A total of 228 patients (34.2%) had >50% myometrial invasion. Thirty-one patients (4.6%) had adnexal metastasis, and 94 (14.1%) had lymph node metastasis. Of the 12 patients with stage IV cancer, 9 had lymph node metastasis. A total of 242 patients (36.3%) underwent pelvic lymphadenectomy alone, and 425 patients (63.7%) underwent pelvic and para-aortic lymphadenectomy. In the postoperative risk assessment, 280 patients (42.0%) were classified as low-risk, 269 (40.3%) were classified as intermediate-risk, and 118 (17.7%) were classified as high-risk. In terms of adjuvant treatment, 346 patients (51.9%) did not receive any treatment, 287 (43.0%) received chemotherapy, and 34 (5.1%) received radiation therapy.

Fig. 2 shows the relationships between several pathologic factors and volume index. The percentage of patients with high-risk histological findings increased as the volume index increased. The percentage of patients with Grade 3 endometrioid carcinoma or non-endometrioid carcinoma was 11% in Group 1, 15% in Group 2, 23% in Group 3, 34% in Group 4, and 43% in Group 5, showing a significant difference ($p < 0.0001$). The percentage of patients with deep myometrial invasion increased as the volume index increased. The percentage of patients with deep myometrial invasion was 15% in Group 1, 28% in Group 2, 44% in Group 3, 54% in Group 4, and 82% in Group 5, showing a significant difference ($p < 0.0001$). The percentage of patients with lymph node metastasis increased as the volume index increased. The percentage of patients with metastatic lymph nodes was 3% in Group 1, 9% in Group 2, 17% in Group 3, 25% in Group 4, and 53% in Group 5, showing a significant difference ($p < 0.0001$). The percentage of patients with extrauterine disease increased as the volume index increased. The percentage of patients with extrauterine disease was 5% in Group 1, 13% in Group 2, 21% in Group 3, 35% in Group 4, and 55% in Group 5, showing a significant difference ($p < 0.0001$).

Fig. 3 shows the relationships between treatment-related factors and volume index. There was no significant difference in the frequencies of para-aortic lymphadenectomy ($p = 0.0119$). The percentage of patients who received any adjuvant treatment increased as the volume index increased. The percentage of patients who received adjuvant radiotherapy or chemotherapy was 25% in Group 1, 41% in Group 2, 66% in Group 3, 77% in Group 4, and 89% in Group 5, showing a significant difference ($p < 0.0001$). Fig. 3 also shows the relationships between volume index and incidence of recurrence. The percentage of patients who suffered recurrence was 8% in Group 1, 12% in Group 2, 26% in Group 3, 31% in Group 4, and 28% in Group 5, showing significant differences ($p < 0.0001$). Logistic regression analysis showed a continuous increase in recurrence with an increased volume index and odds ratio of 1.00316 (95% confidence interval, 1.001–1.005; $p = 0.004$). According to these results, odds ratios for patients with a volume index of 8, 27, 64, and 125 were 1.03, 1.09, 1.22, and 1.48, respectively. Fig. 4 shows the Kaplan–Meier curves and log-rank test results according to the volume index (log-rank test; $p < 0.0001$). The 5-year survival rate was 93.8% in Group 1, 93.5% in Group 2, 83.3% in Group 3, 82.2% in Group 4, and 77.7% in Group 5. Cox regression analysis showed a continuous decrease in OS with an increased volume index and hazard ratio of 1.00284 (95% confidence interval, 1.001–1.005; $p = 0.002$). According to these results, hazard ratios for patients with a volume index of 8, 27, 64, and 125 were 1.02, 1.08, 1.20, and 1.43, respectively.

Table 2 shows the results of Cox regression analysis of prognostic factors for patients with endometrial cancer who received lymphadenectomy. Univariate analysis confirmed that age (58 years or older), histological grade/subtype (Grade 3/non-endometrioid), myometrial invasion (≥50%), large tumor volume (volume index of ≥36), lymph node metastasis (positive), and type of adjuvant therapy (no para-aortic lymphadenectomy) were related to poor survival. Multivariate analysis confirmed that age (hazard ratio [HR]: 1.8, 95% confidence interval [CI]: 1.2–2.8), histological grade/subtype (HR: 1.7, 95% CI: 1.1–2.7), myometrial invasion (HR: 1.8, 95% CI: 1.1–2.9), volume index (HR: 2.0, 95% CI: 1.3–3.1), lymph node metastasis (HR: 2.9, 95% CI: 1.8–4.8), and type of lymphadenectomy (HR: 0.5, 95% CI: 0.3–0.7) were independent prognostic factors.

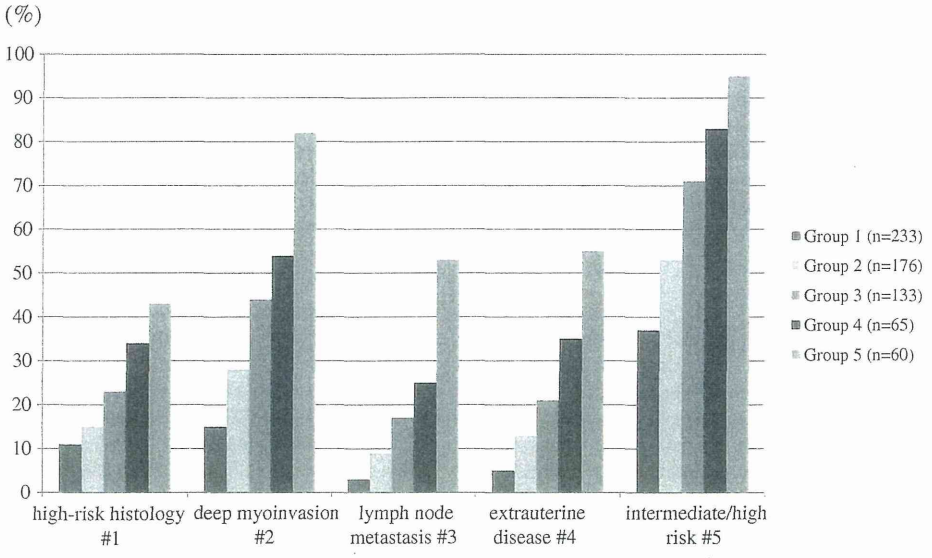
Table 1
Clinical backgrounds of the patients included in the study.

	Number		Number
Age (years)		Lymph node metastasis	
Median	57	Negative	573
(IQR)	(51–63)	Positive	94
FIGO surgical stage (2008)		PLN	87
1	509	PAN	46
2	40	Type of lymphadenectomy	
3	106	PLX	242
4	12	PLX + PALX	425
Tumor grade/histology		Lymph node count	
Endometrioid		Median total (IQR)	57 (34–83)
G1	380	PLX	34
G2	156	PLX + PALX	75
G3	86	Median PLN (IQR)	45 (31–59)
Non-endometrioid		PLX	34
Serous	23	PLX + PALX	52
Clear	10	Median PAN (IQR)	8 (0–25)
Others	3	PLX	0
Carcinosarcoma	9	PLX + PALX	22
Myometrial invasion		Postoperative risk assessment#	
<1/2	439	Low	280
≥1/2	228	Intermediate	269
Adnexal involvement		High	118
Negative	636	Adjuvant therapy	
Positive	31	None	346
		Chemotherapy	287
		Radiation therapy	34

IQR: interquartile range, FIGO: International Federation of Gynecology and Obstetrics, PLN: pelvic lymph node, PAN: para-aortic lymph node, PLX: pelvic lymphadenectomy, PALX: para-aortic lymphadenectomy, # Low: FIGO stage IA disease, grade 1 to 2 tumors, negative peritoneal cytology, and no lymphovascular space invasion, High: FIGO stage III or IV disease, Intermediate: all other tumors except for Low and High.

Discussion

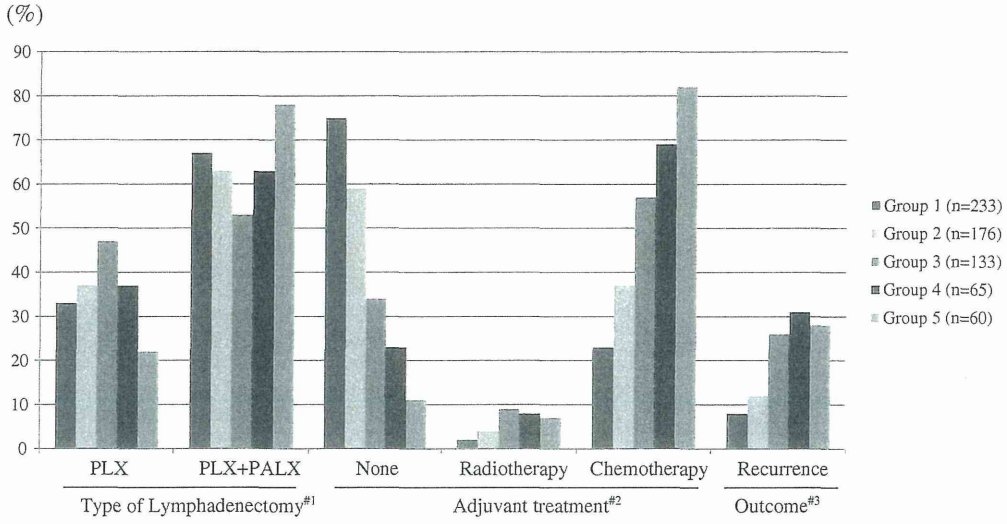
Tumor size is one factor that determines the stage of disease in many types of cancer, including head and neck cancer, breast cancer, lung cancer, renal cancer, uterine cervical cancer, vulvar cancer, uterine sarcoma, melanoma, and soft tissue sarcoma [8]. However, tumor size is not involved in determining the stage of endometrial cancer [8]. The authors focused on tumor volume (size) in the present study because the spectrum theory seems to give a more reasonable explanation for metastatic progression of endometrial cancer than does the systemic theory. Hellman, who is the leader among the spectrum theory supporters, stated that tumor size is the most important parameter indicating metastagenicity, which indicates the ultimate likelihood of distant metastasis and virulence, which indicates the pace of metastatic dissemination. Koscielny et al. showed the relationship between tumor



high-risk histology: Grade 3 endometrioid/Non-endometrioid, #1: p<0.0001, #2: p<0.0001, #3: p<0.0001, #4: p<0.0001, #5: p<0.0001

Fig. 2. Relationship between clinicopathologic factors and volume index.

size and clinical manifestations, including initial metastasis and eventual metastasis, using the data of almost 3000 patients with breast cancer before the routine use of adjuvant chemotherapy [9]. They clearly showed that either initial or eventual metastasis is a continuous function of tumor size. Quiet et al. reported that tumor size is of prognostic importance in patients with fewer than four positive nodes and that four or more positive nodes are an indicator of probable systemic disease according to the data of more than 500 patients with breast cancer and lymph node metastasis [10]. We attempted to demonstrate the metastagenicity and virulence of endometrial cancer and divided the tumor volume measurements into five groups. First, we showed the percentages with which a known prognostic factor is identified in each group. Successive and proportional increases in the percentages were observed, as if arranged in a spectrum, as the tumor volume increased. The percentage of patients who suffered recurrence was 8%, 12%, 26%, 31%, and 28% in Groups 1 to 5, respectively ($p < 0.0001$). The findings suggest initial and eventual metastagenicity with increase of tumor volume in endometrial cancer. Second, we showed the relationship between tumor volume and prognosis. The 5-year survival rate was 93.8%, 93.5%, 83.3%, 82.2%, and 77.7% in Groups 1 to 5, respectively ($p < 0.0001$). These results also showed a spectral arrangement. Nevertheless, the number of patients who underwent para-aortic lymphadenectomy and any adjuvant treatment increased as the volume index increased. These findings suggest virulence of endometrial cancer. The natural history of endometrial cancer does not rapidly worsen at any certain time, but may gradually and successively become aggressive as the tumor volume increases. Moreover, in the present analysis, complete removal of regional lymph nodes was significantly related to improved survival independent of nodal metastasis. This result also suggests that the spectrum theory accounts for the disease properties of endometrial cancer. Mariani et al. reported a significant prognostic difference between tumors of <2 cm and tumors of ≥2 cm in patients with low-risk endometrial cancer and



PLX: pelvic lymphadenectomy, PALX: para-aorticlymphadenectomy, #1: p=0.0119, #2: p<0.0001, #3: <0.0001

Fig. 3. Relationship between treatment/outcome-related factors and volume index.

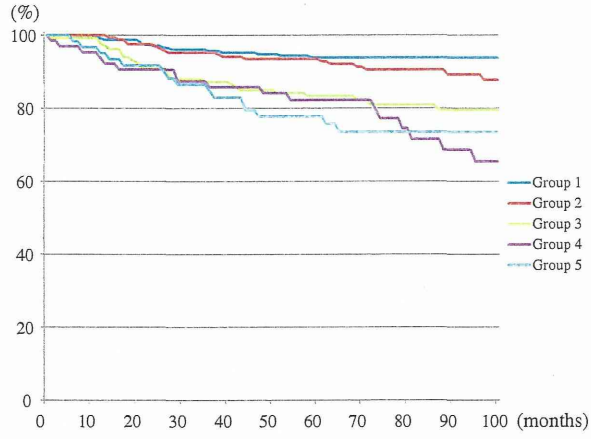


Fig. 4. Overall survival (OS) curves for patients in Group 1 (volume index: <8), patients in Group 2 (volume index: 8 to <27), patients in Group 3 (volume index: 27 to <64), patients in Group 4 (volume index: 64 to <125), patients in Group 5 (volume index: 125 or over). The 5-year survival rate was 93.8% in Group 1, 93.5% in Group 2, 83.3% in Group 3, 82.2% in Group 4, and 77.7% in Group 5 (log-rank test; $p < 0.0001$).

stated that the latter group requires either lymphadenectomy or adjuvant radiation therapy [11]. They stated that lymphadenectomy may be omitted under the following conditions: (1) endometrioid histology (grade 1 or 2), myometrial invasion of <50%, and tumor diameter of <2 cm; or (2) endometrioid histology but no myometrial invasion (independent of grade and tumor diameter). Many gynecologic oncologists regard these conditions to be important and have termed them the Mayo criteria.

Although patients with non-endometrioid histological results were included in the present study, one may consider that uterine papillary serous carcinoma is a systemic disease independent of tumor size. Some previous studies reported that uterine papillary serous carcinoma has a poor prognosis because the 5-year survival rates were less than 50% even in patients with early stage [12–13]. However, most patients did not undergo surgical evaluation of regional lymph nodes in such studies. On the other hand, some studies reported that the 5-year survival rates were more than 80% in FIGO stages I–II uterine papillary serous carcinoma confirmed by surgical evaluation of lymph nodes [14–15]. Thomas et al. showed the possibility that lymphadenectomy has a survival benefit in patients with stage I uterine papillary serous carcinoma [16]. Some patients with early stage uterine papillary serous carcinoma would benefit from complete removal of regional lymph nodes. Further, in the present study, tumor volume was a prognostic factor independent of histological subtype. Although uterine papillary serous carcinoma may be a systemic disease in some patients, it remains local throughout its course in other patients. The spectrum theory may also account for the biological behavior of uterine papillary serous carcinoma.

Tumor volume indicates the ultimate likelihood and pace of metastatic dissemination in endometrial cancer. Therefore, tumor volume has the role of a decision-making tool for surgical treatment. A Gynecologic Oncology Group study reported by Milam et al. confirmed the efficacy of tumor diameter in predicting lymph node metastasis [17]. However, tumor diameter was measured postoperatively using resected uterine specimens. Naturally, postoperative assessment is not available for decision-making of surgical treatment and recruitment into prospective clinical trials. Preoperative assessment is integral to settling this issue. When planning a prospective clinical trial on the therapeutic significance of lymphadenectomy, an adequate population in which low-risk patients are effectively excluded is needed to assess the full benefit of lymphadenectomy. If a population comprises a greater proportion of low-risk patients, the therapeutic significance of lymphadenectomy would be underestimated because low-risk patients do not benefit from lymphadenectomy. The author and colleagues reported that tumor volume preoperatively assessed by MRI, which is called the volume index in this article, is effective for predicting lymph node metastasis [6, 7]. Because it is difficult to evaluate tumor volume in the resected uterus, tumor volume has not been included in previous histopathologic analyses to determine independent risk factors of lymph node metastasis. However, MRI can provide a surrogate marker of tumor volume. Tumor volume preoperatively assessed by MRI can give information on not only the staged prognosis, but also surgical management.

Our analysis has limitations as follows: (1) Implementation of lymphadenectomy was not standardized among some of the institutions. (2) Implementation of para-aortic lymphadenectomy was also not standardized among some of the institutions. (3) Type of adjuvant treatment (radiation or chemotherapy) differed among the 13 institutions. (4) The number of affected lymph nodes was not assessed because of lack of information in several institutions. (5) Reproducibility in regard to measurement of tumor volume, namely interobserver variability has not yet been validated.

Tumor volume successively reflects the state of disease progression in endometrial cancer, suggesting that the spectrum theory accounts for the disease properties of endometrial cancer. If so, locoregional treatment including lymphadenectomy may be integral to improving the prognosis for many patients with endometrial cancer. Because tumor volume has a natural aptitude for preoperative risk assessment, it may have an important role within the framework of treatment strategies for endometrial cancer. Fortunately, an increasing number of patients worldwide are able to undergo MRI examination; therefore, it is becoming easy to obtain information regarding tumor size in a preoperative setting. The cost of routine MRI before surgery for endometrial cancer may be high. If MRI is not available, ultrasound examination may replace MRI.

Table 2
Results of Cox regression analysis of prognostic factors for endometrial cancer patients who received lymphadenectomy.

	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	p-Value	Hazard ratio (95% CI)	p-Value
Age				
57 or younger	1.0			
58 or older	1.71 (1.14–2.58)	0.0096	1.83 (1.21–2.77)	0.0044
Histological grade/subtype				
Grade 1/Grade 2	1.0			
Grade 3/non-endometrioid	3.08 (2.03–4.65)	<0.0001	1.72 (1.11–2.66)	0.015
Myometrial invasion				
Less than half	1.0			
Half or more	3.68 (2.42–5.59)	<0.0001	1.78 (1.09–2.90)	0.020
Volume index				
Less than 36	1.0			
36 or more	3.44 (2.28–5.19)	<0.0001	1.98 (1.25–3.13)	0.0036
Lymph node metastasis				
Negative	1.0			
Positive	5.29 (3.51–8.00)	<0.0001	2.94 (1.80–4.81)	<0.0001
Type of surgery (lymphadenectomy)				
PLX	1.0			
PLX + PALX	0.55 (0.37–0.82)	0.0033	0.45 (0.30–0.68)	0.0002

CI: confidence interval, PLX: pelvic lymphadenectomy, PALX: para-aortic lymphadenectomy.

Conflict of interest statement
The authors declare that they have no conflicts of interest.

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INTRODUCTION TO REVIEW ARTICLES

Recent advances in research on epigenetic alterations and clinical significance of para-aortic lymphadenectomy in endometrial cancer: an introduction

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Endometrial cancer is the most frequent cancer of the female reproductive organs in industrialized countries. In 2012, the numbers of new cases and deaths from endometrial cancer in the US were estimated to be 47,130 and 8,010, respectively [1]. The incidence of endometrial cancer is also increasing steadily in Japan, where the estimated number of new cases in 2007 was 9,104 [2] and the number of deaths in 2011 was 2,034 [3]. Endometrial cancer is a surgically staged disease and post-operative therapy is offered to patients with a high risk of recurrence according to the extent and aggressiveness of the tumor. Current topics in endometrial cancer include: the therapeutic significance of lymphadenectomy, the role of epigenetic alterations, and revision of the International Federation of Gynecology and Obstetrics staging criteria (FIGO 2008) for this disease.

There was a paradigm shift in the treatment strategy for endometrial cancer after the introduction of a surgical staging system (FIGO 1988) that replaced the older clinical staging system. The newer paradigms of extended-surgical staging containing lymphadenectomy with more restricted use of adjuvant therapy and the older paradigm of simple hysterectomy bilateral salpingo-oophorectomy with more frequent use of adjuvant radiotherapy need to be compared prospectively in terms of survival benefits, quality of life, and cost of treatment [4]. Several issues regarding surgical staging need to be clarified. They include: how should suitable patients for complete lymphadenectomy be selected and what is the optimal extent of lymphadenenctomy?

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The therapeutic significance of lymphadenectomy has long been a matter of great debate. In 1964, Lewis suggested a therapeutic effect of pelvic lymphadenectomy in node-positive patients [5]. He employed pelvic lymphadenectomy because endometrial cancer often recurred at the pelvic side wall after conventional hysterectomy and bilateral salpingo-oophorectomy, which suggested inadequate primary surgery. Retrospective studies suggest a therapeutic significance for lymphadenectomy, which is a function of removed lymph node count (thoroughness) and area of dissection (pelvic only versus pelvic and para-aortic lymphadenectomy) [6–8]. However, two prospective randomized controlled trials (RCTs) that intended to prove the therapeutic role of pelvic lymphadenectomy failed to show any survival advantage of pelvic lymphadenectomy versus no lymphadenectomy [9, 10]. However, there has been some criticism about the design of these trials because para-aortic lymphadenectomy was not included in the study arm. A retrospective cohort study which compared pelvic lymphadenectomy with combined pelvic and para-aortic lymphadenectomy revealed survival improvement in the pelvic and para-aortic lymphadenectomy group if this treatment was offered to intermediate-/high-risk endometrial cancer patients [11]. Based on these findings, discussions have begun about the design of future clinical trials to validate the therapeutic significance of lymphadenectomy. Topics for discussion include the eligibility of patients (all patients or selected patients at some risk of nodal metastasis), extent of lymphadenectomy (area: pelvic alone versus pelvic plus para-aortic, thoroughness: number of nodes removed), and type of experimental design (RCT versus cohort study).

The difficulties and pitfalls of RCTs for validating surgical procedures have often been addressed [12–16]. These include the participating surgeons' expertise in experimental

procedures and non-participation of experienced surgeons. Surgeons need to be conversant with both control arm procedures and study arm procedures. If a study arm includes a complex procedure that requires intensive training and if many of the participating surgeons are not familiar with the complex procedure, systemic bias may exist in favor of operations that are in wide use and may favor technically simple procedures [12]. Research in surgery is disadvantaged by the limited quality and quantity of randomized trials of surgical techniques [14]. A preliminary phase 2 surgery study before conducting a RCT or a well-designed prospective cohort study may be a possible solution for this problem [14–16].

Another important issue regarding endometrial cancer is the diversity of aggressiveness of the cancer and its underlying molecular alterations. Histological subtype of endometrial cancer is a strong prognostic factor. Based on the clinicopathological studies, the concept of two different pathogenetic types of endometrial cancer was proposed [17, 18]. Although there may be criticism that this model is an oversimplification, this concept is now widely accepted. Type 1 is represented by endometriod G1/G2 tumors and Type 2 is represented by serous adenocarcinoma and clear cell adenocarcinoma. Type 1 tumors have a relatively favorable prognosis, are related to unopposed estrogen, often coexist with endometrial hyperplasia, and are frequently associated with the phosphatase and tensin homolog (PTEN) mutation. Serous adenocarcinoma, the prototype of the Type 2 tumor, occurs among elderly women, is associated with a poor prognosis, exhibits no estrogen dependency, and is frequently (>90 %) associated with a p53 mutation. There is controversy regarding whether endometriod G3 tumors should be included in the Type 1 or Type 2 category [19, 20]. A p53 mutation has been suggested to be an independent prognostic factor for endometrial cancer and a dominant-negative mutation of the p53 tumor suppressor gene may play a critical role in the poor survival of patients irrespective of the histological subtype of the tumor [21, 22]. The expression profile of microRNA has been shown to be different between Type 1 and Type 2 tumors [23]. In clinical practice, these two types of tumors are treated with different treatment strategies [24, 25]. The malignant phenotypes, such as invasiveness, metastatic potential and resistance to therapy, are related to epithelial–mesenchymal transition (EMT) [26]. Recent studies have suggested that EMT may play an important role in the malignant behavior of endometrial cancer and is related to the invasive potential of endometrial cancer cells in vitro [27–29]. For future directions aimed at more personalized treatment strategies for endometrial cancer, further microRNA studies to establish a highly accurate method for diagnosing the aggressiveness of each tumor, as well as the development of novel molecular targeting therapies, are necessary [30].

In this issue, we have invited three distinguished experts to describe recent advances in research on epigenetic alterations and surgical therapy for endometrial cancer. We hope that this special review session will help oncological researchers and physicians from non-gynecological fields to comprehend some of the most important aspects of endometrial cancer.

Conflict of interest The author declares that he has no conflict of interest.

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Proposal of a concept and design of a randomized phase III trial investigating the survival effect of para-aortic lymphadenectomy in endometrial cancer

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Abstract

Although prospective studies have failed to show the therapeutic effect of lymphadenectomy in the surgical treatment of endometrial cancer, several retrospective studies including the SEPAL study revealed the survival effect of lymphadenectomy. To prospectively investigate the survival benefit of para-aortic lymphadenectomy shown in the SEPAL study, we are proposing a new concept of a randomized phase III trial. An appropriate study population will be selected according to the preoperative assessments (evaluation of myometrial invasion and cervical invasion with magnetic resonance imaging, extrauterine spread with computed tomography, and histological type and grade by pathological evaluation) to estimate the risk of lymph node metastasis. Patients relevant to potential International Federation of Gynecology and Obstetrics (2008) stage IB, II and III diseases will be eligible, and randomly assigned to two arms: pelvic lymphadenectomy alone (control), or pelvic and para-aortic lymphadenectomy (experimental). After initial surgery, patients with postoperative pathological risk factors for recurrence will receive adjuvant chemotherapy. Because we aim to investigate the therapeutic significance of primary treatments, including surgery and adjuvant chemotherapy, the primary end-point could be recurrence-free survival. One of the most important issues to successfully perform this prospective study is to assure the quality of lymphadenectomy (extent and area), which could be evaluated based on the number of harvested nodes and objective evaluation of dissected area by videos and/or photos.

Key words: endometrial cancer, lymphadenectomy, prospective study, SEPAL study, survival.

Introduction

Despite the diagnostic role of lymphadenectomy in endometrial cancer, there has been controversy and debate about the therapeutic relevance of systematic pelvic and para-aortic lymphadenectomy. The current recommendation of the National Comprehensive Cancer Network (NCCN),[1] and Japan Society of Gynecologic Oncology[2] is to perform systematic lymphadenectomy rather than merely nodal sampling. Furthermore, the International Federation of Gynecology and Obstetrics (FIGO) staging system in endometrial cancer has recently been changed.[3] In the revised FIGO staging system, para-aortic node (PAN) involvement has been separated from the single substage IIIC. Stage IIIC is now categorized as IIIC1 (indicating positive pelvic lymph node [PLN]) and IIIC2 (indicating positive PAN with or without positive PLN) and IIIC2

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