MOLECULAR CANCER

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# microRNA 31 functions as an endometrial cancer oncogene by suppressing Hippo tumor suppressor pathway

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## Abstract

**Background:** We aimed to investigate whether MIR31 is an oncogene in human endometrial cancer and identify the target molecules associated with the malignant phenotype.

**Methods:** We investigated the growth potentials of MIR31-overexpressing HEC-50B cells *in vitro* and *in vivo*. In order to identify the target molecule of MIR31, a luciferase reporter assay was performed, and the corresponding downstream signaling pathway was examined using immunohistochemistry of human endometrial cancer tissues. We also investigated the MIR31 expression in 34 patients according to the postoperative risk of recurrence.

**Results:** The overexpression of MIR31 significantly promoted anchorage-independent growth *in vitro* and significantly increased the tumor forming potential *in vivo*. MIR31 significantly suppressed the luciferase activity of mRNA combined with the LATS2 3'-UTR and consequently promoted the translocation of YAP1, a key molecule in the Hippo pathway, into the nucleus. Meanwhile, the nuclear localization of YAP1 increased the transcription of CCND1. Furthermore, the expression levels of MIR31 were significantly increased (10.7-fold) in the patients (n = 27) with a high risk of recurrence compared to that observed in the low-risk patients (n = 7), and this higher expression correlated with a poor survival.

**Conclusions:** MIR31 functions as an oncogene in endometrial cancer by repressing the Hippo pathway. MIR31 is a potential new molecular marker for predicting the risk of recurrence and prognosis of endometrial cancer.

**Keywords:** Endometrial cancer, microRNA 31, LATS2, cyclin D1, Hippo pathway

## Background

Endometrial cancer (EC) is the most common malignancy of the female reproductive tract, the annual incidence of which has been estimated to be 10–20 per 100,000 women [1]. Current therapy for EC includes surgery with adjuvant radiation or chemotherapy [2]. The risk of postoperative recurrence is determined based on several factors, such as the surgical stage [3], differentiation [4], lymph node metastasis and lymphovascular space invasion [5]. The 5-year survival rate for FIGO stage I lesions without grade 3 tumor differentiation, myometrial invasion > 50%, cervical involvement and an adenosquamous histology exceeds 90% [6]. However, the 5-year survival rate of patients with stage III and IV disease is dramatically decreased, ranging from 42% [7] to 79% [8]. EC can be divided into two major categories based on clinicopathologic and molecular genetic features. For example, low-grade carcinomas with PTEN mutations associated with endometrial hyperplasia and estrogenic stimulation, including mucinous or low-grade endometrioid tumors with squamous differentiation, are called type I carcinomas. In contrast, high-grade carcinomas with p53 mutations, such as serous carcinomas and clear cell carcinomas, are referred to as type II cancers [9]. It is important to identify new molecular mechanisms underlying the process of endometrial carcinogenesis and discover molecular targets and novel drugs for improving survival.

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microRNAs (MIRs) are endogenous non-coding RNAs of 18 to 25 nucleotides in length that play important roles in regulating the gene expression. The mature forms of MIRs silence the gene expression by binding to the 3'-untranslated region (UTR) of target mRNAs and initiate the translational repression and/or cleavage of cognate mRNAs [10]. MIRs have frequently been implicated in carcinogenesis [11-14]. In the setting of EC, MIR152 [15], MIR194 [16], MIR34b [17], MIR204 [18], MIR145 [19] and MIR129-2 [20] have been reported to be tumor suppressor genes, and MIR125b [21] has been reported to be an oncogene (oncomir). Furthermore, MIR31 has been reported to be an oncomir in various human cancers, including colorectal [22], esophageal [23], lung [24], oral [25] and head and neck [26] cancer, and a tumor suppressor gene in breast [27] and gastric [28] cancers and malignant mesothelioma [29]. However, little is known about the biological functions of MIR31 in EC.

The Hippo pathway is crucial in regulating the size of organs, and its dysregulation contributes to tumorigenesis [30]. Recently, it was reported that deregulation of the Hippo pathway occurs at a high frequency in a broad range of human cancers, including lung [24], hepatocellular [31], colon [32] and prostate cancer [33], and is often correlated with a poor patient prognosis. LATS2 represents a core component in the kinase cascade of the mammalian Hippo pathway. Interestingly, it has been reported that the Hippo pathway is required for anoikis and that the LATS2 expression levels are significantly downregulated in patients with metastatic prostate cancer [33].

In this study, we aimed to investigate whether MIR31 is an oncomir in human EC and identify the direct target associated with the malignant phenotype of EC.

## Results

### MIR31 is correlated with enhanced colony formation of EC cell lines

In order to investigate whether the MIR31 expression is correlated with the tumorigenesis of EC, we performed colony formation assays. We confirmed the MIR31 expression in three EC cell-lines, HEC-50B, HEC-1A and HEC-108, using qRT-PCR and found that the MIR31 levels were lowest in the HEC-108 cells, followed by HEC-1A and HEC-50B cells (Additional file 1: Figure S1, Lanes 1, 2 and 3). The colony number was increased in the same order as the MIR31 expression under two different serum concentrations (Additional file 2: Figure S2).

### The overexpression of MIR31 enhances tumorigenesis in vitro and in vivo

We established HEC-50B cells overexpressing MIR31 by introducing precursor-MIR31 using lentivirus vectors because the MIR31 expression level of HEC-50B was modest among the several adenocarcinoma cell lines analyzed (Additional file 1: Figure S1) and lentivirus vectors can be efficiently transfected into this cell line (HEC-50B mock and MIR31). The presence of a mature-MIR31 expression was confirmed using qRT-PCR (Figure 1a).

Although MIR31 overexpression did not affect *in vitro* cell proliferation under the standard culture conditions (data not shown), it significantly promoted colony formation under serum starvation (Figure 1b). Additionally, an MIR31-specific inhibitor significantly restrained colony formation (Additional file 3: Figure S3). The MIR31-mediated tumorigenic effects were confirmed in an *in vivo* model. A significant increase in tumor weight was observed in the HEC-50B cells with MIR31 overexpression compared with that noted in the controls in the nude mice subcutaneous tumor model (Figure 1c). These findings demonstrate that MIR31 induces a more aggressive phenotype of EC.

### MIR31 reduces the protein levels of LATS2 by inhibiting translation

In order to elucidate the mechanisms by which MIR31 promotes tumorigenesis, *in silico* prediction models were employed to identify the target mRNAs of MIR31 [10]. Among several candidates, we focused on LATS2 because it is a known tumor suppressor gene that has been previously reported to be a direct target of MIR31 [24,34]. One potential binding site for MIR31 was found in the 3'-UTR region of LATS2 mRNA (Figure 2a).

To confirm that LATS2 is a target of MIR31 in HEC-50B cells, the protein levels of LATS2 were analyzed in HEC-50B cells overexpressing MIR31. We found that LATS2 was downregulated in the MIR31-overexpressing cells, whereas LATS2 was increased by the MIR31-specific inhibitor compared with that observed in the control cells in a Western blot analysis (Figure 2b). We next performed a luciferase reporter assay to assess whether MIR31 inhibits the translation of LATS2. The detection of a normalized luciferase activity revealed that MIR31 significantly suppressed the activity of luciferase combined with wild-type LATS2 3'-UTR in the HEC-50B MIR31 cells, whereas no differences were observed following treatment with the control luciferase and LATS2 3'-UTR possessing a mutation in the putative MIR31-binding site (Figure 2c). As no significant differences in the LATS2 mRNA levels were observed between the HEC-50B control and MIR31-overexpressing cells (Additional file 4: Figure S4), MIR31 does not appear to degrade LATS2 mRNA. These results suggest that MIR31 directly binds to LATS2 mRNA and regulates the LATS2 protein expression via translational inhibition.

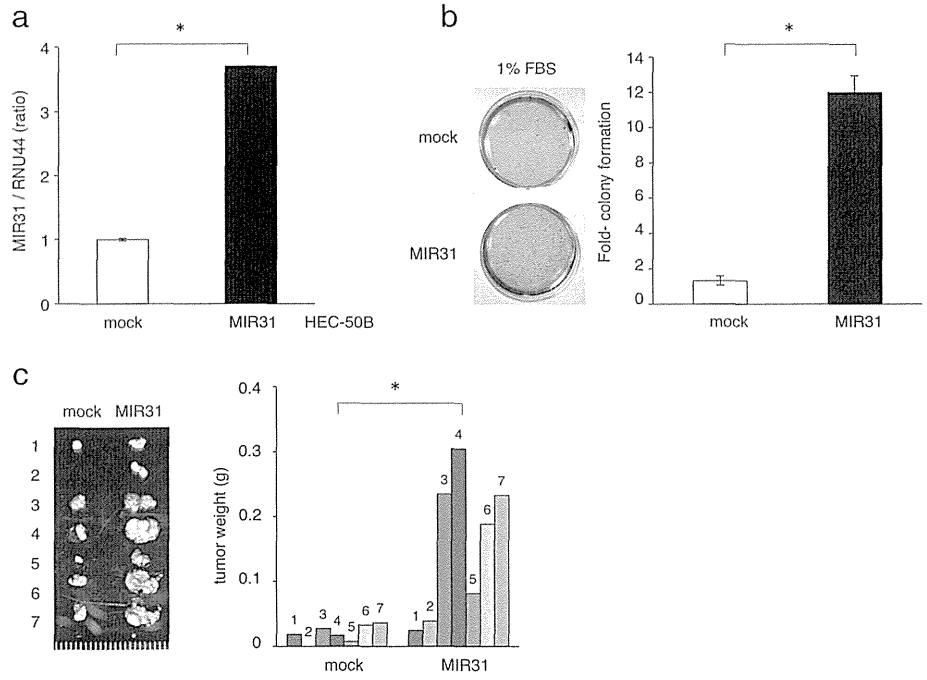


**Figure 1 The overexpression of MIR31 enhanced tumorigenesis *in vitro* and *in vivo*.** **(a)** Establishment of HEC-50B-expressing MIR31 cells. The results of the qRT-PCR analysis of the expression levels of MIR31 are shown in the bar graph. *p < 0.05, unpaired two-tailed Student's *t*-test. The experiments were performed in triplicate. **(b)** Colony formation assay with 1% FBS. Representative stained colonies are displayed in the left panel. *p < 0.05, unpaired two-tailed Student's *t*-test. The experiments were performed in triplicate. **(c)** Subcutaneous tumors in the seven nude mice are displayed in the left panel. The weights of tumors are shown in the right bar graph. *p < 0.05, paired two-tailed Student's *t*-test.

### Downregulation of LATS2 contributes to tumorigenesis in HEC-50B cells

In order to investigate whether the downregulation of LATS2 is responsible for the enhanced colony-forming ability of HEC-50B cells, the expression of LATS2 was suppressed by two different short hairpin RNAs (shRNAs) (Figure 2d bottom), and the treated cells were evaluated for tumorigenesis using a colony formation assay under low serum concentrations. After 12 weeks of severe starvation (incubation with 1% fetal bovine serum (FBS)), increased colony formation was clearly observed in the cells with LATS2 suppression, whereas treatment with nonspecific shRNA did not affect colony formation (Figure 2d top). We observed the same findings following treatment with 5% FBS for four weeks (Additional file 5: Figure S5). These results suggest that the suppression of the LATS2 expression induced by MIR31 contributes to enhanced tumorigenesis.

### MIR31 promotes the transcription of cyclin D1 (CCND1) via dysregulation of the Hippo signaling pathway

The Hippo tumor suppressor pathway regulates several cellular functions, including proliferation, survival and metastasis. In the Hippo pathway, the transcriptional co-activator YAP1 translocates into the nucleus, where it promotes the transcription of several target genes associated with proliferation and anti-apoptosis. LATS2 is the key molecule in this pathway and promotes the phosphorylation of YAP1. The phosphorylation of YAP1 by LATS2 inhibits the translocation of YAP1 into the nucleus and thus prevents the transcription of YAP1 target genes [30]. Therefore, when LATS2 is suppressed by MIR31, it is expected that the translocation of YAP1 into the nucleus would be promoted. In order to confirm the YAP1 translocation, we performed immunofluorescence. The cells successfully transfected with control or precursor MIR31 vectors expressed Green Fluorescent Proteins (Figure 3a top), and YAP1 was stained with Cy5 (Figure 3a bottom). As expected, we found that the nuclear translocation of YAP1 frequently occurred in the MIR31-overexpressing cells compared with that observed in the control cells (Figure 3b). In addition, YAP phosphorylation was either not different or slightly increased in the HEC-50B MIR31 cells compared with that observed in the HEC-50B cells (not significant, Additional file 6: Figure S6). These results suggest that the translocation of YAP1 into the nucleus is the most important effect of MIR31.

Because we hypothesized that nuclear YAP1 promotes the transcription of several anti-apoptosis and pro-proliferation genes, we analyzed the expression levels of several proteins, including CCND1, RAS, XIAP, cyclin E1, MYC, KIT, JNK, AKT, FAS, FADD, FASLG and

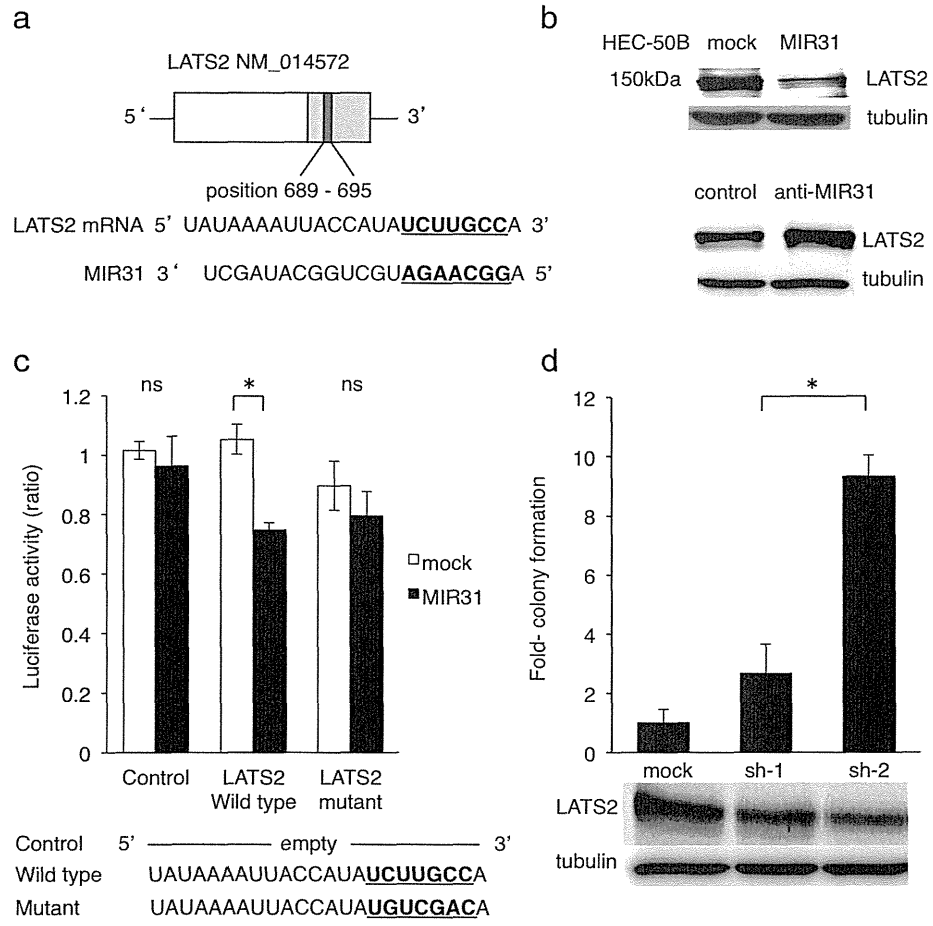


**Figure 2 MIR31 regulates the LATS2 expression by inhibiting translation. (a)** The potential binding site for MIR31 in 3'UTR of LATS2 mRNA. **(b)** The expression level of LATS2 in the mock and MIR31-overexpressing cells (top). The LATS2 expression was increased by the MIR31-specific inhibitor (bottom). The results of immunoblotting for LATS2 and α-tubulin are shown. **(c)** The luciferase activity after transfection of the indicated 3'-UTR-driven reporter constructs. Reporter plasmids containing no oligonucleotides as a Control, the wild-type 3'UTR region of LATS2 as a Wild type and the mutant 3'UTR region as a Mutant. *$p < 0.05$, unpaired two-tailed Student's *t*-test. **(d)** Colony formation assay (bar graph) and immunoblotting for LATS2 and α-tubulin following shRNA transfection (bottom panel). *$p < 0.05$, unpaired two-tailed Student's *t*-test. All experiments were performed in triplicate.

BCL2, in the HEC-50B MIR31-overexpressing and control cells using immunoblotting (Additional file 7: Figure S7). We found the MIR31 overexpression to be associated with increased CCND1, RAS and XIAP expression levels (Figure 3c, Additional file 8: Figure S8a top) and they also decreased in the anti-MIR31-oligonucleotide-induced cells (Figure 3c, Additional file 8: Figure S8a bottom). Because these results strongly suggest that nuclear YAP1, which is increased by the MIR31 expression, promotes the transcription of these targets, we performed luciferase reporter assays in order to investigate the influence of MIR31 on the transcription of CCND1, RAS and XIAP. The detection of a normalized luciferase activity revealed that the MIR31 expression significantly increased the activity of luciferase driven by the CCND1, RAS and XIAP promoters compared with that observed in the control cells (Figure 3d Line 1–2, Additional file 8: Figure S8b). In addition, we focused on the CCND1 expression and investigated the influence of nuclear YAP1 overexpression on the CCND1 expression. HEC-50B cells were transfected with a YAP1 expression vector, the results of which confirmed that nuclear YAP1 was overexpressed on immunofluorescence (Additional file 9: Figure S9). As expected, YAP suppression by anti-YAP siRNA significantly decreased the activity of luciferase driven by the CCND1 promotor, and the expression of CCND1 was increased by YAP1 overexpression (Figure 3d, Lines 2–3, Figure 3e).

We also found a correlation between the MIR31, LATS2 and CCND1 expression *in vivo* (mouse 7 in Figure 1c). The LATS2 expression was increased in the control cell tumors compared with that observed in the MIR31-expressing tumor cells, and the CCND1 levels were increased in the tumors formed from MIR31-expressing cells (Figure 3f).

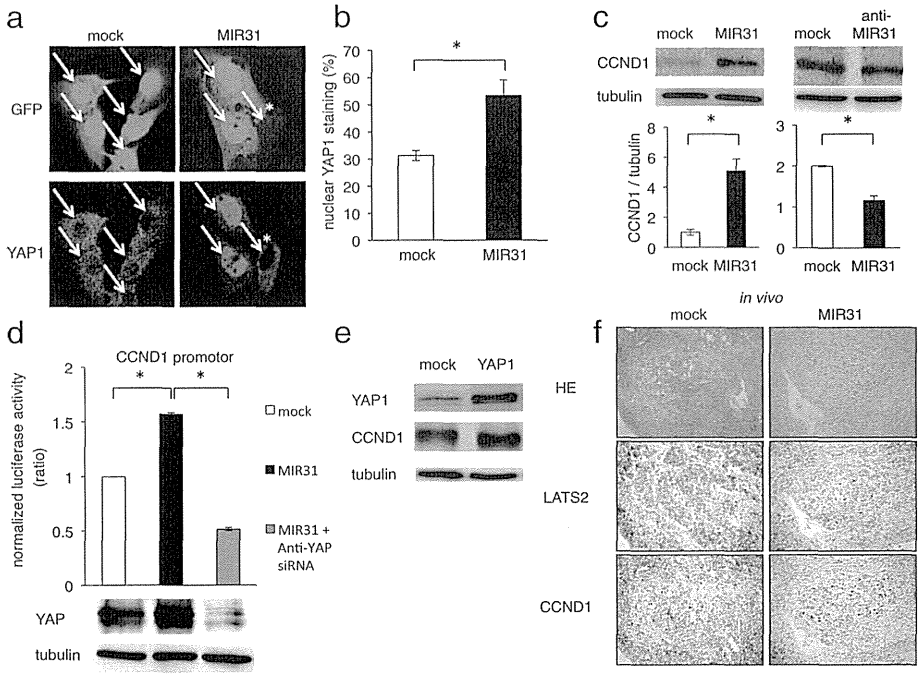


**Figure 3 MIR31 promotes the translocation of YAP1 into the nucleus and promotes the transcription of CCND1. (a)** Representative cells of immunofluorescence for GFP and YAP1, x600. The nuclei are indicated by white arrows. *The translocation of YAP1 into the nucleus was not observed in the cells unsuccessfully transfected with pre-MIR31. The cells were cultured under standard conditions with 5% fetal bovine serum. **(b)** Ratio of nuclear YAP1 staining. *$p < 0.05$, unpaired two-tailed Student's *t*-test. **(c)** The CCND1 levels were increased by MIR31 overexpression and the CCND1 levels were decreased by the MIR31-specific inhibitor on immunoblotting for CCND1 and α-tubulin. The ratio of CCND1/α-tubulin is shown in the bar graph. **(d)** The CCND1 levels were increased by YAP1 overexpression. Immunoblotting for YAP1, CCND1 and α-tubulin. **(e)** The luciferase activity after transfection of the reporter constructs containing the LATS2 promotor region normalized to the GAPDH promotor region (top). Immunoblotting for YAP and α-tubulin following siRNA transfection (bottom). Mock and MIR31 cells were transfected with non-targeting siRNA. *$p < 0.05$, unpaired two-tailed Student's *t*-test. **(f)** Correlation between the MIR31 expression and results of the immunohistochemical analysis of LATS2 and CCND1 *in vivo*. Representative results are shown in micrographs, x100. All experiments were performed in triplicate.

### Correlations between the MIR31, LATS2 and CCND1 expression in EC

We compared the MIR31 expression quantified by qRT-PCR and the immunohistochemical expression of LATS2 and CCND1 in 34 EC patients who underwent surgery as their initial treatment (Table 1, Lane 1). When we divided the 34 patients into two groups according to the MIR31 expression (MIR31/RNU44 = 15), the MIR31 expression levels were found to be low in the LATS2-positive (73%) and CCND1-negative (27%) tumors and high in the LATS2-negative (25%) and CCND1-positive (75%) tumors. (Figure 4a; Two representative cases are shown in Figure 4b). The MIR31 expression was lowest in the LATS2-positive and CCND1-negative groups and highest in the LATS2-negative and CCND1-positive groups (Figure 4c).

### The MIR31 expression is increased in high-risk human endometrial cancers

We defined low-risk patients as those who satisfied all of the following criteria: pT1a, pN0, M0, grade1 or 2 without lymphovascular space invasion (Table 1, Lanes 2 and 3). We found that the expression of MIR31 was significantly increased in the high-risk patients (Figure 4d). All patients with recurrent disease were classified as high-risk patients. Since most tumors in the recurrent disease patients were of grade 2 (Table 1, Lane 4), we focused on the prognosis of the 13 patients with grade 2 tumors (Additional file 10: Table S1). As expected, the progression-free survival was significantly worse among the patients with high MIR31 tumors (> = 0.8) than among those with low MIR31 tumors (<0.8) (Figure 5). These results suggest that MIR31 is related to the aggressiveness of EC.

## Discussion

In this study, we demonstrated that MIR31 functions as an oncomir in EC. MIR31 is an oncomir in several human cancers and a tumor suppressor gene in others. We speculate that MIR31 has a specific function in each type of malignancy, and several mechanisms, including methylation-dependent silencing [35] and local deletion [29], may explain its different roles in different tumor types. However, little is known about the MIR31 status

**Table 1 Clinical features of human endometrial cancer**

| | Total | Low-risk | High-risk | Recurrence |
|---|---|---|---|---|
| n (%) | 34 (100) | 7 (21) | 27 (79) | 7 (21) |
| Age, mean (range) | 59 (38–78) | 58 (38–75) | 60 (45–78) | 56 (45–67) |
| FIGO stage, n (%) | | | | |
| I | 15 (44) | 7 (100) | 8 (30) | 1 (14) |
| II | 3 (9) | 0 (0) | 3 (11) | 1 (14) |
| III | 14 (41) | 0 (0) | 14 (52) | 3 (43) |
| IV | 2 (6) | 0 (0) | 2 (7) | 2 (29) |
| Grade, n (%) | | | | |
| 1 | 15 (44) | 5 (71) | 10 (37) | 0 (0) |
| 2 | 13 (38) | 2 (29) | 11 (41) | 6 (86) |
| 3 | 6 (18) | 0 (0) | 6 (22) | 1 (14) |
| Lymphovascular space invasion, n (%) | | | | |
| + | 10 (29) | 0 (0) | 10 (37) | 2 (29) |
| - | 24 (71) | 7 (100) | 17 (63) | 5 (71) |
| MIR31, mean | 18.40 | 2.05 | 21.90 | 9.15 |
| (range) | (0.15 - 284.06) | (0.33 - 5.98) | (0.15 - 284.06) | (0.70 - 27.77) |

in patients with EC. In previous studies that reported MIR31 to be an oncomir, MIR31 was found to regulate RAS p21 GTPase Activating Protein 1 (RASA1) [22] and RhoBTB1 [36] in colorectal cancer, LATS2 and PP2A regulatory subunit B alpha isoform (PPP2R2A) [24] in lung cancer and factor-inhibiting hypoxia-inducible factor (FIH) [26] in head and neck carcinomas. It is plausible that MIR31 represses the expression of tumor suppressor genes, such as LATS2, to act as an oncomir and indirectly promotes the transcription of genes related to cell cycle control and tumorigenesis (Figure 6).

We herein demonstrated that the Hippo pathway is involved in EC tumorigenesis and correlates with a poor patient prognosis. Although no significant relationships were observed between the immunohistochemical expression of LATS2 and clinical risk factors, including lymph node metastasis, cervical invasion or lymphovascular space invasion, such relationships deserve further investigation in a larger patient cohort. It is reasonable to postulate that the Hippo pathway regulates the CCND1 expression via YAP1 translocation into the nucleus in EC, as the cyclin family is known to be a major target of the Hippo pathway [37-39]. The overexpression of CCND1 may not be an independent factor causing tissue overgrowth, since it is suggested that the overexpression of all known yorkie targets fails to mimic the effect of yorkie itself in driving tissue growth in drosophila [40]. As MIR31 tends to block the cell apoptosis induced by ultraviolet treatment in HEC-50B cells (data not shown), we speculate that other transcriptional targets of YAP1 associated with apoptosis, such as XIAP, may be coordinately regulated with CCND1. The Hippo pathway is known to be related to the p53 activity, for example, LATS2 tumor suppressor augments p53-mediated apoptosis by promoting the nuclear proapoptotic function of ASPP1 [41,42]. On the other hand, a p53 mutation is found in some patients with aggressive histologic subtypes of endometrial cancer [43]. Our findings suggested the existence of a possible connection between MIR31 and p53 mutation in EC which thus induces the aggressiveness of EC. Additionally, the connection between MIR31 and the p53 mutation could therefore explain the reason why MIR31 either promotes or suppresses different cancers.

As mentioned above, recommendations for postoperative adjuvant therapy for EC are based on the risk assessment of recurrence for each individual patient. In this study, we divided 34 EC patients into two groups according to the criteria generally used to determine whether postoperative adjuvant therapy is required and found a strong correlation between the MIR31 expression and these clinical risk factors. These results suggest that MIR31 is potentially a new molecular marker for distinguishing the risk of recurrence combined with histological findings. However, the small sample size of the present study limits the robustness of our findings, and further investigation in a larger patient cohort is necessary.

## Conclusions

In conclusion, we herein demonstrated that MIR31 promotes EC tumorigenesis. MIR31 indirectly promotes the translocation of YAP1 into the nucleus by repressing the tumor suppressor gene LATS2.

## Methods

### Human endometrial tumor tissues

Tumor specimens were obtained from patients with EC treated at Hokkaido University Hospital under institutional review board approval (registration ID: 011–0157). Informed consent was obtained from each subject. Patients treated at Hokkaido University Hospital between 2006 and 2012 were eligible for inclusion. All samples were obtained at the initial surgery. RNA was extracted using the RecoverAll™ Total nucleic Acid Isolation Kit (Ambion, Austin, TX, USA) from formalin-fixed, paraffin-embedded tissues. We set the samples on the slide glass and microscopically recognized the malignantly transformed epithelial lesion, then cored out the epithelial lesion. MIR31 was detected using quantitative real-time PCR (qRT-PCR). All experiments were performed three times, and ratio of the mean MIR31 level relative to the endogenous control RNU44 level was calculated.

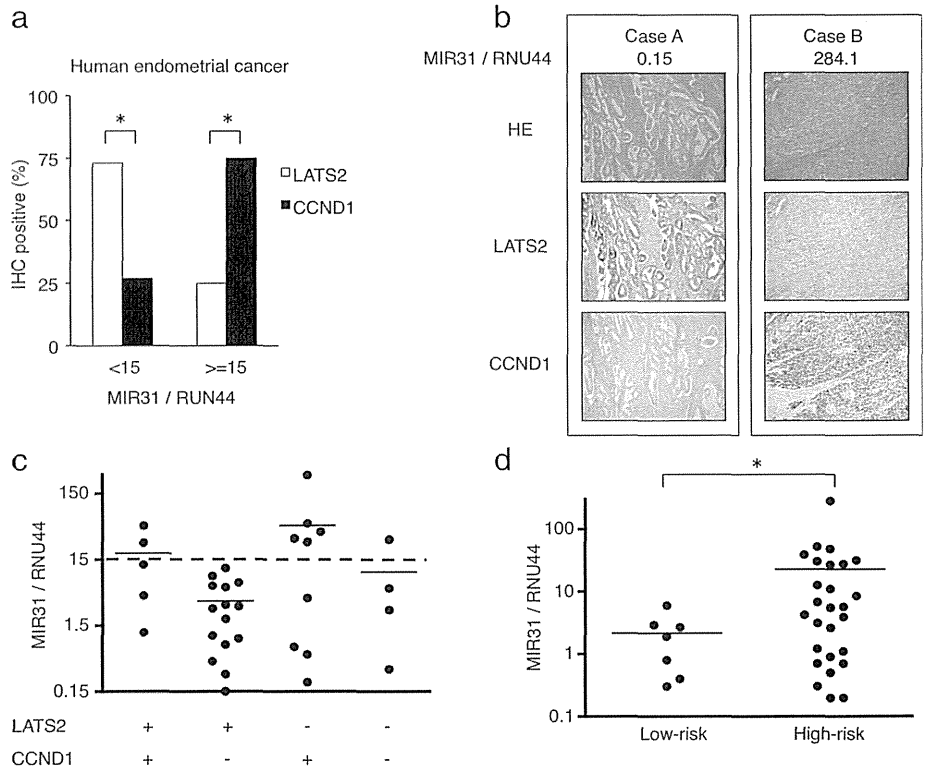


**Figure 4 Correlation between the MIR31 expression and the immunohistochemical detection of LATS2 and CCND1 in human EC cells.** **(a)** Proportion of patients with positive staining for LATS2 and CCND1 classified into the MIR31 > =15 and MIR31 < 15 groups. *p < 0.05, Chi-square test. **(b)** The MIR31 expression assessed using qRT-PCR with RNU44 as the endogenous control (top) and representative micrographs, x100. **(c)** The MIR31 expression levels in all patients classified according to staining for LATS2 and CCND1 are shown in the scatter diagram. The horizontal solid lines indicate the mean. **(d)** The expression levels of MIR31 in the human EC cells were analyzed using qRT-PCR. The horizontal lines demonstrate the mean. *p < 0.05, Mann-Whitney's U-test.

### qRT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The MIR31 and RNU44 levels were quantified using qRT-PCR with the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and TaqMan® MicroRNA Assays (Applied Biosystems) according to the manufacturer's instructions. We assessed the RNA expression according to relative quantification using the $2^{-\Delta\Delta Ct}$ method [44] to determine the fold change in the expression.

### Cell lines

The human EC cell lines HEC-50B, HEC-1-A and HEC-108 were obtained from RIKEN BioResource Center (Tsukuba, Japan) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, 2 mM L-glutamine and 100 U/ml of penicillin and streptomycin in a 6-cm dish. SK-OV-3 and OVCAR-3 were obtained from the ATCC (Manassas, VA, USA) and maintained in McCoy's 5a Medium with 10% FBS, 2 mM L-glutamine and 100 U/ml of penicillin and streptomycin and in Roswell Park Memorial Institute medium 1640 with 20% FBS, 0.01 mg/ml of bovine insulin, 2 mM L-glutamine and 100 U/ml of penicillin and streptomycin.

### Overexpression of MIR31

Precursor-MIR31 was transfected into HEC-50B using the BLOCK-iT™ Lentiviral miR RNAi Expression System (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol, as previously described [45]. After transfection, we performed blasticidin selection at a concentration of 2.5 μg/ml for 10 days.

### Colony formation assay

A total of $1.0 \times 10^5$ cells were seeded in a layer of 0.4% noble agar/DMEM/1% FBS/0.5 μg/ml of puromycin or 0.4% noble agar/DMEM/5% FBS over a layer of 0.5% bacto agar/DMEM/1% or 5% FBS in a 6-cm dish. The colonies were stained using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (Sigma-Aldrich, St. Louis, MO, USA) and counted.

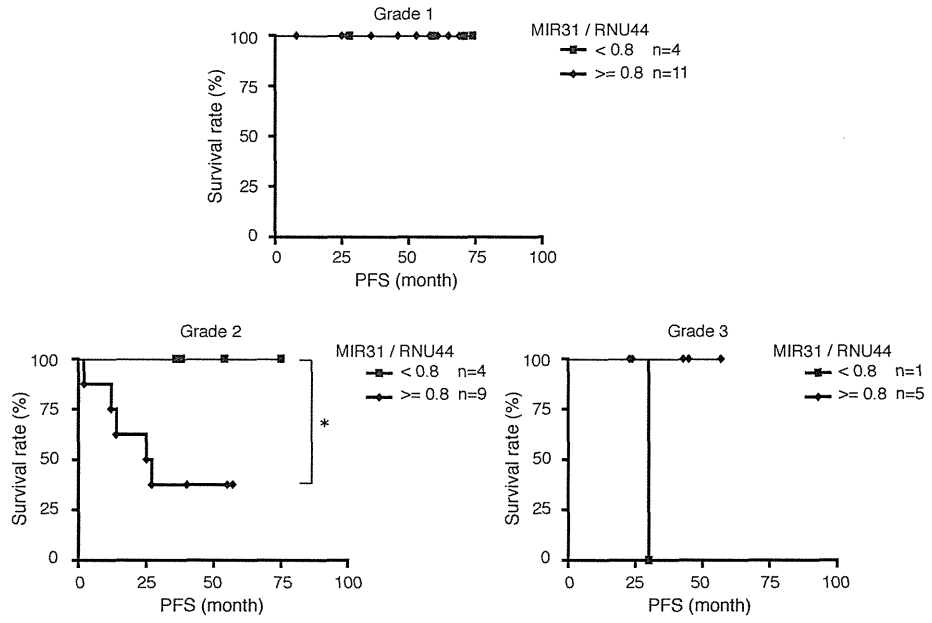


**Figure 5 Prognosis of patients classified into the high and low MIR31 groups.** Kaplan–Meier curves depicting the progression-free survival (PFS). *p < 0.05, log-rank test.

### Analysis of the tumor-forming potential in vivo

All experiments were conducted in accordance with guidelines authorized by the Animal Research Committee of Hokkaido University. Six-week-old BALB/c nude mice (Clea, Tokyo, Japan) were injected subcutaneously into their flanks with 2 x $10^7$ HEC-50B mock or HEC-50B MIR31 cells bilaterally in 200 μl of normal culture medium. All mice were sacrificed on day 28, and the tumor weight was measured.

### Immunoblotting

SDS–PAGE and immunoblotting were carried out as described elsewhere [45]. Briefly, filters were incubated with rabbit polyclonal antibodies against LATS2, mouse monoclonal antibodies against α-tubulin (1:1,000 dilution, Abcam, Cambridge, UK), rabbit polyclonal antibodies against CCND1 (1:500 dilution, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal antibodies against YAP1 and phospho-YAP (Ser127) (1:1,000 dilution, Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal antibodies against RAS (1:1,000 dilution, BD Biosciences, San Jose, CA, USA) and rabbit polyclonal antibodies against XIAP (1:100 dilution, Abnova, Taipei City, Taiwan).

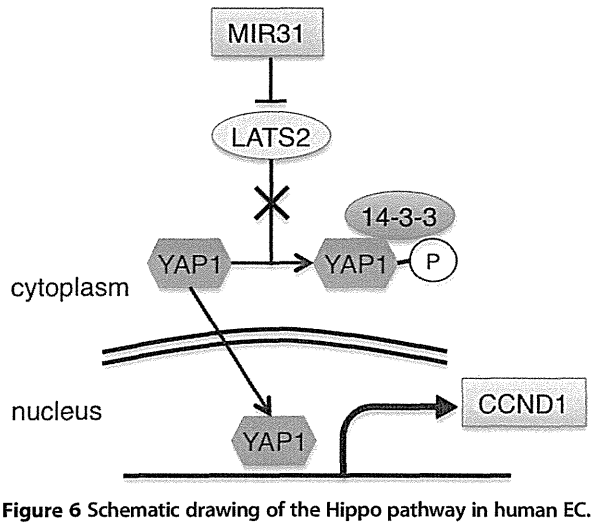


**Figure 6 Schematic drawing of the Hippo pathway in human EC.**

### Luciferase reporter assay

To investigate the translation of LATS2, luciferase reporter assay was carried out as described elsewhere [45]. The wild-type (NM_014572) or mutant LATS2 3'-UTR sequence was inserted downstream of the firefly luciferase reporter gene, which was controlled by the SV40 enhancer for expression in mammalian cells, whereas no oligonucleotides were inserted in the control vector (Genecopoeia, Rockville, MD, USA). Renilla luciferase was used as a tracking indicator for successful transfection. In order to investigate the transcription of CCND1, HRAS, KRAS and XIAP, luciferase reporter constructs for the promoters of these molecules and a positive control of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained (SwitchGear Genomics, Menlo Park, CA, USA). The luciferase activity was measured using Light-Switch Assay Reagent (SwitchGear Genomics) according to the manufacturer's instructions. Briefly, 1.0 to 1.5 × $10^4$ cells were seeded in white 96–well plates on day 1 and

transfected with reporter constructs on day 2 using FuGENE HD (Promega). The luciferase activity was measured using assay reagent 48 hours after transfection.

### RNA interference for LATS2 and YAP1

Two shRNA lentiviruses against LATS2 (Sigma-Aldrich) and non-targeting shRNA (Sigma-Aldrich) were transfected into HEC-50B cells in 48-well plates according to the manufacturer's instructions. The multiplicity of infection (MOI, number of transducing lentiviral particles per cell) was 5. We performed puromycin selection at a concentration of 0.5 μg/ml for 10 days. siRNAs against YAP and non-targeting siRNA (Santa Cruz Biotechnology) were transfected using HiPerFect transfection reagent (Qiagen, Tokyo, Japan) in 12-well plates, and the cells were harvested after 48 hours.

### Reverse transcription-PCR

Reverse transcription-PCR was carried out as described elsewhere [45]. The primers used for the expression analysis were as follows: pre- MIR31 - forward, 5'-GGAGAGGAGGCAAGATGCTG-3'; pre- MIR31 - reverse, '-GGAAAGATGGCAATATGTTG-3': GAPDH - forward, 5'-CTCATGACCACAGTCCATGC-3': GAPDH - reverse, 5'-TTACTCCTTGGAGGCCATGT-3': LATS2 - forward, 5'-TAGAGCAGAGGGCGCGGAAG -3': LATS2 - reverse, 5'- CCAACACTCCACCAGTCACAGA-3'.

### Immunofluorescence

Cells were grown on 35-mm glass-based dishes (Asahi Glass, Tokyo, Japan), fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100/PBS before blocking with 1% BSA. The cells were incubated with rabbit polyclonal antibodies against YAP1 (1:500 dilution, Cell Signaling Technology) and secondary antibodies, including goat antibodies to rabbit coupled to Alexa 594 (1:250 dilution, Invitrogen). All samples were examined using laser-scanning confocal microscopy (Fluoview™, Olympus, Tokyo, Japan).

### MIR inhibitor

A total of 200 nM of miRIDIAN microRNA Hairpin Inhibitor and its negative control (Thermo Scientific Dharmacon, Lafayette, CO, USA) were employed to transiently inhibit MIR31 and transfected 48 hours prior to seeding with Oligofectamine (Invitrogen).

### Overexpression of Yes-associated protein 1 (YAP1)

The human YAP1 expression vector, p2xFLAG-YAP1 and negative control were kindly provided by Dr. Sudol [46]. HEC-50B cells at 80% confluence in 6-well plates were transfected with 4 μg of YAP1 vector using FuGENE HD (Promega).

### Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissues were used to detect the LATS2 and CCND1 expression. The sections were incubated with anti-LATS2 rabbit polyclonal antibodies (Abcam, Cambridge, UK) at 1:300 dilution and anti-CCND1 rabbit monoclonal antibodies (Dako, Glostrup, Denmark) at 1:50 dilution. A semi-quantitative scoring system was used to evaluate the intensity of staining: low (proportion: 0 to 50%, intensity: no staining to weak) and high (proportion: more than 50%, intensity: intermediate to strong).

### Statistical analysis

The data are presented as the mean ± SEM. The unpaired two-tailed Student's *t*-test, Mann-Whitney's U-test and Chi-square test were used for comparisons, with a p value of $< 0.05$ considered to be significant (*).

## Additional files

**Additional file 1: Figure S1.** qRT-PCR analysis of the MIR31 expression in five adenocarcinoma cell lines of the female genital tract.

**Additional file 2: Figure S2.** Colony formation assay. *$p < 0.05$, unpaired two-tailed Student's *t*-test compared with HEC-108.

**Additional file 3: Figure S3.** Colony formation assay, four weeks. *$p < 0.05$, unpaired two-tailed Student's *t*-test.

**Additional file 4: Figure S4.** Detection of LATS2 and GAPDH mRNA using RT–PCR.

**Additional file 5: Figure S5.** Representative results of the colony formation assays with 5% FBS for four weeks.

**Additional file 6: Figure S6.** MIR31 is not involved in YAP phosphorylation. Immunoblotting for phospho-YAP and α-tubulin.

**Additional file 7: Figure S7.** Immunoblotting for putative targets of YAP1.

**Additional file 8: Figure S8.** (a) The expression levels of RAS and XIAP in the mock and MIR31-overexpressing cells (top). The RAS and XIAP levels were decreased by the MIR31-specific inhibitor (bottom). Results of immunoblotting for RAS, XIAP and α-tubulin. (b) The luciferase activity after transfection of the reporter constructs containing the HRAS, KRAS and XIAP promotor region normalized to the GAPDH promotor region. *$p < 0.05$, unpaired two-tailed Student's *t*-test.

**Additional file 9: Figure S9.** Representative immunofluorescence analysis of YAP1, x600.

**Additional file 10: Table S1.** Correlation between the MIR31 expression and risk of postoperative recurrence in the patients with grade 2 tumors.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

TM, HW, HN, ST and NS designed the experiment, interpreted the data and prepared the manuscript. TM, LW, HK, MK, MKH, TK, MT conducted the experiment, collected the data and helped to prepare the manuscript. All authors read and approved the final manuscript.

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# Distribution of Lymph Node Metastasis Sites in Endometrial Cancer Undergoing Systematic Pelvic and Para-Aortic Lymphadenectomy: A Proposal of Optimal Lymphadenectomy for Future Clinical Trials

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## ABSTRACT

**Purpose.** The aim of this study was to demonstrate the precise mapping of lymph node metastasis (LNM) sites in endometrial cancer.

**Methods.** A total of 266 patients who underwent primary radical surgery including systematic pelvic and para-aortic lymphadenectomy for endometrial cancer from 1993 to 2010 were enrolled in this study. We removed lymph nodes from the femoral ring to the para-aortic node up to the level of renal veins. We analyzed the distribution of positive-node sites according to their anatomical location.

**Results.** Overall, 42 of 266 patients (15.8 %) showed LNM. The median number of nodes harvested was 62.5 (range 40–119) in pelvic nodes (PLN), and 20 (range 3–47) in para-aortic nodes (PAN). Among 42 cases with positive-nodes, 16 cases (38.1 %) showed positive PLN alone, 7 cases (16.7 %) in PAN alone, and 19 cases (45.2 %) in both PLN and PAN. The most prevalent site of positive-nodes was PAN (9.8 %) followed by obturator nodes (9.4 %), internal iliac nodes (7.1 %), and common iliac nodes (5.6 %). Six of 19 cases (31.6 %) of positive PAN above the inferior mesenteric artery (IMA) showed negative PAN below IMA. Metastasis to the deep inguinal nodes was found to be extremely rare (0.38 %). Single-site LNM was the most frequently observed in obturator nodes, followed by PAN above IMA.

**Conclusion.** Routine resection of deep inguinal nodes is not recommended, whereas para-aortic lymphadenectomy should be extended up to the level of renal veins for endometrial cancer.

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 recommendations of the American College of Obstetricians and Gynecologists,[1] the National Comprehensive Cancer Network,[2] and the Japan Society of Gynecologic Oncology[3] 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.[4] 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 nodes [PLN]) and IIIC2 (indicating positive PAN with or without positive PLN). However, in clinical practice, it is not clearly defined who should undergo a systematic para-aortic lymphadenectomy.

The association between the extent of lymph node involvement and survival has been demonstrated in most solid tumors. Chan et al.[5,6] recently reported that the extent of lymph node resection improves the survival of patients

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with an intermediate/high risk of endometrial cancer. We routinely performed complete systematic pelvic and para-aortic lymphadenectomy in all operable patients with endometrial cancer in our institute because (i) nodal status is the most important prognosticator; and (ii) results of lymphadenectomy allow tailoring of postoperative adjuvant treatment.[7,8] Consequently, we have recently demonstrated the result of a retrospective cohort study that para-aortic lymphadenectomy combined with pelvic-node dissection improves survival of endometrial cancer patients with intermediate/high risk for recurrence (the SEPAL study).[9] Thus, one of the most important determinants for prognosis of patients with advanced endometrial cancer is the quality of surgery (lymphadenectomy) because the absolute count of lymph nodes harvested, and the extent of lymphadenectomy in the pelvic and para-aortic areas might be potential indicators of the quality of lymphadenectomy.[10] At the same, we attempted to create a scoring system to select patients who are at low risk of PAN metastasis and in whom para-aortic lymphadenectomy can be safely omitted.[11]

Regarding all current status on lymphadenectomy for endometrial cancer described above, we aimed to demonstrate the precise mapping of lymph node metastasis (LNM) in patients with endometrial cancer who underwent systematic pelvic and para-aortic lymphadenectomy up to the level of renal veins. Fotopoulou et al.[12] previously reported the lymph node mapping in 13 node-positive patients with endometrial cancer but, as far as we know, our study is the largest series of lymph node mapping in endometrial cancer in the literature. We discuss the importance of quality assurance of lymphadenectomy for future prospective clinical trials aimed at demonstrating the therapeutic effect of lymphadenectomy in endometrial cancer.

## MATERIALS AND METHODS

### *Patients*

A total of 266 patients with endometrial cancer underwent primary radical surgical treatment from 1993 to 2010 at the Department of Obstetrics and Gynecology, Hokkaido University Hospital, Japan. All subjects underwent hysterectomy (either extrafascial, modified radical, or radical hysterectomy), bilateral salpingo-oophorectomy, and systematic retroperitoneal lymphadenectomy which consisted of complete dissection of PLN and PAN from the femoral ring to the level of the renal vein, as previously described.[7,8] All lymphatic tissues that surrounded the arteries and veins were completely removed. All patients were treated with an adjuvant chemotherapy of six cycles of platinum-based regimens, including CAP (cyclophosphamide 350 mg/m$^2$, adriamycin 40 mg/m$^2$ and cisplatin 50–70 mg/m$^2$) or TC (paclitaxel 175 mg/m$^2$, carboplatin AUC5) every 3 weeks, as previously described.[7,8] This retrospective study was conducted after approval from the Institutional Review Committee of Hokkaido University Hospital.

### *Definition and Prevalence of Lymph Node Metastasis Sites*

PLN sites were classified into nine sites, as previously described,[13] with some modifications: circumflex iliac nodes distal to the external iliac nodes (CINDEIN), circumflex iliac nodes distal to obturator nodes (CINDON), external iliac nodes (EIN), internal iliac nodes (IIN), obturator nodes (ON), para-uterine artery nodes (PUN), cardinal ligament nodes (CLN), sacral nodes (SN), and common iliac nodes (CIN). CINDEIN and CINDON were categorized as deep inguinal nodes.

The definitions of each site are as follows. EIN are located outside a lateral iliac artery and below the level of bifurcation of the common iliac artery; CINDEIN are the most distal EIN; IIN are located on the anterior side of a medial iliac artery below the level of bifurcation of the common iliac artery, between both iliac arteries (therefore, this definition of IIN includes the medial part of the external iliac node); and ON are located on the anterior side of an obturator nerve and under IIN. CINDON are the most distal ON, being located below an anonymous vein. PUN are located along the uterine artery or uterine vein. CLN are located on the posterior side of an obturator nerve and might usually be called deep ON. SN are located inside a medial iliac artery and below the level of bifurcation of the aorta. CIN are located between the level of bifurcation of the common iliac artery and the level of bifurcation of the aorta. CIN were further separated into three categories, superficial external CIN (SECIN), deep external CIN (DECIN), and internal CIN (ICIN). Para-aortic lymph node (PAN) metastasis was further investigated by dividing the metastatic nodes according to the sites above and below the inferior mesenteric artery (IMA). Figure 1 illustrates the locations of lymph node sites defined above.

The incidence rate of the LNM site was expressed as the ratio of the number of LNM sites to the total number of patients (266 patients), as previously described.[14]

### *Survival Analysis*

Patient survival was calculated using the Kaplan–Meier method. The significance of the survival difference was

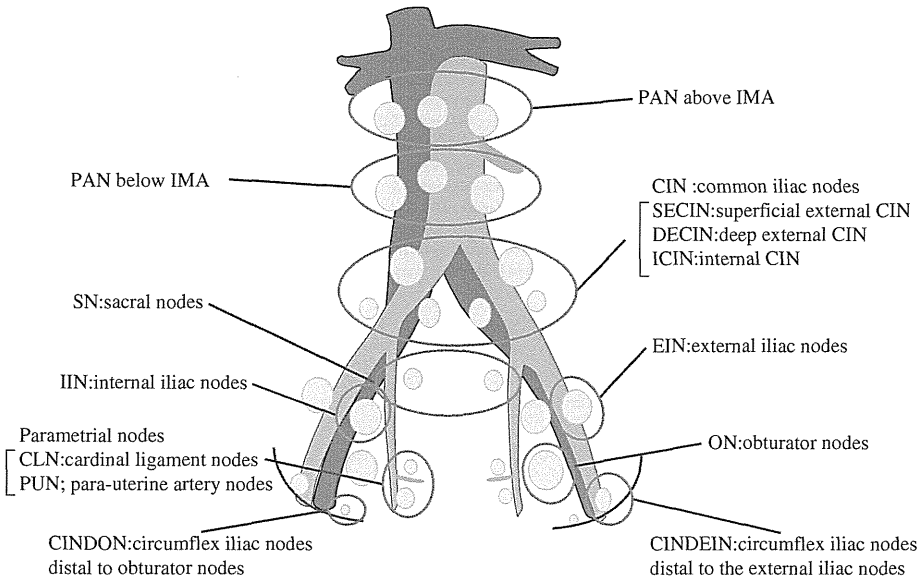



**FIG. 1** Definition of LNM sites in endometrial cancer

**TABLE 1** Pathological characteristics of node-positive patients with endometrial cancer

| Factor | N (%) |
|---|---|
| FIGO 2008 stage | |
| IIIC1 | 17 (40.5) |
| IIIC2 | 21 |
| IVB | 4 |
| Histology | |
| Endometrioid | 35 (83.3) |
| Non-endometrioid | 7 (16.7) |
| Grade | |
| G1 | 6 (14.3) |
| G2 | 22 (52.4) |
| G3 | 14 (33.3) |
| Myometrial invasion | |
| ≤1/2 | 8 (19.0) |
| >1/2 | 34 (81.0) |
| Lymphvascular space invasion | |
| (−)/(+) | 18 (42.9) |
| (++)/(+++) | 24 (57.1) |
| Cervical stromal invasion | |
| (−) | 30 (71.4) |
| (+) | 12 (28.6) |
| Ovarian metastasis | |
| (−) | 34 (81.0) |
| (+) | 8 (19.0) |

examined using the log-rank test. A $p$ value $< 0.05$ was considered statistically significant. Statistical analyses were performed using the StatView software package (SAS Institute Inc., Cary, NC, USA).

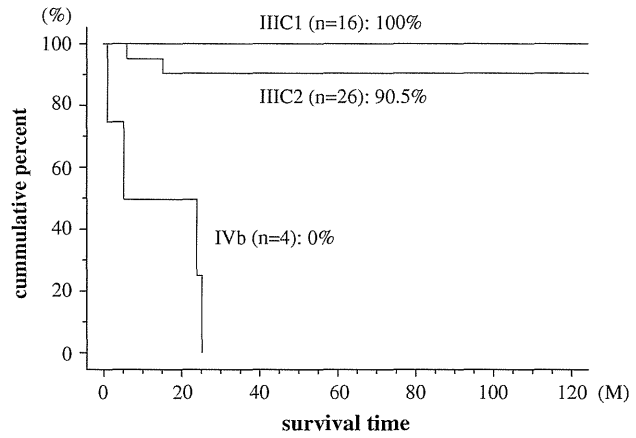



**FIG. 2** Kaplan–Meier survival curve of node-positive patients with endometrial cancer

## RESULTS

### *Patients' Characteristics*

Among 266 patients, 42 (15.8 %) showed positive lymph nodes. The pathologic characteristics of these 42 patients are listed in Table 1. Median age of the node-positive patients was 58 years (range 24–77 years), and median follow-up period was 57 months at the time of writing (range 1–211 months). The FIGO 2008 stage of the patients was as follows: 16 (16/42, 38.1 %) stage IIIC1, 26 (26/42, 61.9 %) stage IIIC2, and 4 (4/42, 9.5 %) stage IVB. Stage IV disease with distant metastasis (liver or lung metastasis) was excluded from the survival analysis. Estimated 5-year survival rate was 100 % for stage IIIC1 and 90.5 % for stage IIIC2. Patients with stage IVB disease all died within 2 years (Fig. 2).

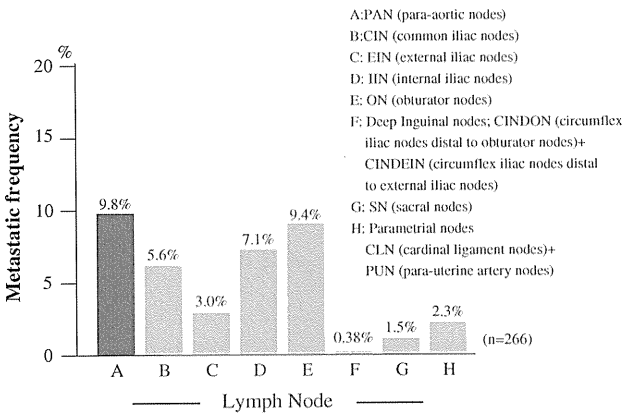



**FIG. 3** Distribution and prevalence of LNM sites in surgically-staged endometrial cancer

*Status of Lymph Node Metastatis (LNM)*

The median number of extirpated PLN was 62.5 (range 40–119), and the median number of extirpated PAN was 20 (range 3–47). Among the 42 cases with positive-nodes, 16 cases (16/42, 38.1 %) showed positive PLN alone, 7 cases (7/42, 16.7 %) showed positive PAN alone, and 19 cases (19/42, 45.2 %) showed both positive PLN and PAN. The incidence of PLN metastasis and PAN metastasis was 13.1 % (35/266) and 9.7 % (26/266), respectively.

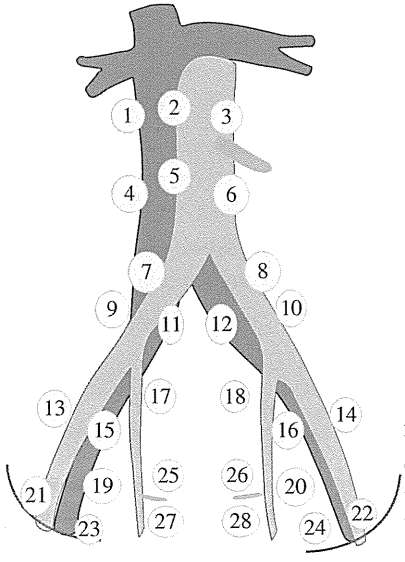
Figure 3 demonstrated the distribution of LNM sites. The most prevalent site of LNM was PAN (9.8 %, 26/266), followed by ON (9.4 %, 25/266), IIN (7.1 %, 19/266), CIN (5.6 %, 15/266), EIN (3.0 %, 8/266), parametrial nodes (CLN and PUN; 2.3 %, 6/266), and SN (1.5 %, 4/266). Metastasis to the deep inguinal nodes (CINDEIN or CINDON) was extremely rare (1/266, 0.38 %).

When we look at the detail of positive-nodes in CIN, the incidence rate of positive SECIN, DECIN, ICIN was 3.4 % (9/266), 3.8 % (10/266), and 3.0 % (8/266), respectively. In the positive PAN, positive PAN below IMA was found in 7.5 % (20/266), and positive PAN above IMA up to the renal veins was found in 7.1 % (19/266). Among deep inguinal nodes, CINDEIN was not affected in this study. Figure 4 summarizes the details of precise mapping of LNM sites in our patient cohort.

Six of 19 cases (31.6 %) of PAN above IMA showed negative PAN below IMA. Nine of 19 cases (47.4 %) of positive PAN above IMA revealed negative CIN.

*Single-Site LNM*

We then categorized LNM sites into five groups (group A: PAN above IMA; group B: PAN below IMA; group C: CIN; group D: PLN except CIN; group E: CINDEIN + CINDON) according to the anatomical locations. The incident rate of LNM was 9.8 % in group A+B, 6.0 % in group C, 12.0 % in group D, and 0.4 % in group E. (Electronic Supplementary Fig. 1) Single-site LNM was the most frequently observed in group D (eight cases), followed by four cases in group A, one in groups B and C, and none in group E. In group D, ON was the most prevalent single-site LNM (Electronic Supplementary Fig. 2).




1:Rt PAN above IMA 2.6%
2:Medain PAN above IMA 1.1%
3:Lt PAN above IMA 7.3%
4:Rt PAN below IMA 4.1%
5:Median PAN above IMA 1.1%
6:Lt PAN below IMA 4.5%
7:Rt SECIN 2.3%
8:Lt SECIN 2.6%
9:Rt DECIN 1.9%
10:Lt DECIN 2.6%
11:Rt ICIN 1.9%
12:Lt ICIN 1.5%
13:Rt EINI 1.5%
14:Lt EINI 1.5%
15:Rt IIN 4.9%
16:Lt IIN 3.8%
17:Rt SN 1.1%
18:Lt SN 1.1%
19:Rt ON 4.9%
20:Lt ON 6.8%
21:Rt CINDEIN 0.0%
22:Lt CINDEIN 0.0%
23:Rt CINDON 0.0%
24:Lt CINDON 0.38%
25:Rt PUN 0.75%
26:Lt PUN 0.75%
27:Rt CLN 0.75%
28:Lt CLN 0.75%

PAN;para-aortic nodes
IMA;inferior mesenteric artery
CIN; common iliac nodes
SECIN; superficial external CIN
DECIN; deep external CIN
ICIN; Internal CIN
EIN; external iliac nodes
IIN; internal iliac nodes
SN; sacral nodes
ON; obturator nodes
CINDEIN; circumflex iliac nodes distal toEIN
CINDON; circumflex iliac nodes distal to ON
PUN; para-uterine artery nodes
CLN; cardinal ligament nodes

**FIG. 4** Precise mapping of LNM sites in node-positive endometrial cancer

## DISCUSSION

Lymphadenectomy is currently one of the most controversially discussed topics in the management of endometrial cancer. Since FIGO introduced surgical staging of endometrial cancer in 1988, essential questions have remained unanswered, including the extent of an optimal lymphadenectomy, and which subgroup of patients would benefit.[15–17] Additionally, the FIGO staging system in endometrial cancer has recently been changed in 2008.[4] In the revised FIGO staging, PAN involvement has been separated from the single substage IIIC. Stage IIIC is now categorized as IIIC1 (indicating positive PLN) and IIIC2 (indicating positive PAN with or without positive PLN). We have routinely performed systematic pelvic and para-aortic lymphadenectomy for endometrial cancer in our institute. The current study clearly demonstrated that PAN was frequently affected as well as PLN in endometrial cancer, re-confirming that systematic lymphadenectomy is essential for accurate staging purposes. The incidence of PLN metastasis and PAN metastasis was 13.1 % (35/266) and 9.7 % (26/266), respectively, which is comparable to the incidence previously reported in other studies (11.9 % [100/834] and 17 % [76/456] of PLN metastasis, and 5.2 % [43/834] and 12 % [49/425] of PAN metastasis) in the literature.[18,19]

In many cases, the decision to perform systematic pelvic and/or para-aorticlymphadenectomy reflects individual surgeons' preferences based on their personal experience, as well as potential technical difficulties derived from the high body mass index and the advanced age of the affected women, factors that often applyunfavorably to the typical patients with endometrial cancer.[20] These factors often lead to an incomplete lymphadenectomy limited to the pelvis or to the area below the IMA,[21] since the dissection of PAN, especially in the infrarenal area, requires higher operative skills.

Although some retrospective series have suggested a benefit in terms of survival with the addition of lymphadenectomy,[5,9,22,23] two randomized trials reported that there is not a therapeutic role.[24,25] However, in the (A Study in the Treatment of Endometrial Cancer) ASTEC trial, lymph node dissection in the 'node' group appears to be inadequate as 8 % in the lymphadenectomy group had no nodes removed and over one-third had nine or less lymph nodes removed. Therefore, almost half of the patients randomized to the lymphadenectomy group had no nodes or what would be considered inadequate lymphadenectomy by current cooperative group standards. Our retrospective cohort study (the SEPAL study) clearly demonstrated survival effect of para-aortic lymphadenectomy in intermediate-/high-risk patients, but not in low-risk patients.[9] In the SEPAL study, the median number of nodes removed was 34 in the PLN dissection group, and 82 nodes (59 PLN and 23 PAN) in the PLN and PAN dissection group.[9] Thus, it would be a good time to conduct a prospective study on the therapeutic role of lymphadenectomy for endometrial cancer.

To propose an appropriate protocol for prospective clinical trials on the therapeutic role of lymphadenectomy in endometrial cancer, we must consider some critical issues regarding quality assurance of lymphadenectomy. Otherwise, we may draw incorrect conclusions even from prospective randomized studies. One of the most important points is to clearly define the extent of lymphadenectomy. The number of nodes harvested, and dissected regions, should be included as indicators of the quality of lymphadenectomy. Among PLN, dissection of ON, IIN, CIN, and EIN is mandatory since metastasis to those PLN was found to be frequent in this study. Para-aortic lymphadenectomy must be completed up to the level of renal veins because prevalence of PAN metastasis above IMA is similar to PAN metastasis below IMA and PLN (ON, IIN) in this study. Additionally, we may need to ask each participating institution to submit operation records and a pathology report to obtain information of the absolute count of nodes harvested, and also ask to submit videos or pictures of the dissected area after lymphadenectomy to evaluate the extent of lymphadenectomy.

To enrol endometrial cancer patients into an appropriate prospective study, we need to establish the optimal method for preoperative assessment to predict the risk of LNM. According to the criteria proposed by us and others [26–28] we can estimate the risk of LNM in each patient preoperatively. Then, for patients with intermediate- or high-risk for LNM, we can conduct the randomized trial to prove the therapeutic role of para-aortic lymphadenectomy.

The current study demonstrated that single-site LNM was the most frequently identified in ON followed by PAN above IMA, indicating that ON and PAN above IMA are sentinel nodes in endometrial cancer. Studies on sentinel nodes have been reported in endometrial cancer, and Niikura et al.[29], demonstrated that sentinel nodes are frequently detected in the PAN area. Although sentinel node sampling is not yet widely performed in clinical practice, resection of PAN above IMA as well as PLN must be performed for staging purposes at this moment.

The current study demonstrated that metastasis to CINDEIN was extremely rare in node-positive endometrial cancer patients who underwent even systematic pelvic and para-aortic lymphadenectomy. It has recently been reported that adjuvant whole pelvic external beam radiation therapy, resection of more than 31 lymph nodes and removal of CINDEIN were significantly related to the occurrence of postoperative lower-extremity lymphedema in endometrial cancer.[30] However, adjuvant radiation therapy was not

commonly used in our patient cohort. Thus, removal of CINDEIN can be one of the critical risk factors for postoperative lower-extremity lymphedema. Abu-Rustum et al.[31] suggested that removal of CINDEIN is likely to be a factor contributing to the risk of postoperative lower-extremity lymphedema. Hareyama et al.[32] recently reported that preservation of CINDEIN can reduce or prevent the incidence of lower extremity lymph edema after systematic lymphadenectomy for patients with gynecologic malignancies. Therefore, the number of patients who had postoperative lower-extremity lymphedema can possibly be reduced if CINDEIN can be preserved. Todo et al.[13] reported that removal of CINDEIN can be safely eliminated in patients with specific characters, such as G1 or G2 endometrial cancer who have no pelvic-node metastasis. Taken together, we conclude that routine resection of CINDEIN is not recommended for endometrial cancer patients in terms of extremely low frequency of metastasis and its adverse effect on the occurrence of lymphedema.

## CONCLUSIONS

The current study demonstrated that PAN metastasis is commonly observed, as well as ON, IIN, and CIN, while CINDEIN metastasis is extremely rare in endometrial cancer. We must consider the optimal lymphadenectomy according to the risk for LNM, and the quality of lymphadenectomy for future prospective clinical trials to investigate the therapeutic role of lymphadenectomy for endometrial cancer.

**DISCLOSURE** We declare no conflict of interest for this study.

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# Tailoring lymphadenectomy according to the risk of lymph node metastasis in endometrial cancer

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## Abstract

It has been strongly suggested that patients with endometrial cancer with low risk of lymph node metastasis do not benefit from lymphadenectomy and that intermediate-risk/high-risk endometrial cancer patients benefit from complete pelvic and para-aortic lymphadenectomy. This hypothesis needs to be validated by prospective studies. For randomized controlled trials (RCT), heterogeneity of intervention compromises internal validity and non-participation of experienced doctors compromises external validity. As these situations easily occur in randomized surgical trials (RST) intended for high-risk patients, the effects of complicated surgery, such as full lymphadenectomy, might be underestimated in RST. In a famous RST, data for all eligible patients implied that survival outcome for the non-randomized group was significantly better than that for the randomized group. One plausible explanation is that physicians' judgment and experience produce better treatment decisions than do random choices. Although two RCT from European countries showed negative results of lymphadenectomy on prognosis, valuing the care of individual patients may be more important than uncritically adopting the results of RCT. In endometrial cancer, lymphadenectomy must be tailored to maximize the therapeutic effect of surgery and minimize its invasiveness and adverse effects. Two strategies are: (i) to remove lymph nodes most likely to harbor disease while sparing lymph nodes that are unlikely to be affected; and (ii) to perform full lymphadenectomies only on patients who can potentially benefit from them. Here, we focus on the second strategy. Preoperative risk assessments used in Japan and Korea to select low-risk patients who would not benefit from lymphadenectomy are discussed.

**Key words:** cancer of the endometrium, gynecologic imaging, gynecologic oncology, gynecology.

## Reasons for Tailor-made Surgery

It is well known that uniform treatment for patients with the same disease is not always appropriate. Although the term 'personalized medicine' was coined in the context of genetics, this notion makes sense also in the context of surgical therapy. In the evidence-based medicine era, results of randomized controlled trials (RCT) tend to be uncritically accepted. In a famous RCT called the Emory Angioplasty versus Surgery Trial (EAST), the outcomes of percutaneous transluminal coronary angioplasty (PTCA) and coronary angioplasty bypass grafting (CABG) surgery were compared.[1] Of the 842 eligible patients, 392 (46.6%) agreed to participate, but 450 (53.4%) were not approached due to the attending or referring physician's refusal to participate ($n = 353$) or refusal by the patient ($n = 97$). Two interesting results were provided by EAST: (i) there was no survival difference between the PTCA group and the CABG group on the basis of data for 392 patients included in the trial; and (ii) survival outcome for the non-randomized group was

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significantly better than that for the randomized group on the basis of data for all 842 eligible patients.[2] Two plausible explanations can be provided to account for the result of the latter. One is that prognosis of patients in the non-randomized group may have been better than that of patients in the randomized group. The other is that physicians' judgment based on experience may be more important for treatment decision-making than a random choice. CABG generally tends to be performed for patients who have three-vessel disease or proximal left anterior descending artery stenosis. Therefore, the right treatment may have been conducted in the right disease status on the basis of physicians' appropriate experience. Valuing the care of individual patients may be more important than uncritically adopting the results of RCT.

Two reports in *The Lancet*[3,4] strongly suggest that pelvic lymphadenectomy (PLX) has no survival benefit for patients with endometrial cancer with low risk of lymph node metastasis and that combined pelvic and para-aortic lymphadenectomy (PLX + PALX) improves survival of patients with intermediate-risk/high-risk endometrial cancer. The former report was based on a randomized controlled trial by A Study in the Treatment of Endometrial Cancer (ASTEC), while the latter report was based on a retrospective cohort study. Some gynecologists seem to have been skeptical about the efficacy of lymphadenectomy in endometrial cancer based on the results of the ASTEC trial. Some physicians have believed that standard surgery for endometrial cancer does not include lymphadenectomy even though many previous reports suggested the efficacy of lymphadenectomy. Such an idea is an overgeneralization of the results of the ASTEC trial because the study population included only a small number of patients with high-risk endometrial cancer. If lymphadenectomy has a survival benefit for high-risk patients and lymphadenectomy is excluded from standard surgery in endometrial cancer, high-risk patients would not be able to receive optimal treatment. On the other hand, full lymphadenectomy was shown to have a survival benefit for patients with intermediate-risk/high-risk endometrial cancer in the Survival Effect of Para-aortic Lymphadenectomy (SEPAL) study.[4] Although omission of lymphadenectomy can be applied to patients with clinical stage I endometrial cancer according to the results of the ASTEC trial, clinical stage I includes not only low-risk patients but also intermediate-risk and high-risk patients. The range of application for omission of lymphadenectomy should probably be limited to patients with low-risk endometrial cancer. Although the results of these two studies in *The Lancet* are referred to as contradictory statements, they can be compatible. We need to deepen discussions regarding tailoring of lymphadenectomy in endometrial cancer.

## A Problem Inherent in Surgical Studies in High-risk Cancer

The SEPAL study was based on a retrospective observational study.[4] Another observational study from the Mayo Clinic also showed the effectiveness of full lymphadenectomy for patients with high-risk endometrial cancer.[5] Some physicians have underestimated these results due to the study design inherent in a retrospective cohort study. However, the authors believe that study design is not grounds for underestimating the value of the SEPAL study. Well-designed cohort studies may in fact be more appropriate formats than RCT for assessing optimal surgery in high-risk cases. Special difficulties are encountered in randomized surgical trials intended for high-risk patients. Some physicians would decline participation in a randomized controlled trial in which pelvic lymphadenectomy versus combined pelvic and para-aortic lymphadenectomy is compared for patients with high-risk endometrial cancer because they might be familiar with para-aortic lymphadenectomy and its benefits and would be reluctant to perform pelvic lymphadenectomy alone. Conversely, doctors with limited experience may be assigned the task of performing complicated surgery. However, they might not achieve the optimal desired outcome due to inadequate experience. Both scenarios create a situation where quality control of treatment might be reduced in the para-aortic lymphadenectomy group. The situation easily occurs in randomized surgical trials intended for high-risk patients. It is generally accepted that RCT are internally valid. However, non-participation of experienced doctors is a threat to external validity. Heterogeneity of intervention is also a threat to internal validity. Should we stick to randomized surgical trials intended for high-risk patients? A high-risk group is not suitable for a randomized surgical trial. In my humble opinion, a prospective cohort study is an option for assessing the role of lymphadenectomy in high-risk endometrial cancer because it would promote homogeneity of surgical intervention.

There are two interesting reports published in the *New England Journal of Medicine* in which results of