colon, gastric and endometrial cancer cells *in vitro* (9,11,12), but has not been studied in cervical cancer. Therefore, we investigated this relationship in cervical cancer cells, with the goal of establishing a new molecular marker for selection of therapy for cervical cancer.

Materials and methods

Subjects and cytologic specimens. Samples were obtained from 20 normal cervical smears and 40 cervical cancer smears. After obtaining informed consent, cervical smears were collected using the ThinPrep collecting system (Cytyc Corporation, Boxborough, MA) and kept in preservation fluid (PreservCyt Solution, Cytyc Corp.) (13). Pathological diagnosis was confirmed by cervical histology, and the cytological and histological results were consistent for all 60 smears. Of the 40 cervical cancer smears, 26 were squamous carcinoma and 14 were adenocarcinoma. The histological type and stage were determined according to the General Rules for Clinical Cervical Cancer in Japan published by the Japan Society of Obstetrics and Gynecology.

Cultured cell lines. The human cervical squamous cell carcinoma-derived cell lines, SKG-I, SKG-II, SKG-IIIa and SKG-IIIb, and the human cervical adenocarcinoma-derived cell lines, HeLa and TCO-I, were used in the study. HeLa cells were incubated in DMEM (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS) (Sanko Junyaku Co., Ltd., Tokyo, Japan) and TCO-I cells were incubated in MEM medium (Sigma) with 10% FBS. All other cell lines were incubated in F12 medium (Sigma) with 10% FBS. Cells were incubated in 10-cm dishes at 37°C in a 5% CO_2 atmosphere.

DNA extraction and methylation-specific PCR (MSP) assay of the CHFR gene. DNA was extracted from 60 cervical smears and 6 cervical carcinoma-derived cell lines using a Get Pure DNA Kit (Dojin Glocal Corporation, Kumamoto, Japan). DNA (1 μg) extracted from cervical smears was diluted with 50 μl of distilled water and incubated in 5.5 μl of 3 N NaOH at 37°C for 15 min. To this solution, 30 μl of 10 mM hydroquinone (Sigma) and 520 μl of 3 M sodium bisulfite (prepared at pH 5.5 with 10 N NaOH, Sigma) were added with mixing. Mineral oil was laid over the solution to prevent evaporation, and the solution was incubated overnight at 50°C. The lower layer of the reaction solution was mixed with 1 ml of Clean-up Resin (Promega Corporation, Madison, WI) and then injected into a column. After rinsing with 2 ml of 80% isopropanol, the mixture was centrifuged at 15,000 rpm for 3 min to completely remove isopropanol. Hot (70°C) distilled water (50 μl) was added, and the mixture was centrifuged at 15,000 rpm for 2 min to elute DNA. The DNA was then incubated with 5.5 μl of 2 N NaOH at 37°C for 20 min. Next, 66 μl of 5 N ammonium acetate and 243 μl of 95% ethanol were added and the mixture was incubated at -80°C for 1 h and centrifuged at 15,000 rpm for 30 min to precipitate DNA. Supernatant exceeding 50 μl was removed, 1 ml of 60% ethanol was added, and the mixture was centrifuged at 15,000 rpm for 30 min and rinsed. The precipitated DNA was dried in air and dissolved in 20 μl of distilled water. DNA solution (2 μl) was used as the MSP template. In the PCR assay, AmpliTaq Gold and 10x PCR buffer/$MgCl_2$ with dNTP (Applied Biosystems, Foster City, CA) were used and the results were analyzed with a GeneAmp PCR System 9700 (Applied Biosystems). The PCR conditions and primer sequence have been described previously (12). DNA extracted from the cultured cell lines was prepared similarly for use in MSP analysis of the *CHFR* gene.

RNA extraction and RT-PCR assay of CHFR expression. Total RNA from 6 cervical cancer-derived cell lines was extracted using an RNeasy mini-Kit (Qiagen, Valencia, CA). cDNA was synthesized from 1 μg of total RNA using SuperScriptII Reverse Transcriptase (Invitrogen, Carlsbad, CA). *CHFR* expression was analyzed in an RT-PCR assay using 1 μl of first-strand cDNA as template. AmpliTaq Gold and 10x PCR buffer/$MgCl_2$ with dNTP were used in the PCR assay, with analysis using a GeneAmp PCR System 9700 (Applied Biosystems). The PCR conditions and primer sequence have been described previously (12).

Demethylation treatment. Cervical carcinoma-derived HeLa cells with aberrant methylation of *CHFR* were plated on a 10-cm dish at 10^6 cell/dish and incubated for 72 h. 5-aza-dC (Sigma), a demethylating agent, was then added at a final concentration of 1 μM in culture medium. After 48 h of incubation, 5-aza-dC was added again and DNA and RNA were extracted 24 and 72 h after the second addition of 5-aza-dC.

Cell-cycle analysis using flow cytometry. Cervical-carcinoma derived SNG-IIIa and HeLa cells were plated on a 10-cm dish at $5x10^5$ cell/dish and incubated until the cells reached 80% confluence. Paclitaxel (supplied by the Bristol-Myers Squibb Company) was added to the culture medium at a final concentration of 1.0 μg/ml. The cells were trypsinized 48 h later and rinsed twice with PBS. Supernatant was separated from the cell pellets by centrifugation at 15,000 rpm for 5 min, and 500 μl of PBS was added to the pellets and the mixture was pipetted well. As the mixture was vortexed, 1 ml of cool 100% ethanol was added. The mixture was then incubated at room temperature for 30 min for cell fixation. The cells were rinsed twice with PBS and 500 μl of RNase was added to the pellets after supernatant removal. The cells were then incubated at room temperature for 20 min. Subsequently, 500 μl of propidium iodide (PI) solution was added, the mixture was poured into a cell strainer, and the cell cycle was determined by flow cytometry using an EpicsXL MCL (Beckman Coulter, Inc, Fullerton, CA).

In vitro test of sensitivity to anticancer agents. The sensitivity to anti-cancer agents of 6 cervical carcinoma-derived cell lines was determined using the collagen gel droplet embedded culture drug sensitivity test (CD-DST) (14). Cervical carcinoma-derived cells were pretreated with cell dispersion enzyme EZ (Nitta Gelatin Inc., Tokyo, Japan) for 2 h, followed by centrifugation to collect the cells. In a flask containing collagen gel, the cells were pre-incubated for 24 h and surviving cells that adhered to collagen gel were collected. Cellmatrix Type CD solution was added to the

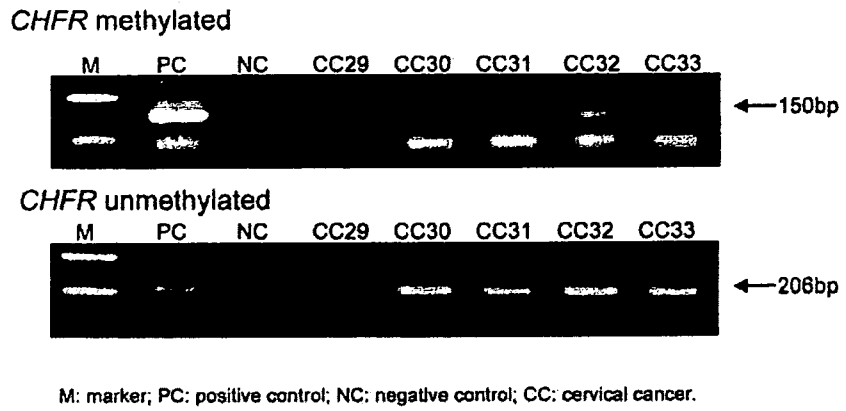

Figure 1. MSP analysis of the *CHFR* gene in cervical cancer cytologic specimens. MSP analysis was examined with DNA extracted from cervical cancer cytologic specimens. A band due to aberrant methylation is observed at CC32.

collected cells, and the suspension of cells and collagen gel was dropped onto a 6-well plate to prepare 3 drops of 30 μl each. The suspension was left to stand in an incubator at 37°C in a 5% CO_2 atmosphere for 1 h for gelling and then overlaid with 4 ml/well of medium. An anticancer agent was then added to the suspension: cisplatin, doxorubicin, paclitaxel, docetaxel, 5-fluorouracil and etoposide at final concentrations of 2.0, 0.02, 1.0, 0.1, 1.0 and 1.0 μg/ml, respectively. After 24 h, the drugs were removed by rinsing and the cells were incubated without serum at 37°C in 5% CO_2 for 7 days. The cells were dyed with Neutral Red, fixed with formalin and dried. Images were collected by scanning using an image analyzer and the ratio of surviving cells in the anticancer agent-treated group (T) to that in the non-treated group (C) (T/C ratio) was determined. In general, cells are considered to be highly sensitive to the agent when the T/C ratio is ≤40%.

Transfection of small interfering RNA (siRNA). SKG-IIIa cells were plated on a 60-mm dish at $4x10^5$ cell/dish and transfected 48 h later with siRNA using siFECTOR (B-Bridge International Inc, CA). In this procedure, 4.5 μl of siRNA stock solution (100 μM) and 295.5 μl of serum-free MEM were mixed in a test tube. In another tube, 13.5 μl of siFECTOR and 286.5 μl of serum-free MEM were mixed. The solutions from the two tubes were mixed and incubated at room temperature for 30 min. Each dish containing SKG-IIIa cells was rinsed twice with 2 ml of serum-free MEM and 2.4 ml of serum-free MEM was then added. The incubated siRNA mixture solution was added to the dish at 0.6 ml/dish and incubated at 37°C in 5% CO_2 for 6 h. After incubation, 3 ml of MEM containing 20% serum was added to the dish. S20C-0600 (B-Bridge International) was used as negative control siRNA. The siRNA sequence corresponding to the *CHFR* gene was 5'-GGAAAAACAUGUUGACCGAdTdT-3'. The expression levels of mRNA and protein were determined 48 h after siRNA addition. Anticancer agents were added 48 h after siRNA addition and the sensitivity of the cells to each agent was analyzed using the CD-DST.

Immunoblotting. SKG-IIIa cells were rinsed with PBS, trypsinized and centrifuged at 15,000 rpm for 5 min at 4°C. Protein was extracted using a Mammalian Cell Extraction Kit (Bio Vision Research Products, CA) according to the manufacturer's protocol. The sample (200 μg of protein) was mixed with sample buffer (Bio-Rad Laboratories, CA) containing the equivalent volume of 5% ß-mercaptoethanol (Bio-Rad Laboratories) and the mixture was boiled for 5 min. After boiling, the mixture was electrophoresed on a 10% polyacrylamide gel and the proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were soaked in PBS containing 1% BSA and 0.1% Tween-20 and incubated at room temperature for 1 h for blocking. They were then reacted with anti-ß-actin antibody (A5316 Sigma-Aldrich Inc, St. Louis, MO, 5,000-fold diluted) and anti-CHFR antibody (ab13773, Abcam, Cambridge, UK, 500-fold diluted) at 4°C overnight, followed by rinsing three times with PBS containing 0.1% Tween (PBS-T) for 10 min each. The anti-ß-actin and anti-CHFR antibodies were reacted with anti-mouse IgG antibody (PK-6102, Vector Laboratories, Inc., CA) and anti-goat IgG antibody (BA-5000, Vector Laboratories, 250-fold diluted), respectively, at room temperature for 1 h. The membranes were rinsed with PBS-T three times and reacted with ABC complex (PK-6102, Vector Laboratories, pre-reacted at 4°C for 30 min) at room temperature for 1 h. The membranes were rinsed with PBS-T twice and PBS once, and visualized with DAB (Sigma).

Results

Results from MSP analysis of cervical cancer cytologic specimens are shown in Fig. 1. Aberrant hypermethylation of the *CHFR* gene in the promoter region was detected in 14.3% (2/14) of adenocarcinoma specimens, whereas there was no aberrant DNA hypermethylation in normal cervical cells and squamous cell carcinoma cells (Fig. 1, Tables I and II). Aberrant hypermethylation of *CHFR* was also analyzed in 6 human cervical carcinoma-derived cell lines and was detected in SKG-IIIb and HeLa cells. RT-PCR analysis confirmed that expression of mRNA for *CHFR* was reduced in SKG-IIIb and HeLa cells (Fig. 2). The sensitivity of the cell lines to paclitaxel and docetaxel was determined using the CD-DST, and SKG-IIIb and HeLa cells showed much

Table I. Aberrant methylation of the *CHFR* gene in cervical cancer cytologic specimens.

No.	Tissue type	Stage	*CHFR*
CC1	SCC	Ib1	U
CC2	SCC	Ib1	U
CC3	SCC	Ib1	U
CC4	SCC	Ib1	U
CC5	SCC	IIa	U
CC6	SCC	IIa	U
CC7	SCC	Ib2	U
CC8	SCC	IIb	U
CC9	SCC	Ib1	U
CC10	SCC	Ib1	U
CC11	SCC	Ib1	U
CC12	SCC	Ib2	U
CC13	SCC	Ib1	U
CC14	SCC	Ib1	U
CC15	SCC	Ib1	U
CC16	SCC	IIa	U
CC17	SCC	Ib2	U
CC18	SCC	Ib2	U
CC19	SCC	Ib1	U
CC20	SCC	Ib2	U
CC21	SCC	Ib1	U
CC22	SCC	Ib1	U
CC23	SCC	Ib2	U
CC24	SCC	Ib1	U
CC25	SCC	Ib1	U
CC26	SCC	IIb	U
CC27	MAD	Ib1	U
CC28	MAD	IIa	M
CC29	MAD	Ib1	U
CC30	MAD	Ib1	U
CC31	MAD	Ib1	U
CC32	MAD	Ib1	M
CC33	MAD	IIa	U
CC34	MAD	Ib1	U
CC35	MAD	Ib1	U
CC36	MAD	Ib1	U
CC37	MAD	Ib2	U
CC38	MAD	Ib1	U
CC39	MAD	Ib1	U
CC40	MAD	IIb	U

CC, cervical cancer: SCC, squamous cell carcinoma; MAD, mucinous adenocarcinoma (endocervical type).

Table II. Aberrant methylation frequency of the *CHFR* gene in cervical cancer cytologic specimens.

	CHFR	
	M (%)	U (%)
NCE	0 (0)	20 (100)
SCC	0 (0)	26 (100)
MAD	2 (14.3)	12 (85.7)

NCE, normal cervical epithelium; SCC, squamous cell carcinoma; MAD, mucinous adenocarcinoma (endocervical type); M, methylated; U, unmethylated.

higher high sensitivity to these agents, compared to other cells (Table III).

Recovery of *CHFR* expression by treatment with 5-aza-dC was examined in HeLa cells (which showed aberrant *CHFR* hypermethylation), and increased CHFR expression following 5-aza-dC treatment was confirmed by RT-PCR (Fig. 3). Changes in the sensitivity of HeLa cells and SKG-IIIa cells (which did not show aberrant CHFR hypermethylation) to 6 anticancer agents were determined before and after 5-aza-dC addition, using the CD-DST. Anticancer agents other than taxanes (5-fluorouracil, etoposide, cisplatin and doxorubicin) showed almost no change in the T/C ratio before and after 5-aza-dC addition and regardless of aberrant *CHFR* hypermethylation. In contrast, the T/C ratios of HeLa cells treated with paclitaxel and docetaxel increased significantly after 5-aza-dC addition, indicating a significant decrease in sensitivity (Table IV).

Changes in cell cycle were determined using flow cytometry in SKG-IIIa and HeLa cells treated with paclitaxel alone or a combination of paclitaxel and 5-aza-dC. In SKG-IIIa cells (no aberrant *CHFR* methylation), cells in G2/M phase markedly increased to 73.9% after paclitaxel treatment and G2 arrest was observed. In contrast, in HeLa cells (aberrant *CHFR* hypermethylation), the percentage of G2/M cells remained low (8.3%) after paclitaxel treatment and Sub-G1 cells increased to 13.4%, higher than that of controls, suggesting that paclitaxel treatment induced apoptosis. However, combined treatment with paclitaxel and 5-aza-dC resulted in 73.9% of cells in the G2/M phase and a marked decrease in Sub-G1 cells to 2.2%, showing a similar pattern to paclitaxel treatment of SKG-IIIa cells (Fig. 4).

SKG-IIIa cells were transfected with siRNA for *CHFR* and the expression levels of *CHFR* mRNA and protein decreased to approximately half of the control levels (Fig. 5). Under these conditions, changes in sensitivity to anticancer agents were determined using the CD-DST. The T/C ratios for paclitaxel and docetaxel were significantly decreased compared with those for non-taxane anticancer agents, indicating that reduction of CHFR expression specifically increases sensitivity to taxanes (Fig. 6).

Discussion

Aberrant hypermethylation of the *CHFR* gene has been reported in endometrial, gastrointestinal and lung cancers (12,15-17). A similar effect has not been studied in cervical cancer, and the relationship between aberrant *CHFR* hypermethylation and the biological characteristics of cervical

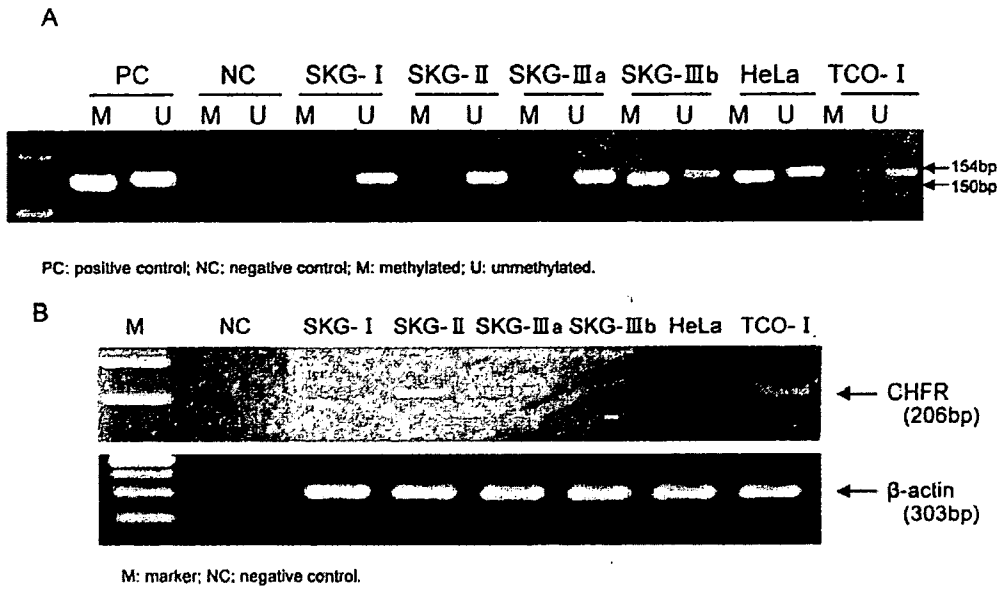

Figure 2. (A) MSP analysis of the *CHFR* gene in cervical cancer-derived cell lines. Aberrant hypermethylation of the *CHFR* gene was observed in SKG-IIIb and HeLa cells. (B) Analysis of *CHFR* expression in cervical cancer-derived cell lines using RT-PCR. CHFR expression was decreased in SKG-IIIb and HeLa cells, which had aberrant hypermethylation of the *CHFR* gene.

Table III. Sensitivity (T/C ratio) of cervical cancer-derived cells to various anticancer agents, assessed using the CD-DST.

Cell line	*CHFR*	Cisplatin (%)	Doxorubicin (%)	Paclitaxel (%)	Docetaxel (%)
SKG-I	U	75.9	89.6	39.5	41.3
SKG-II	U	97.8	91.6	55.5	49.6
SKG-IIIa	U	94.5	90.1	69.2	63.1
SKG-IIIb	M/U	93.2	77.2	14.0	14.0
HeLa	M/U	75.2	79.1	9.8	9.7
TCO-I	U	96.9	66.7	33.2	35.1

M, methylated; U, unmethylated.

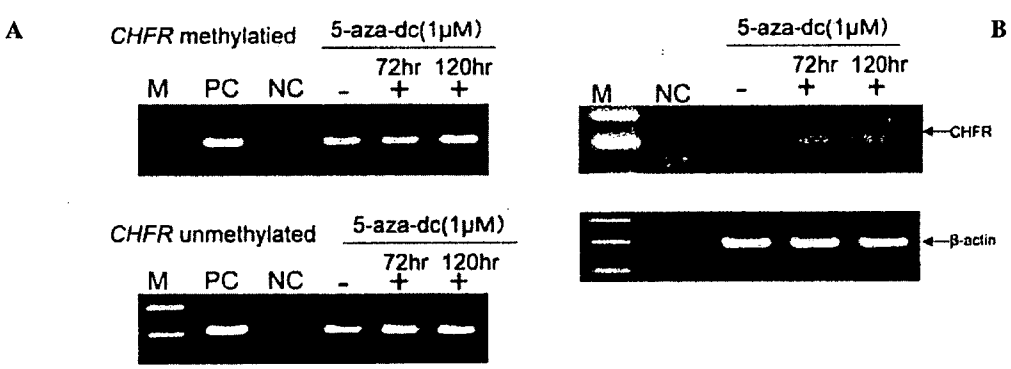

Figure 3. Demethylation analysis of the *CHFR* gene in HeLa cells. (A) MSP analysis after 5-aza-dC treatment. (B) *CHFR* expression recovered 72 h after 5-aza-dC retreatment (RT-PCR).

cancer, including sensitivity to taxanes, is unclear. In this study, aberrant hypermethylation of *CHFR* was observed in adenocarcinoma cells at a rate of 14.3%, but not in normal cervical cells and squamous cell carcinoma cells. Epigenetic

Table IV. Changes in sensitivity (T/C ratio) of cervical cancer-derived cells to various anticancer agents by treatment with a demethylation agent.

Cell line	*CHFR*	5-FU (%)		Etoposide (%)		Cisplatin (%)		Doxorubicin (%)		Paclitaxel (%)		Docetaxel (%)	
		5aza (-)	5aza (+)	5aza (-)	5aza (+)	5aza (-)	5aza (+)	5aza (-)	5aza (+)	5aza (-)	5aza (+)	5aza (-)	5aza (+)
SKG IIIa	U	88.5	83.2	76.0	84.2	94.5	80.7	90.1	98.6	69.2	87.0	63.1	88.5
HeLa	M/U	71.4	84.6	46.3	55.9	75.2	70.9	79.1	81.2	9.8	51.4	9.7	57.1

5aza, 5-aza-dc; M, methylated; U, unmethylated.

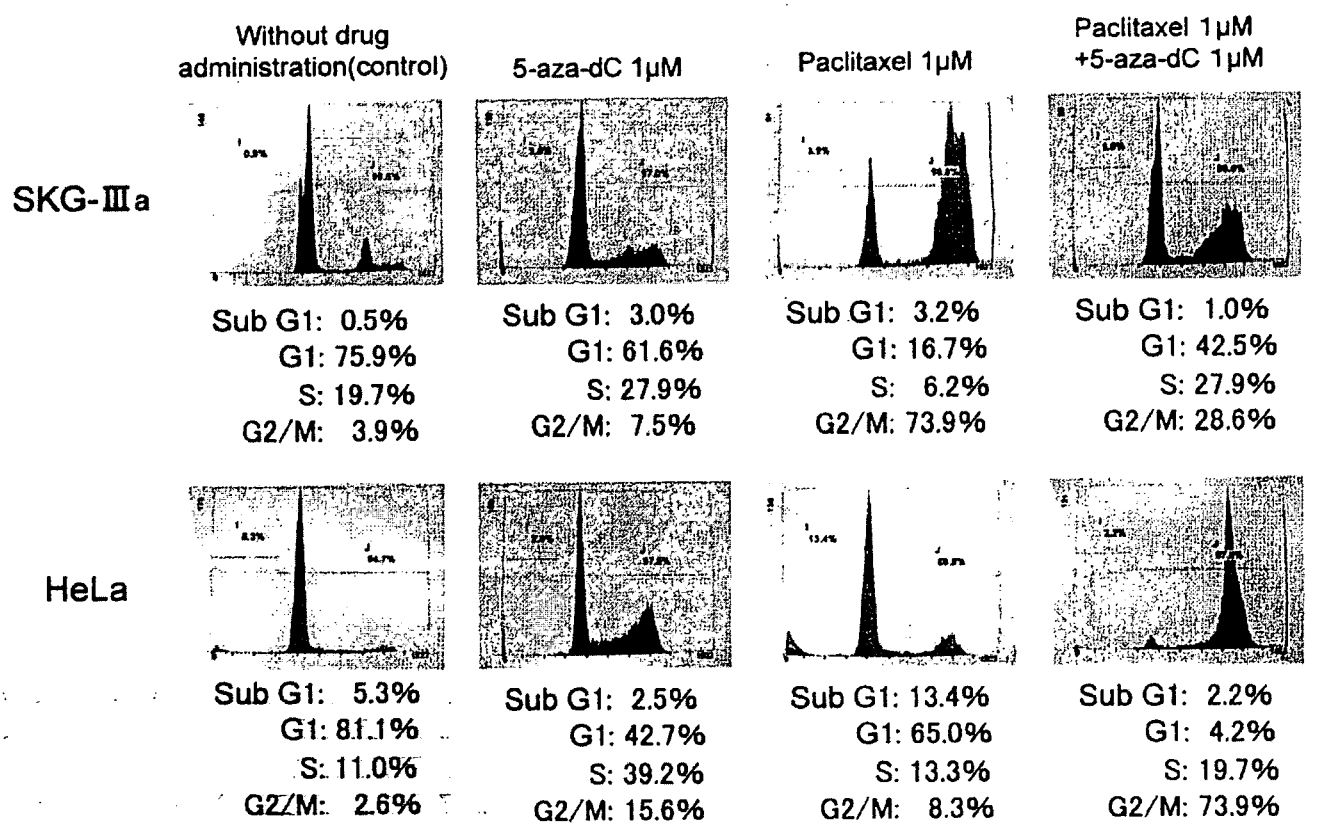

Figure 4. Cell-cycle analysis of SKG-IIIa and HeLa cells using flow cytometry. In SKG-IIIa cells after treatment with paclitaxel alone, the percentage of cells in the G2/M phase was high and that of cells in the Sub-G1 phase did not change markedly. In HeLa cells with paclitaxel alone, the percentage of cells in the G2/M phase was low and that of cells in the Sub-G1 phase increased. In contrast, after treatment with a combination of paclitaxel and 5-aza-dC, cells in the G2/M phase markedly increased and those in the Sub-G1 phase decreased to a level similar to that of the control.

inactivation of *CHFR* has also been observed in endometrial cancer cells, suggesting that aberrant hypermethylation may play an important role in development of uterine cancer, and specifically in adenocarcinoma. There has been a recent increase in cases of cervical cancer, especially in women aged up to 35 years (18-20), and cervical adenocarcinoma has markedly different biological characteristics from squamous cell carcinoma; these characteristics include high nodal metastasis, a refractory nature, poor outcome, and severe malignancy (21-23). The *CHFR* gene negatively regulates the *Aurora-A* gene, a mitotic kinase; hence, suppression of *CHFR* expression increases Aurora-A expression (24). Aurora-A overexpression is reported to induce chromosomal instability (CI) and lead to a poor prognosis in ovarian, breast and bladder cancers (25-27), and a similar mechanism might underlie the characteristics of cervical adenocarcinoma.

Cell-cycle analysis of cervical cancer-derived cells using flow cytometry showed an increase in G2/M cells after paclitaxel treatment in cells with a normal *CHFR* gene. In cells with *CHFR* inactivated epigenetically by aberrant hypermethylation, paclitaxel treatment alone resulted in only a small number of G2/M cells, whereas treatment with a combination of paclitaxel and a demethylation agent caused a marked increase in G2/M cells. These results strongly

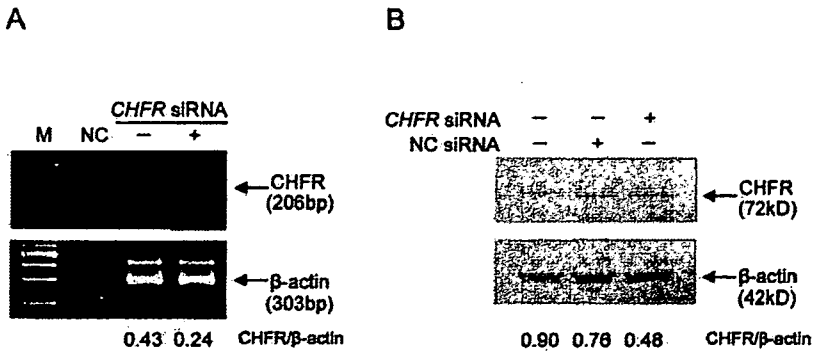

Figure 5. siRNA-induced suppression of CHFR expression in SKG-IIIa cells. (A) RT-PCR, (B) Western blotting. siRNA suppressed expression of mRNA and protein to approximately 50% of control levels.

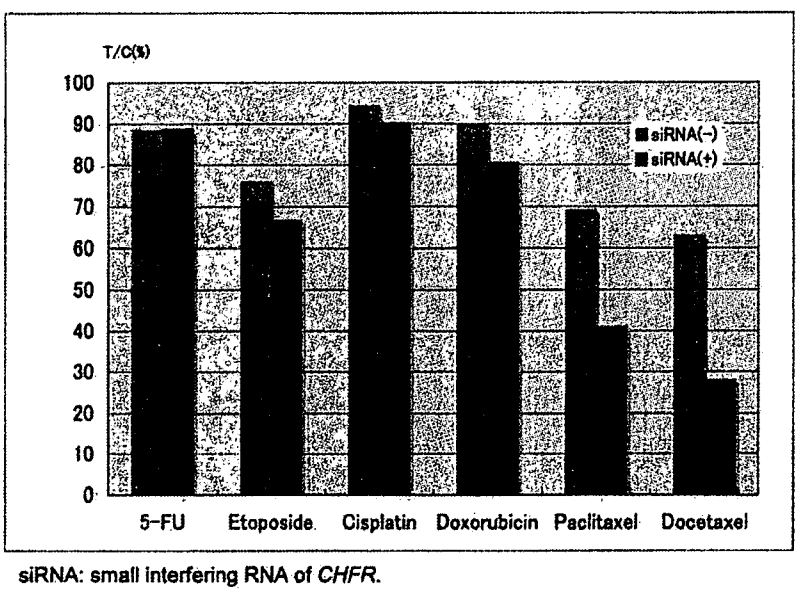

Figure 6. siRNA-induced changes in sensitivity (T/C ratio) of SKG-IIIa cells to various anticancer agents. After suppression of *CHFR* expression, the sensitivity to taxanes alone was increased.

support the hypothesis that when tumor cells are treated with taxane, cells with normal *CHFR* expression undergo arrest in G2 phase to repair damaged DNA and are resistant to taxane, whereas cells with an inactivated *CHFR* gene due to aberrant hypermethylation cannot detect DNA damage and proceed to mitosis, thereby showing high sensitivity to taxane. This mechanism was apparent in cells with inactivated *CHFR* genes following paclitaxel treatment, which caused an increase in Sub-G1 cells, rather than G2/M cells, indicating progression to mitosis and subsequent cell death due to the mitotic catastrophe.

In CD-DST analysis of the sensitivity of HeLa cells to anticancer agents, demethylation significantly reduced the sensitivity to taxanes. Treatment with 5-aza-dC is likely to demethylate various genes, in addition to *CHFR*. However, we also confirmed that suppression of *CHFR* in siRNA-transfected SKG-IIIa cells did not alter sensitivity to cisplatin and doxorubicin, but specifically to taxanes (paclitaxel and docetaxel). This result suggests that epigenetic inactivation of the *CHFR* gene specifically contributed to taxane sensitivity. Therefore, aberrant hypermethylation of *CHFR* may be a molecular marker for prediction of the sensitivity of cervical cancer (and especially cervical adenocarcinoma) to taxane therapy. As discussed above, cervical adenocarcinoma is more refractory and shows a poorer response to anticancer agents compared with squamous carcinoma. Clinical responses of cervical adenocarcinoma are 20% with cisplatin, 14% with 5-fluorouracil, and 12% with etoposide, which are slightly lower than those for squamous carcinoma (28). However, in cervical adenocarcinoma with higher epigenetic inactivation of CHFR gene compared to squamous carcinoma, the clinical response to paclitaxel alone is 31%, 17% higher than with any other agent (29), and these findings are consistent with our results.

The CD-DST can be used to test chemosensitivity to anticancer agents using a small number of cells in a three-dimensional culture, and can be analyzed by cultured cells and specimens in the same system. This method has also been reported to show a strong correlation with clinical response in gynecological tumors (14). Therefore, the aberrant hypermethylation of the *CHFR* gene may be useful for a molecular marker for selection of therapy for cervical cancer. Furthermore, transfection of siRNA for *CHFR* increased the sensitivity of cervical squamous carcinoma to taxanes without affecting the sensitivity to other anticancer agents. This approach may be applicable to preoperative chemotherapy for stage Ib and IIb patients, and may offer a new therapeutic strategy for cervical cancer.

Acknowledgments

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A validation study of a scoring system to estimate the risk of lymph node metastasis for patients with endometrial cancer for tailoring the indication of lymphadenectomy☆

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Abstract

Objective. The aim of this study was to verify whether a preoperative scoring system to estimate the risk of lymph node metastasis (LNM) in endometrial carcinoma is clinically useful for tailoring the indication of lymphadenectomy.

Study design. LNM score was set up using volume index, serum CA125 level, and tumor grade/histology, which were found to be independent risk factors for LNM in a pilot study. Based on the LNM score before a validation study was started, the estimated rates of LNM (para-aortic LNM) were 3.4% (0.0%) in a low risk group, 7.7% (5.8%) in an intermediate group, 44.4% (30.6%) in a high risk group and 70.0% (50.0%) in an extremely high risk group. The validation study was carried out using data for 211 patients with endometrial carcinoma for whom three risk factors were preoperatively confirmed. Logistic regression analysis was used to determine whether these factors remain valid. The actual rate of LNM was investigated according to the LNM score.

Results. Volume index, serum CA125 level, and tumor grade/histology were found to be independent risk factors for LNM in the cohort of this study. The actual rates of LNM (para-aortic LNM) were 3.2% (1.0%) in the low risk group, 15.3% (11.9%) in the intermediate group, 30.2% (23.8%) in the high risk group and 78.6% (57.1%) in the extremely high risk group.

Conclusion. The actual rate of LNM for each score was fairly consistent with the estimated rate of LNM. Para-aortic lymphadenectomy may not be necessary in cases of a low risk group. A large prospective multicenter clinical trial needs to be conducted to establish the clinical usefulness of our preoperative scoring system.

Keywords: Endometrial carcinoma; Lymph node metastasis; MRI; CA125

Introduction

Retroperitoneal lymph node metastasis (LNM) is a critical prognostic factor for patients with endometrial carcinoma [1]. Lymphadenectomy has become accepted as the standard treatment for women with endometrial cancer but still has some issues of debate. Many gynecologists would agree that patients with endometrial carcinoma who have grade 1 tumor with no myometrial invasion do not need lymphadenectomy [2]. However, there has not been a gold-standard method for selecting patients who do not need lymphadenectomy in a preoperative setting. It has been suggested that para-aortic lymphadenectomy has a potential therapeutic role in node-positive endometrial cancer [3]. However, a consensus has not been reached regarding the issue of whether to extend the

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application field of lymphadenectomy to the para-aortic area. We previously reported that volume index, which is a substitute for tumor volume, preoperative serum CA125 level, and histologic grade 3 tumor or serous adenocarcinoma determined by preoperative endometrial biopsy were independent risk factors for LNM [4]. Using these indexes, we formulated a scoring system to estimate the risk of LNM in endometrial cancer. The aim of this study was to determine whether this scoring system is valid in a different cohort of patients with endometrial cancer. Indication for lymphadenectomy is also discussed in this report.

Materials and methods

Study design

We designed a series of clinical studies to establish a scoring system that can determine the risk of LNM in an individual patient with endometrial cancer. These studies comprise of as follows: 1) a pilot study to define the independent risk factor of LNM that will be incorporated in the scoring system, 2) a validation study in which the scoring system will be applied to a different cohort of patients, and 3) a large multi-institutional observational study to verify the generalizability and applicability of the scoring system. This is a report of the validation study.

A pilot study for formulating lymph node metastasis score (LNM score)

The lymph node metastasis score (LNM score) was formulated based upon the data of 214 patients with endometrial cancer who underwent extensive surgical staging including systematic pelvic and para-aortic lymphadenectomy during the period between January 1993 and March 2000, which was reported in a previous paper [4]. In a pilot study, all patients with endometrial cancer underwent systematic lymphadenectomy. The pelvic lymph node groups that were dissected included the common iliac, external iliac, internal iliac, obturator, medial deep inguinal, lateral deep inguinal, parametrial, and sacral node group in the pelvic area. 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 level of the renal vessels were dissected. The clinicopathologic characteristics of the patients are shown in Table 1. Among the 214 patients, 31 (14.5%) had LNM and 19 (8.9%) had para-aortic LNM.

The scoring system includes volume index, serum CA125 level, and tumor grade/histology. Volume index was defined as the product of the maximum longitudinal diameter along the uterine axis, the maximum anteroposterior diameter (thickness) in a sagittal section image, and the maximum horizontal diameter in a horizontal section image. The results of measurements were used to obtain receiver operating characteristic (ROC) curves for LNM. Although we separately showed the ROC curve for pelvic LNM and that for para-aortic LNM in a previous report, the revised ROC curve for LNM that includes both pelvic LNM and para-aortic LNM is shown in Fig. 1. When determined on the curve, cut-off value for LNM was 36. The serum CA125 level was determined using a RIA kit (Fujirebio Diagnostics, Malvern, PA). The patient population was divided into two groups by age. Although we separately showed the ROC curve for pelvic LNM and that for para-aortic LNM in a previous report, the revised ROC curves for LNM that includes both pelvic LNM and para-aortic LNM are shown in Fig. 1. Using these curves, two cut-off values (70 U/ml for patients aged less than 50 years and 28 U/ml for patients aged 50 years or over) divided patients into low and high CA125 groups for LNM. Preoperative endometrial biopsy specimens were evaluated for tumor grade and histologic variant (three grades according to the 1988 FIGO criteria). Tumor grade/histology, which put two factors of tumor grade and histologic type together, was used as an independent variable in the revised analysis. In a previous report, we separately showed the results of logistic regression analysis in which pelvic LNM was used as a dependent variable and that in which para-aortic LNM was used as a dependent variable. Table 2 shows the results of revised logistic regression analysis in which LNM including both pelvic LNM and para-aortic LNM was used as a dependent variable.

Table 1
Characteristics of a cohort of 214 patients with endometrial carcinoma to produce LNM score in the pilot study

Study design	Retrospective cohort study			
Number of institution	3			
Number of patients	214			
Age, median (range)	56 (23–80)			
Number of resected lymph nodes, mean±SD	71.2±34.0			
	No.	LNM	PLNM	PANM
pT (TNM classification)				
1a	54	0	0	0
1b	62	5	5	1
1c	44	8	8	6
2a	10	3	2	1
2b	8	5	5	4
3	31	5	4	4
4	5	5	5	3
Histologic type (preoperative diagnosis)				
Endometrioid	207	27	25	17
(G1)	(134)	(10)	(9)	(7)
(G2)	(55)	(10)	(10)	(4)
(G3)	(18)	(7)	(6)	(6)
Serous	7	4	4	2

These data were not published in a previous paper [4].
LNM: lymph node metastasis, PLNM: pelvic lymph node metastasis, PANM: para-aortic lymph node metastasis.

The impact of the LNM score was determined according to the number of independent risk factor which was confirmed in Table 2. All patients were classified into low risk group (with no risk factor), intermediate risk group (with one risk factor), high risk group (with two risk factors) and extremely high risk group (with all risk factors). Of the 214 patients, 116 (54%) were included in the low risk group, 52 (24%) were included in the intermediate risk group, 36 (17%) were included in the high risk group and 10 (5%) were included in the extremely high risk group. Based on the LNM score, the estimated rates of LNM (para-aortic LNM) were 3.4% (0.0%) in the low risk group, 7.7% (5.8%) in the intermediate group, 44.4% (30.6%) in the high risk group and 70.0% (50.0%) in the extremely high risk group.

Validation study of LNM score

Among patients with endometrial carcinoma treated in the Department of Obstetrics and Gynecology, Hokkaido University Hospital and twelve affiliated hospitals during the period from July 2000 to April 2005, 216 patients who underwent extensive surgical staging including systematic lymphadenectomy were entered in this study. The patients underwent pelvic MRI, endometrial biopsy, and serum CA125 level determination as preoperative examinations. Although lymphadenectomy was not carried out in some affiliated hospitals for some patients who were preoperatively diagnosed as having grade 1 tumor without myometrial invasion, all patients entered in the validation study underwent hysterectomy, bilateral salpingo-oophorectomy, and systematic lymphadenectomy as initial treatment. For a medical complication, para-aortic lymphadenectomy was not performed in one patient. Five cases with an admixture of sarcomatous component and carcinomatous component in a preoperative endometrial biopsy were excluded from this study. A total of 211 patients were included in the study. The clinicopathologic characteristics of those patients are shown in Table 3. The ages of the patients ranged from 24 to 77 years (median age, 57 years). 21 patients were in pT1a (TNM classification), 91 were in pT1b, 50 were in pT1c, 4 were in pT2a, 17 were in pT2b, 26 were in pT3, and 2 were in pT4. The lymphadenectomy specimens included a median of 77 lymph nodes (mean±SD, 79.4±29.6) evaluated for each patient. 36 (17.1%)

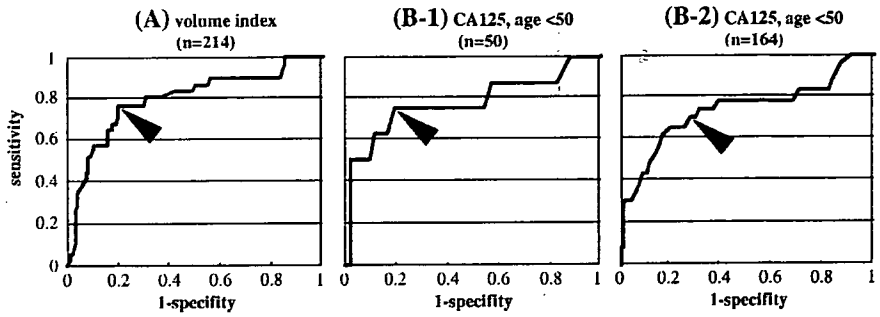

Fig. 1. (A) Receiver operating characteristic curve obtained from the relationships between volume index and LNM in the pilot study. Cut-off value (arrow) for LNM was 36. (B) Receiver operating characteristic curves obtained from the relationships between serum CA125 level and LNM. Cut-off value (arrow) for LNM was 70 U/ml for patients aged less than 50 years. Cut-off value (arrow) for LNM was 28 U/ml for patients aged 50 years or over. (These data were not published in a previous paper [4].)

patients had LNM and 26 (12.3%) had para-aortic LNM. Logistic regression analysis was used to determine whether the factors incorporated into the model of LNM score are still valid in a validation study. Volume index, MRI-based myometrial invasion, serum CA125 level, and tumor grade/histology were used as independent variables, and LNM was used as a dependent variable. The 211 patients were classified into a low risk group, an intermediate risk group, a high risk group and an extremely high risk group. The actual rates of LNM were compared to the estimated rates of LNM according to the model of LNM score.

Statistical analysis

Logistic regression analysis was used to select the risk factors for LNM. Variables that achieved statistical significance in univariate analysis were subsequently included in a multivariate analysis. The statistical significance level was set at .05. Statistical analyses were performed with StatView J-5.0 PPC (SAS Institute, Cary, NC).

Results

Cases with invasion of less than one half the myometrium had a sensitivity of 83%, a specificity of 83%, and an accuracy of 83%. Cases with the diagnosis of G1/G2 histopathology had a sensitivity of 97%, specificity of 74% and accuracy of 92%. Only 5.7% of cases with G1/G2 histopathology diagnosed by endometrial biopsy were upgraded to G3/serous adenocarcinoma after hysterectomy.

Table 4 shows the results of logistic regression analysis. Univariate analysis revealed that all factors were significantly related to LNM. Multivariate analysis confirmed that only high volume index, high serum CA125, and G3/serous adenocarcinoma were independent risk factors for LNM for this cohort of patients. MRI-based myometrial invasion had an odds ratio of 5.4 (95% confidence interval [CI]=2.4–11.9) before adjusting other factors but an odds ratio of 2.7 (95% CI=1.1–6.5) after adjusting volume index. Eventually it had an odds ratio of 2.0 (95% CI=0.8–5.3) after adjusting volume index, serum CA125, and tumor grade/histology.

LNM frequencies according to the LNM score are shown in Table 5. The rates of LNM were 3.2% (3/95) in the low risk group, 15.3% (9/59) in the intermediate group, 30.2% (13/43) in the high risk group and 78.6% (11/14) in the extremely high risk group. The rates of para-aortic LNM were 1.0% (1/95) in the low risk group, 11.9% (7/59) in the intermediate group, 23.8% (10/42) in the high risk group and 57.1% (8/14) in the extremely high risk group.

7 (6.2%) of the 113 patients who had G1/G2 tumor as assessed by endometrial biopsy and invasion of less than half of the myometrium as assessed by MRI had LNM. On the other hand, 3 (3.2%) of the 95 patients with low risk according to the LNM score had LNM. 5 (4.4%) of the 113 patients who had G1/

Table 2
Correlation between the factors incorporated into the LNM score in the pilot study

Factor	LNM		Univariate analysis	Multivariate analysis			
	n/N	%	*p*-value	*β*	SE	OR (95% CI)	*p*-value
Preoperative tumor grade/histology							
G1/G2	20/189	10.6					
G3/serous	11/25	44.0	<0.0001	1.2	0.55	3.5 (1.2–10.2)	<0.05
Volume index							
<36	7/153	4.6					
≥36	24/61	39.3	<0.0001	1.8	0.53	5.7 (2.0–16.1)	<0.001
MRI myo-invasion							
<1/2	7/118	5.9					
≥1/2	24/96	25.0	<0.0005	0.7	0.53	2.1 (0.7–5.8)	NS
Serum CA125 level							
Low	9/146	6.2					
High	22/68	32.4	<0.0001	1.2	0.49	3.5 (1.3–9.1)	<0.05

These data were not published in a previous paper [4].
LNM: lymph node metastasis, PLNM: pelvic lymph node metastasis, PANM: para-aortic lymph node metastasis.

Table 3
Characteristics of a cohort of 211 patients with endometrial carcinoma included in the validation study

Study design	Retrospective cohort study			
Number of institution	13			
Number of patients	211			
Age, median (range)	57 (24–77)			
Number of resected lymph nodes, mean±SD	79.4±29.6			
	No.	LNM	PLNM	PANM
pT (TNM classification)				
1a	21	0	0	0
1b	91	11	10	10
1c	50	15	12	7
2a	4	1	1	1
2b	17	4	4	3
3	26	3	3	3
4	2	2	2	2
Histologic type (preoperative diagnosis)				
Endometrioid	205	33	30	24
(G1)	(124)	(13)	(12)	(8)
(G2)	(51)	(7)	(6)	(5)
(G3)	(30)	(13)	(12)	(11)
Serous	6	3	2	2

LNM: lymph node metastasis, PLNM: pelvic lymph node metastasis, PANM: para-aortic lymph node metastasis.

G2 tumor as assessed by endometrial biopsy and invasion of less than half of the myometrium as assessed by MRI had para-aortic LNM. On the other hand, only 1 (1.0%) of the 95 patients with low risk according to LNM score had para-aortic LNM.

Fig. 2 shows LNM frequencies for the combined cohorts of 425 patients with endometrial carcinoma according to LNM score. The rates of LNM were 3.3% (95% CI=0.9–5.7) in the low risk group, 11.7% (95% CI=5.7–17.7) in the intermediate group, 36.7% (95% CI=26.1–47.3) in the high risk group and 75.0% (95% CI=57.7–92.3) in the extremely high risk group. The rates of para-aortic LNM were 0.5% (95% CI=0.0–1.4) in the low risk group, 9.0% (95% CI=3.7–14.3) in the intermediate group, 27.6% (95% CI=17.6–37.7) in the high risk group and 54.2% (95% CI=34.2–74.1) in the extremely high risk group. Of the 211 patients in low risk group, 1.7% (95% CI=0.0–4.1) with grade 1 carcinoma and invasion of less than half of the myometrium as assessed by MRI had LNM. On the other hand, 5.2% (95% CI=0.8–9.7) with grade 2 carcinoma or invasion of more than half of the myometrium as assessed by MRI had LNM.

Table 5
LNM frequencies according to LNM score

LNM score		LNM (%)		PLNM (%)		PANM (%)	
		Pilot study	Validation study	Pilot study	Validation study	Pilot study	Validation study
Low risk	RF=0	3.4	3.2	3.4	3.2	0.0	1.0
Intermediate risk	RF=1	7.7	15.3	7.7	15.3	5.8	11.9
High risk	RF=2	44.4	30.2	38.9	23.3	30.6	23.8
Extremely high risk	RF=3	70.0	78.6	70.0	71.4	50.0	71.4

LNM: lymph node metastasis, PLNM: pelvic lymph node metastasis, PANM: para-aortic lymph node metastasis, RE: risk factor (MM tumor volume, serum CA125 level, tumor grade/histology).

Discussion

(G1) The FIGO 2001 annual report showed that the 5-year overall survival rate of patients with carcinoma of the endometrium has increased by 13.5% in the past 30 years [5]. The main change in the therapeutic paradigm for endometrial cancer in the past 30 years is the introduction of surgical staging. About 91% of patients have been surgically staged [5]. The treatment strategy including surgical staging may have resulted in improvement of the 5-year survival rate. Recent scientific publications have confirmed the relative safety of surgical staging including lymphadenectomy when performed by subspecialty trained surgeons, and this procedure has become accepted as the standard treatment for women with endometrial cancer [6]. However, there are still some issues of debate about lymphadenectomy in endometrial cancer. There has not been a gold-standard method for selecting patients who do not need lymphadenectomy in a preoperative setting, although many gynecological oncologists would agree that patients with endometrial carcinoma who have grade 1 tumor with no myometrial invasion do not need lymphadenectomy. A consensus

Table 4
Correlation between the factors incorporated into the LNM score and LNM in the validation study

Factor	LNM		Univariate analysis	Multivariate analysis			
	n/N	%	*p*-value	*β*	SE	OR (95% CI)	*p*-value
Preoperative tumor grade/histology							
G1/G2	20/175	11.4					
G3/serous	16/36	44.4	<0.0001	1.6	0.47	4.8 (1.9–12.0)	<0.001
Volume index							
<36	9/137	6.6					
≥36	27/74	36.5	<0.0001	1.2	0.51	3.4 (1.3–9.4)	<0.05
MRI myo-invasion							
<1/2	10/128	7.8					
≥1/2	26/83	31.3	<0.0001	0.7	0.49	2.0 (0.8–5.3)	NS
Serum CA125 level							
Low	11/134	8.2					
High	25/77	32.5	<0.0001	1.0	0.47	2.7 (1.1–6.9)	<0.05

LNM: lymph node metastasis, PLNM: pelvic lymph node metastasis, PANM: para-aortic lymph node metastasis.

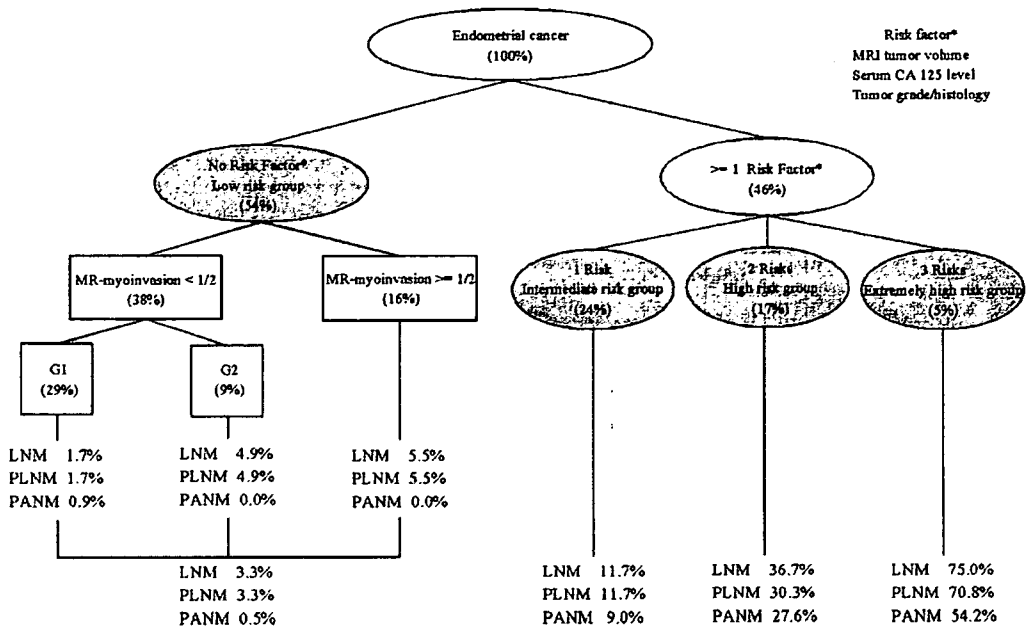

Fig. 2. Rate of LNM for the combined cohorts of 425 patients with endometrial carcinoma according to LNM score and distribution map of patients estimated from a previous paper [4].

has not been reached regarding the issue of whether to extend the application field of lymphadenectomy to the para-aortic area, although it has been suggested para-aortic lymphadenectomy has a potential therapeutic role in node-positive endometrial cancer. The establishment of a gold-standard method for selecting patients with endometrial carcinoma who have little risk for para-aortic LNM would be clinically very useful.

Classically, the risk of LNM has been classified according to the depth of myometrial invasion and histological grade. The methods used to evaluate those parameters in a preoperative setting are MRI and endometrial biopsy. Patients without myometrial invasion who have histologic grade 1 would be candidates for elimination of lymphadenectomy. Although myometrial invasion is indirectly evaluated by MRI in many institutions in Japan, the level of accuracy still has limitations. MRI-based evaluation used for diagnosis of deep invasion in a multi-institutional cooperative study had a sensitivity of 54% and specificity of 89%, indicating that results of previous single institutional studies might have been biased [7]. The level of accuracy for diagnosis of the presence or absence of myometrial invasion using MRI is poorer than the level of accuracy for diagnosis of deep (> 1/2) invasion. Pathological examination using frozen sections obtained during the operation improves the level of accuracy [8,9], but even its level of accuracy is not sufficient. It has been reported that only 60%–72% of patients who were diagnosed as having no myometrial invasion by pathological examination using frozen sections obtained during the operation were diagnosed as having no myometrial invasion by examination of resected specimens [8,9]. There is also the question of whether myometrial invasion is an independent risk factor of LNM. Since 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 LNM. However, tumor volume can be estimated by using MRI. There has not been sufficient study to determine which is a more significant risk factor of LNM, myo-invasion or tumor volume. We showed in this validation study that myometrial invasion assessed by MRI might be a confounding factor of the volume index but not an independent risk factor for LNM, as we found in our previous study [4]. Although histological grade is evaluated by endometrial curettage in many institutions, the level of accuracy also has limitations. Larson et al. reported that the use of office endometrial biopsy had a sensitivity of 67%, specificity of 73% and accuracy of 70% for the diagnosis of G1 histopathology and that 37% of cases with G1 histopathology diagnosed by office endometrial biopsy were upgraded to G2/G3 adenocarcinoma after examination of specimens obtained from hysterectomy [10]. However, the level of accuracy for diagnosis of histologic grade would be higher and the number of upgraded cases would decrease if the category of diagnosis including both G1 and G2 is investigated. Larson et al. reported that the use of endometrial biopsy had an accuracy of 86% for the diagnosis of G1/G2 histopathology and that only 1.7% of cases with G1/G2 histopathology diagnosed by office endometrial biopsy were upgraded to G3 adenocarcinoma after examination of specimens obtained from hysterectomy [10].

According to our LNM score, the rate of para-aortic LNM in the low risk group was 0.5%. If treatment strategy including para-aortic lymphadenectomy rescued all patients with para-aortic LNM, the results shown in Fig. 2 suggest that performing para-aortic lymphadenectomy in all cases of endometrial cancer would result in improvement of the 5-year survival rate by about 10%. On the other hand, only 0.5% of patients with no risk factor would benefit from para-aortic lymphadenectomy. According to our

LNM score, the rate of LNM in the low risk group with histologic grade 1 and with invasion of less than half of the myometrium as assessed by MRI was only 1.7%. The rate of LNM increases in proportion to the number of resected lymph nodes [11]. Conversely, cases false-negative for LNM will increase if there are only a few resected lymph nodes. Since the number of resected lymph nodes in this study was much more than that in previous studies, the reported rates of LNM may be reliable.

To conclude, our LNM score may offer useful information for stratification of risk of LNM, and our results suggest that para-aortic lymphadenectomy can be eliminated in cases with no risk factors for LNM in the LNM score and that lymphadenectomy itself may be eliminated in some cases with no risk factors for LNM in the LNM score.

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Diabetes mellitus is a multivariate independent prognostic factor in endometrial carcinoma: A clinicopathologic study on 313 patients

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Summary

Objective: The aim of this study was to analyse the influence of diabetes mellitus as a prognostic factor for overall survival in endometrial cancer. *Materials and Methods:* Charts were reviewed from patients with endometrial carcinoma from 1985 to 2003. Data on clinicopathologic variables, adjuvant treatment, site of recurrence and survival were collected. The chi-square test was used to examine associations between variables. The Kaplan-Meier method was used for survival analysis and Cox's proportional hazards model for multiple regression analysis. *Results:* Multivariate analysis revealed that diabetes mellitus, FIGO stage and depth of myometrial invasion were significantly associated with overall survival.

Key words: Endometrial cancer; Diabetes mellitus; Prognostic factor; Overall survival.

Introduction

With approximately 10,100 new cases each year in Germany and an incidence rate of 18/100,000, endometrial cancer is the most common gynaecological malignancy (Robert Koch Institute, 2006). In most cases it has a favourable prognosis. A wide variety of prognostic factors (including histological type, stage, grade, depth of myometrial invasion, steroid receptor status, DNA index, peritoneal cytology, p53, and MIB-1) have been described and evaluated in detail. As recently reported, personal and lifestyle characteristics also impact survival [1, 2], but confirmation of this is needed. We therefore studied multivariate independent factors predicting death following a diagnosis of endometrial cancer.

Materials and Methods

This retrospective study includes 313 patients with histologically proven endometrial carcinoma. Of these 269 were treated between 1985 and 2000 at the Department of Obstetrics and Gynaecology of the University Hospital, Mainz, Germany and data from 44 patients were available who were treated between 1992 and 2003 at the Department of Obsterics and Gynaecology of the University of Hokkaido, Sapporo, Japan. Based on information from hospital records, including surgical notes and pathologic reports, a database was generated. Histological tumour type and tumour grade, weight, height and age of the patients, comorbidity (such as diabetes mellitus), FIGO stage, type of surgery and pathologic TNM classification were included, The FIGO stage followed the surgical staging system for endometrial carcinoma of 1988 [3]. The follow-up of all patients was recorded between 2001 and 2002. Data on survival and recurrence-free interval were included in the database. All tumours were classified according to the WHO/ISGPY classification [4]. Tumour grade was evaluated including architectural and nuclear grading [5]. Depth of myometrial invasion was described as the inner, middle, and outer one-third [6]. The standard surgical procedure at Mainz University was abdominal hysterectomy and bilateral salpingo-oophorectomy. Lymph node dissection as far as possible was performed in cases where intraoperative frozen section showed myometrial infiltration of the outer third of the myometrium and in cases of cervical involvement, according to factors of general morbidity of the patient. The standard surgical procedure at Hokkaido University included pelvic and paraaortal lymph node dissection as a standard operative procedure. Postoperative treatment at Mainz University included radiation; at Hokkaido University chemotherapy was also included in selected cases. Statistical analysis was performed using the SPSS (release 6.1.3) system. Analysis of differences between proportions and survival curves was performed with the chi-square test. Recurrence-free survival and overall survival were calculated from the date of surgery, and distributions utilised the product-limit method of Kaplan and Meier. For multivariate regression modelling with Cox's proportional hazards (forward/backward) regression model was used; p values of less than 0.05 were considered statistically significant.

Results

A total of 313 patients with endometrial carcinoma, 147 (47.7%) with tumour grade 1, 102 (33.1% with tumour grade 2, and 59 (19.2%) with grade 3 tumours were included in the study. The median age at diagnosis was 63.95 years (range 32-91). Two hundred and eleven

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patients (67.4%) were initially diagnosed as FIGO Stage I, 35 patients (11.2%) as FIGO Stage II, 50 patients (16.0%) as FIGO Stage III, and 17 patients (5.4%) as FIGO Stage IV. The majority of our patients were obese. Of the tumour patients 27.4% had a body mass index (BMI) of more than 29, 39.5% had a BMI between 25 and 29, and 33.1% of the patients had a BMI between 17 and 24. Sixty-four (23.2%) suffered from diabetes mellitus, 43% were treated by oral medication, 28% were treated with insulin, and 21% did not take any medication. There was no differentiation made between type I and type II diabetes mellitus. The mean follow-up time was 1,353 days (median 1,096 days) and 75 patients died (23.9%). Forty-one patients died from unrelated diseases and were counted as missing cases and not included in the Kaplan-Meier procedure. In 34 patients (12.8%), recurrent disease could be observed and eight patients (3.0%) showed immediate progression of disease without a disease-free interval. Histopathologic tumour type was adenocarcinoma in 265 cases (86.3%), and other tumour types as adenoakanthoma, adenosquamous carcinoma, papillary and clear cell carcinoma occurred in 13.7%. The estimated overall survival was 78.1% for patients with adenocarcinoma. This was significantly higher than in other tumour types (64.3%). The p value was 0.0037 for overall survival and 0.021 for recurrence-free interval. The univariate model revealed diabetes mellitus ($p \leq 0.001$), FIGO stage ($p \leq 0.001$) and depth of myometrial invasion ($p \leq 0.001$) as the strongest prognostic factors (Table 1). These factors were included in a multivariate setting using the forward/backward LR-model. Cox regression analysis was done for 297 cases (94.9%); 16 patients (5.1%) were excluded as missing cases. Multivariate analyses revealed that diabetes mellitus ($p = 0.049$), FIGO stage ($p \leq 0.001$) and depth of myometrial invasion ($p = 0.004$) as multivariate independent prognostic factors. Hazards ratio was 1.880 for Diabetes mellitus (CI 1.107-3.193) (Table 2). There was no influence between the two different institutions in different countries. The different treatment strategies were also not significantly associated with overall survival.

Table 1. — *Univariate analysis.*

Variable	Likelihood ratio (p value)
Diabetes mellitus	0.049
FIGO stage	0.000
Myometrial invasion	0.004

Table 2. — *Cox's proportional hazards regression (backward/forward LR-model).*

Variable	Hazard ratio	CI	CI
Diabetes mellitus	1,880	1,107	3,193
FIGO stage			
Stage I			
Stage II	1,425	0,681	2,983
Stage III	1,705	0,826	3,518
Stage IV	9,347	4,067	21,483
Invasion			
Only endometrium			
inner 1/3	0,904	0,346	2,361
middle1/3	1,632	0,610	4,364
outer 1/3	2,784	1,148	6,751

Discussion

The major new result from this retrospective clinical study – based on two different institutions in two different countries – was that women with diabetes as a comorbidity had a significantly higher risk of death from endometrial cancer than nondiabetic women. The association between diabetes and shorter survival was not explained by other important prognostic variables, such as the extent or grade of endometrial cancer at time of diagnosis or by differences in initial course of treatment, different institutions or nations.

The variability in overall survival and recurrence-free survival of patients with endometrial cancer has prompted numerous studies examining several clinical and pathologic factors as prognostic factors. There are various clinical and pathologic variables which are reported to be of prognostic significance in univariate or multivariate analysis. Up to now, we have identified six studies with multivariate analyses of more than one or two prognostic factors concerning all four FIGO stages [7-14]. As recently reported, personal and lifestyle characteristics also impact survival [1, 2] We have already published a study with multivariate analyses in a group of 189 patients [2]. These patients were included in the present study. Knowing the limitation of biases and confounding factors it is worth while analysing epidemiological results [1] in a clinical series of patients. In the present study we analysed multivariate independent prognostic factors in a total of 313 patients with endometrial cancer. Our study represents a large group of patients with endometrial cancer who were treated under comparable conditions. In our univariate analysis we identified diabetes mellitus, FIGO stage and depth of myometrial invasion as the strongest factors. This is comparable to our data from the 189 patients in the previous study. The prognostic evidence for FIGO stage and depth of myometrial invasion is no doubt beyond dispute and has been previously published by different authors [7-14]. Multivariate analysis using the forward/backward LR Cox regression model revealed now in a total of 313 patients that diabetes mellitus, FIGO stage and depth of myometrial invasion are independent prognostic factors for overall survival. The hazards ratio was 1.880 for diabetes mellitus (CI 1.107-3.193) and endometrial cancer mortality. There was no influence of nation, institution or treatment strategy. Currently there is now only one epidemiological study, which evaluates mortality risk for patients with endometrial cancer in association with diabetes mellitus. Folsom *et al.* [1] reported in an epidemiological setting a comparable hazard ratio of 2.38 (CI 1.05-5.37) for endometrial cancer mortality. In the literature,

between 6% [15] and 19% [16] of patients affected with endometrial cancer suffered from diabetes mellitus; in an unaffected population 4.3% would be affected [17]. In the present study 23.2% of patients suffered from diabetes mellitus. Unfortunately diabetes type I and type II were mixed, thus a differentiation was not possible retrospectively and HbA1c levels were not available. As is known cancer-stromal interactions initiate endometrial cancer invasion, therefore depth of myometrial invasion is one of the multivariate independent prognostic factors. Since these results are independent of age, extent of cancer at diagnosis, tumour grade, and initial treatment, it might be possible that diabetes, hyperglycemia, or hyperinsulinemia could contribute directly to late effects of endometrial cancer. This could be a reasonable explanation because recent studies have demonstrated diabetes [18] and greater glucose concentrations [19] to be risk factors for endometrial cancer. Additionally laboratory results showed that endometrial cancer cells in vitro have high-affinity binding sites for insulin and proliferate in response to insulin exposure [20].

In summary, we have described diabetes mellitus as a multivariate independent prognostic factor for overall survival of patients with endometrial cancer in a retrospective analysis of 313 patients. The significant hazard ratio in endometrial cancer mortality suggests an influence of endocrine disorders, e.g., diabetes mellitus, on the aggressiveness of endometrial cancer. Examination of endometrial cancer molecular biology under the influence of endocrine disorders like diabetes mellitus and/or under the influence of steroid hormones could offer a better understanding of this association and its possible mechanism.

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Para-aortic lymphadenectomy may improve disease-related survival in patients with multipositive pelvic lymph node stage IIIc endometrial cancer

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Abstract

Objective. The purpose of this study was to determine whether para-aortic lymphadenectomy improves disease-related survival (DRS) in stage IIIc endometrial cancer.

Methods. A total of 63 patients with stage IIIc endometrial carcinoma underwent primary radical surgery in the Tohoku Gynecologic Cancer Unit from 1993 to 2004. All patients had modified radical hysterectomy, bilateral salpingo-oophorectomy, systemic pelvic lymph node (PLN) adenectomy, and with or without para-aortic lymph node (PAN) adenectomy, followed by adjuvant chemotherapy. DRS was analyzed using Kaplan–Meier curves and the log-rank test. Independent prognostic factors were determined by multivariate Cox regression analysis using a forward stepwise selection.

Results. There were no statistical differences in age distribution and histopathological prognostic factors between PLN adenectomy group ($n=25$) and PLN+PAN adenectomy group ($n=38$). On univariate analysis, architectural grade ($p=0.026$), peritoneal cytology ($p=0.033$), and the number of PLN positive sites (≤ 1 or ≥ 2) ($p=0.010$) were related to poor DRS. On multivariate Cox regression analysis, the number of positive PLN sites was related to DRS ($p=0.040$). In positive PLN≥ 2 sites group ($n=33$), PAN adenectomy significantly improved DRS compared to PLN adenectomy alone ($p=0.011$). The incidence of initial PAN recurrence was higher in the PLN adenectomy group (6/25) than in the PLN+PAN adenectomy group (1/38) ($p=0.013$, Odds Ratio=11.68).

Conclusions. The number of positive PLN site is an independent prognostic factor in stage IIIc endometrial cancer. PAN adenectomy decreased the incidence of PAN recurrence and may improve DRS in patients with ≥ 2 positive PLN sites. A large prospective clinical trial needs to be conducted to establish the strategy of PAN adenectomy before or intra-operative treatment.

Keywords: Endometrial carcinoma; Prognostic factor; Stage IIIc; The number of positive pelvic lymph node site; Para-aortic lymph node adenectomy

Introduction

Retroperitoneal lymph node metastasis (LNM) is a critical prognostic factor for patients with endometrial carcinoma. The FIGO (1988) surgical staging system classifies endometrial carcinoma with metastasis to the pelvic and/or para-aortic lymph nodes as stage IIIc. However, various procedures have been used to assess pelvic and/or para-aortic lymph nodes in endometrial cancer patients; they include biopsies only from enlarged nodes, selective nodal sampling from multiple sites, pelvic lymph node (PLN) adenectomy, and both PLN and para-

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aortic lymph node (PAN) adenectomy. Obviously, both PLN adenectomy and PAN adenectomy are the most accurate methods for assessing lymph node metastases. However, PLN adenectomy and PAN adenectomy are not considered to be the standard surgical procedure for endometrial cancer because the therapeutic relevance of the procedure has not yet been sufficiently demonstrated. There are many reports in the literature that suggest the importance of PLN adenectomy [1–6]. However, only limited information is available about the importance of PAN adenectomy [7]. The aim of this study was to determine whether PAN adenectomy improves disease-related survival (DRS) in patients with stage IIIc endometrial cancer.

Material and methods

Patients

A total of 602 patients with endometrial carcinoma had primary radical surgery treatment in the Tohoku Gynecologic Cancer Unit, which consist of 6 Universities, from 1993 to 2004. In 2 Universities, only PLN adenectomy was performed during 1993 through 2000, and PLN+PAN adenectomy from 2001. In other 4 Universities, PLN+PAN adenectomy was performed from 1993 to 2004. All patients had modified radical hysterectomy, bilateral salpingo-oophorectomy, and systemic PLN adenectomy with or without systemic PAN adenectomy. Systemic PAN adenectomy was performed in 297 patients. Among 602 patients, there were 63 patients with stage IIIc disease. We defined a node group by its laterality and location. PLN adenectomy included right and left common iliac, external iliac, suprainguinal, internal iliac, obturator, sacral, and parametrial nodal chains. PAN adenectomy included the nodes located from the bifurcation of the aorta to the level of the renal vein. All lymphatic tissues that surrounded the arteries and veins were completely removed. The median number of nodes removed during pelvic and para-aortic lymphadenectomy was 51 (20–73) and 21 (10–58), respectively. Patients receiving pre-/post-operative radiation or neoadjuvant chemotherapy were excluded from this study. All stage IIIc patients were treated with adjuvant chemotherapy: 74.6% (47/63) of the patients received a combination of adriamycin (40 mg/m^2), cisplatin (75 mg/m^2), with or without cyclophosphamide (500 mg/m^2), every 3 weeks for 5 to 6 cycles from 1993 to 2001; and 25.4% (16/63) received a combination of paclitaxel (175 mg/m^2) and carboplatin (AUC=5) every 3 weeks for 5 to 6 cycles from 2000–2004.

All pathology was reviewed centrally. One of the authors reviewed 63 cases of stage IIIc endometrial carcinoma that had been initially diagnosed by the gynecological pathologists of each institute with respect to histological subtype, architectural grade, lymph-vascular space invasion (LVSI), depth of myometrial invasion, cervical invasion, peritoneal cytology, ovarian metastasis, PLN metastasis, and PAN metastasis. Architectural grade was determined according to the criteria proposed by Kurman et al. [8] LVSI was considered to be present when tumor cells were noted within a vascular or lymphatic space lined by flattened endothelial cells. LVSI was recorded based on the general rules of the Japanese Research Society for Gastric Cancer (1985) [9], which classifies it into 4 degrees: nil, minimal, moderate, and prominent. The degree of LVSI was classified as: nil (−) or minimal (+) when only a few lymph-vascular channels were involved on the border of the invading front of the tumor; moderate (+), when more vessels were involved in a wider area surrounding the invading tumor; and prominent (++), when many vessels were diffusely involved in the deeper part of the myometrium.

The following histopathological prognostic factors were included in the survival analysis: histological subtype, architectural grade, LVSI, depth of myometrial invasion, cervical invasion, peritoneal cytology, ovarian metastasis, PLN and PAN metastasis, and the number of positive PLN and PAN sites.

Statistics

The correlation between the variables was analyzed using Fisher's exact test and the chi-square test. Patient survival was calculated using the Kaplan–Meier method. The significance of the difference in survival was examined by the log-rank test. Univariate and multivariate survival analyses were performed using the Cox regression model with DRS as the outcome measure. A forward stepwise procedure was used to select the independent variables for multivariate analysis. $p<0.05$ was considered statistically significant. Statistical analyses were done using the StatView software package (SAS Institute, Inc, Cary, NC, USA).

Results

A total of all 63 stage IIIc endometrial cancer patients' characteristics are shown in Table 1. The mean age of the PLN adenectomy group was 60.4±8.4 years, and that of the PLN+PAN adenectomy group was 56.3±9.3 years ($p=0.085$). There were no significant differences between the groups in histopathological prognostic factors: histological subtype ($p=0.420$); architectural grade ($p=0.282$); lymph-vascular space invasion ($p=0.075$); myometrial invasion ($p=0.603$); cervical invasion ($p=0.591$); peritoneal cytology ($p=0.219$); ovarian metastasis ($p=0.308$); and the number of PLN positive sites ($p=0.140$). In the PLN+PAN adenectomy group, incidence of PLN metastasis alone, PAN metastasis alone, and both PLN and PAN metastasis were 36.8% (14/38), 10.5% (4/38), and 52.6% (20/38), respectively.

Comparisons of surgical complications between PLN and PLN+PAN adenectomy group are listed in Table 2. Comparing

Table 1
Clinical characteristics of patients with stage IIIc endometrial cancer

	PLN ($n=25$)	PLN+PAN ($n=38$)	p value
Age (year)	60.4±8.4	56.3±9.3	0.085
Prognostic factor			
Histological subtype			
Endometrioid	23	33	0.420
Serious/Clear	2	5	
Architectural grade			
Grade 1/2	19	25	0.282
Grade 3	6	13	
Lymph-vascular space invasion			
Nil/minimal	7	4	0.075
Moderate/prominent	18	34	
Myometrial invasion			
≤1/2	6	9	0.603
>1/2	19	29	
Cervical invasion			
Negative/cervical gland	20	30	0.591
Stromal invasion	5	8	
Peritoneal cytology			
Negative	16	29	0.219
Positive	9	9	
Ovarian metastasis			
Negative	23	32	0.308
Positive	2	6	
The number of positive PLN sites			
0	0	4	0.140
1	13	13	
≥2	12	21	
The number of positive PAN sites			
0	(−)	14	(−)
1	(−)	16	
≥2	(−)	8	

Table 2
Comparison of surgical complications in PLN and PLN+PAN adenectomy group

	PLN (n=25)	PLN+PAN (n=38)	p value
Mean operative time (min)	160.2±49.9	274.0±54.2	<0.0001
Mean estimated blood loss (ml)*	375.5±191.5	657.4±199.0	<0.0001
Ureteral injury	0	1	0.414
Intestinal injury	0	1	0.414
Lymphocyst			
Pelvis	2	3	0.989
Para aorta	(·)	1	(·)
Lymphedema	1	4	0.348
Ileus	1	3	0.535
Wound infection	1	0	0.214

PLN: pelvic lymph node, PAN: para-aortic lymph node.
There were statistical significance in mean operative time (p<0.0001) and mean estimated blood loss (p<0.0001) compared with PLN group and PLN+PAN group.
* Including lymphatic effusion.

these two groups, there were significant differences in mean operative time (160.2±49.9 vs. 274.0±54.2 min, p<0.0001) and mean estimated blood loss including lymphatic effusion (375.5± 191.5 vs. 657.4±199.0 ml, p<0.0001), respectively. However, there were no significant differences in ureteral injury (p=0.414), intestinal injury (p=0.414), lymphocyst (p=0.989), lymphedema (p=0.348), ileus (p=0.535), and wound infection (p=0.214).

Since there were no significant differences in the clinicopathological distribution between the PLN and the PLN+PAN adenectomy group (Table 1), the prognostic risk factors were analyzed in all 63 patients (Table 3). On univariate analysis, the architectural grade (p=0.026), peritoneal cytology (0.033), and the number of PLN positive sites (p=0.010) were related to poor survival. Lymph-vascular space invasion (p=0.198), histological subtype (p=0.150), myometrial invasion (p=0.539),

Table 3
Univariate and multivariate Cox regression analyses for disease-related survival in stage IIIc endometrial carcinoma

	Univariate	Multivariate		
	p value	Risk ratio	95% CI	p value
PAN adenectomy	0.191	–	–	NS
Lymph vascular space invasion	0.198	–	–	NS
Architectural grade	0.026	–	–	NS
Histological subtype	0.150	–	–	NS
Myometrial invasion	0.539	–	–	NS
Cervical invasion	0.979	–	–	NS
Peritoneal cytology	0.033	–	–	NS
Ovarian metastasis	0.529	–	–	NS
The number of positive PLN site	0.010	3.236	1.1-9.9	NS

PAN: para-aortic lymph node.
PAN adenectomy: (−) vs. (+).
Lymph-vascular space invasion: (−)/(+) vs. (++)(+++).
Architectural grade: Grade 1/2 vs. Grade 3.
Histological subtype: endometrioid vs. serous/clear cell.
Myometrial invasion: ≤1/2 vs. >1/2.
Cervical invasion: (−)/cervical gland vs. cervical stroma.
Peritoneal cytology: negative vs. positive.
Ovarian metastasis: (−) vs. (+).
PLN metastasis: ≤1 vs. ≥2.

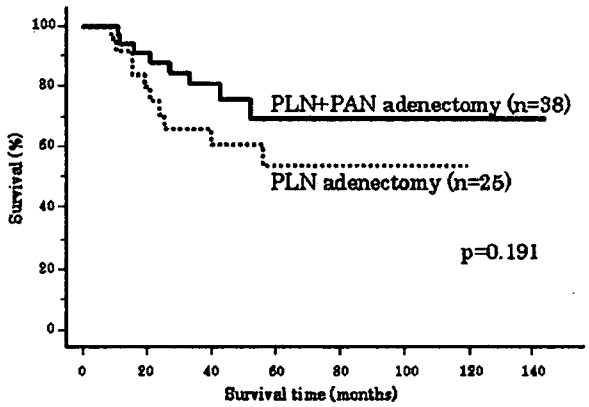

Fig. 1. Disease-related survival in stage IIIc endometrial cancer patients: comparison between the PLN and PLN+PAN adenectomy groups. PLN: pelvic lymph node, PAN: para-aortic lymph node. There was no significant difference in DRS between the PLN and PLN+PAN adenectomy groups (p=0.191). Five-year DRS was 53.9% in PLN adenectomy group and 69.1% in PLN+PAN adenectomy group.

cervical invasion (p=0.979), and ovarian metastasis (p=0.529) were not related to survival. The DRS between the PLN and the PLN+PAN adenectomy groups was also compared (Table 3, Fig. 1); there was no difference in DRS between these two groups (p=0.191); 5-year DRS was 53.9% in the PLN adenectomy group and 69.1% in the PLN+PAN adenectomy group. We also analyzed whether PAN metastasis affects the prognosis in PLN+PAN adenectomy group (Fig. 2). Five-year DRS was 82.4% in the PAN negative group and 43.5% in the PAN positive group (p=0.039).

The multivariate analysis, which included the prognostic factors that were statistically significantly related to DRS on univariate analysis, was done using a forward stepwise procedure (Table 3). On multivariate analysis, only the number of positive PLN sites was an independent prognostic factor for DRS (p=0.040).

Therefore, further analysis was performed to determine whether the number of positive PLN sites had an effect on

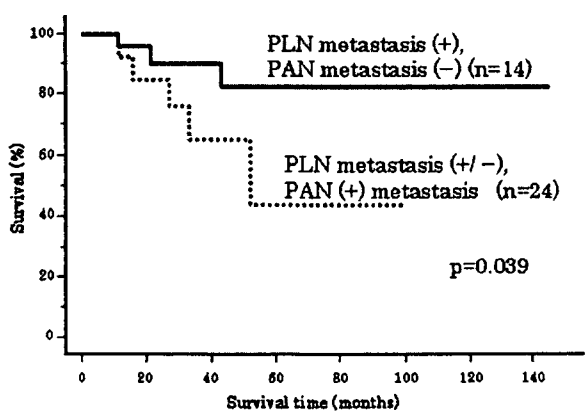

Fig. 2. Survival of patients with stage IIIc endometrial carcinoma by PAN metastasis in PLN+PAN adenectomy group. Five-year disease-related survival of patients without or with PAN metastasis was 82.4%, 43.5%, respectively. The difference was statistically significant (p=0.039).

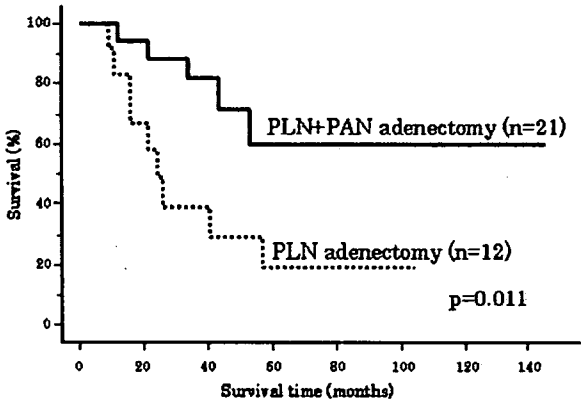

Fig. 3. Disease-related survival of stage IIIc endometrial cancer patients with ≥2 positive PLN sites by PAN adenectomy. PAN adenectomy significantly improved DRS compared to PLN adenectomy alone (p=0.011). Five-year DRS was 19.4% in the PLN group and 59.6% in the PLN+PAN group.

whether PAN adenectomy improves DRS. In the ≥2 positive PLN sites group (n=33), PAN adenectomy significantly improved DRS compared to PLN adenectomy alone (p=0.011) (Fig. 3). The 5-year DRS was 19.4% in the PLN group (n=12) and 59.6% in the PLN+PAN group (n=21). PAN adenectomy did not improve DRS (p=0.408) in the ≤1 positive PLN site group (n=30; PLN adenectomy group: n=13, PLN+PAN adenectomy group: n=17).

Therefore, we also analyzed the incidence of PAN metastasis by the number of positive PLN sites in the PAN adenectomy group (n=38) (Table 4). In the ≤1 positive PLN site group, 16 of 17 patients (94.1%) had ≤1 positive PAN site, and only 1 of 17 patients (5.9%) had ≥2 positive PAN sites; in the ≥2 positive PLN site group, 14 of 21 patients (66.7%) had ≤1 positive PAN site, and 7 of 21 patients (33.3%) had ≥2 positive PAN sites. There was a statistically significant difference in the incidence of ≥2 positive PAN sites between patients who had ≤1 positive PLN site and those who had ≥2 positive PLN sites (p=0.045, Odds Ratio=8.00).

Since our current retrospective analysis demonstrated that PAN adenectomy was therapeutically relevant in patients with ≥2 positive PLN sites (Fig. 3), we examined the clinicopathological factors related to ≥2 positive PLN sites (Table 5). The mean age of the ≤1 positive PLN site group was 56.9±10.0 years, while that of the ≥2 positive PLN sites group was 58.9±8.3 years (p=0.373). There were no statistically significant differences between the two groups in the histopathological prognostic factors: histological subtype (p=0.555), architectural grade (p=0.383), lymph-vascular space invasion (p=0.423), myometrial invasion (p=0.584), cervical invasion (p=0.057), peritoneal cytology (p=0.276), and ovarian metastasis (p=0.300).

The incidence of initial recurrence of PAN between the PLN adenectomy group (n=25) and the PLN+PAN adenectomy group (n=38) was compared. Although there was no significant

Table 4
The relationship between the number of positive PLN and PAN sites in the PAN adenectomy group

	Number of positive PAN sites		Total
	≤1	≥2	
Number of positive PLN sites			
≤1	16	1	17
≥2	14	7	21
Total	30	8	38
			p=0.045 Odds ratio=8.00

PLN: pelvic lymph node, PAN: para-aortic lymph node.
There was a statistically significant difference in the incidence of ≥2 positive PAN sites between patients who had ≤1 positive PLN site and those who had ≥2 positive PLN sites (p=0.045, odds ratio=8.00).

Table 5
Comparison of clinicopathological characteristics between the ≤1 positive PLN site group and the ≥2 positive PLN site group

	≤positive PLN site	≥2 positive PLN sites	p value
Age (year)	56.9±10.0	58.9±8.3	0.373
Prognostic factor			
Histological subtype			
Endometrioid	27	29	0.555
Serious/Clear	3	4	
Architectural grade			
Grade 1/2	22	22	0.383
Grade 3	8	11	
Lymph-vascular space invasion			
Nil/minimal	6	5	0.423
Moderate/prominent	24	28	
Myometrial invasion			
≤1/2	7	8	0.584
>1/2	23	25	
Cervical invasion			
Negative/cervical gland	28	25	0.057
Stromal invasion	2	8	
Peritoneal cytology			
Negative	23	22	0.276
Positive	7	11	
Ovarian metastasis			
Negative	25	30	0.300
Positive	5	3	

PLN: pelvic lymph node.

Table 6
The incidence of PAN recurrence in the PLN and PLN+PAN adenectomy group

	PLN adenectomy	PLN+PAN adenectomy	Total
PAN recurrence			
(-)	19	37	56
(+)	6	1	7
Total	25	38	63
			p=0.013 Odds ratio=11.68

PLN: pelvic lymph node, PAN: para-aortic lymph node.
The incidence of initial recurrence of PAN was significantly higher in the PLN adenectomy group than in the PLN+PAN adenectomy group (24.0% vs.2.6%, 6/25 vs.1/38, p=0.013, Odds ratio=11.68).