Results

As shown in the trial profile (Fig. 1), the initial enrollment was 475 patients, 41 of whom were ineligible due to myometrial invasion of less than 50%, histological diagnosis of sarcoma, or rapid progression of disease after enrollment. An additional 49 patients with non-endometrioid histology were excluded. As a result, 385 patients were eligible for this trial. Seven patients in the PRT group did not receive PRT and 4 patients in the CAP group did not receive CAP.

As shown in Table 1, the study groups were well balanced for patient characteristics including age, postmenopausal status, co-morbidity, type of hysterectomy, postoperative stage, tumor grade, myometrial invasion, lymphovascular space invasion, cervical involvement, parametrial invasion, peritoneal cytology, adnexal metastasis, pelvic lymph node metastasis, and para-aortic lymph node metastasis. None of these characteristics was significantly different between groups in univariate analysis. The distribution of postoperative stages was 61.0% IC, 13.8% II, 13.0% IIIA, and 11.9% IIIC. Pelvic lymphadenectomy was performed in 96.1% of the patients, and paraaortic lymphadenectomy was performed in 28.6% of the patients.

The analysis was performed using data finalized on April 14, 2005. The median follow-up periods in the PRT and CAP groups were 59.5 (2.2–60.8) months and 60.8 (5.0–60.8) months, respectively.

Protocol compliance

Treatment was completed in 98.9% (184/186) and 97.3% (183/188) of the patients in the PRT and CAP groups, respectively. We regarded pelvic radiation as being completed when the total radiation dose reached 40 Gy and regarded chemotherapy as being completed when the number of CAP courses reached three. The median total doses were 50 Gy of pelvic irradiation and 1309 mg/m^2 cyclophosphamide, 120 mg/m^2 doxorubicin, and 180 mg/m^2 cisplatin. The median number of CAP courses was 3, ranging from 1 to 7. The median duration of treatment was 5.1 weeks and 11.4 weeks in the PRT and CAP groups, respectively.

Table 3
Sites of initial recurrence

Recurrence sites*	PRT	CAP
	n=193	n=192
Pelvis	11	5
Vagina only	2	9
Intrapelvic recurrence	13 (6.7%)	14 (7.3%)
Peritoneal cavity	2	2
Liver	3	1
Lung	11	15
Paraaortic lymph node	3	10
Others	7	3
Extrapelvic recurrence	26 (13.5%)	31 (16.1%)
Total recurrent cases	30 (15.5%)	33 (17.2%)

*Including multiple recurrence.
CAP: cyclophosphamide, doxorubicin, and cisplatin.
PRT: pelvic radiation treatment.

Adverse effects

G3 and G4 toxicities were experienced in 1.6% (3/193) of the PRT and 4.7% (9/192) of the CAP groups. Bowel obstructions were the main complication in the PRT group, and myelosuppression was detected in the CAP group. No treatment-related deaths occurred in either group.

Prognostic factors

We performed univariate analyses to detect prognostic factors in all eligible patients. The statistically significant prognostic factors predicting worse PFS were age (≧60 years vs. <60 years), co-morbidity, clinical staging (IIIA vs. II vs. IB vs. IA), tumor grade (G2/3 vs. G1), myometrial invasion (beyond serosa vs. serosa vs. ≧2/3 to < serosa vs. ≧1/2 to <2/3), pelvic lymph node metastasis, adnexal involvement, cervical involvement, peritoneal cytology, and surgical staging (IIIC vs. IIIA vs. IIB vs. IIA vs. IC). For OS, the statistically significant prognostic factors were age, co-morbidity, clinical staging, tumor grade, myometrial invasion, pelvic lymph node metastasis, lymphovascular space invasion, and surgical staging.

Table 2
Multivariate analysis of prognostic factors

Prognostic factors	PFS				OS			
	Hazard ratio	95% confidence interval		P-value	Hazard ratio	95% confidence interval		P-value
		Lower	Upper			Lower	Upper	
Treatment (CAP vs. PRT)	1.07	0.651	1.762	0.788	0.72	0.399	1.290	0.268
Age (≧60 vs. <60)	1.92	1.142	3.210	0.014	3.30	1.634	6.646	0.001
Co-morbidity	1.61	0.974	2.647	0.063	2.24	1.226	4.109	0.009
Tumor grade	1.55	1.125	2.137	0.007	1.64	1.115	2.418	0.012
Cervical involvement	2.28	1.352	3.829	0.002	n.d.	n.d.	n.d.	n.d.
Peritoneal cytology	2.07	1.091	3.920	0.026	n.d.	n.d.	n.d.	n.d.
Pelvic lymph node metastasis	n.d.	n.d.	n.d.	n.d.	4.25	2.235	8.072	<0.001

CAP: cyclophosphamide, doxorubicin, and cisplatin.
PRT: pelvic radiation treatment.
n.d.: not done.

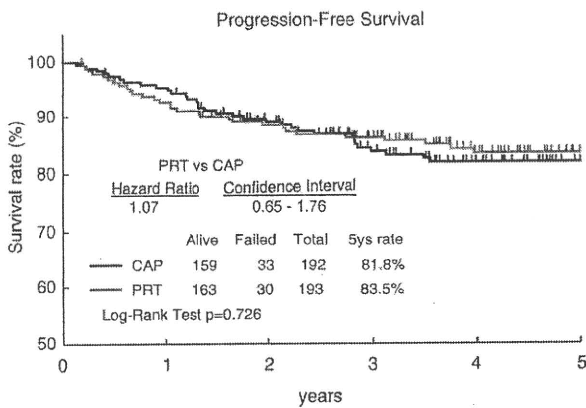

Fig. 2. Progression-free survival rates of all patients in the PRT (pelvic radiation treatment) group and CAP (cyclophosphamide, doxorubicin, and cisplatin) group. Kaplan–Meier analysis. Data for both groups nearly overlap, with no statistical difference.

The significant prognostic factors were used to perform a multivariate analysis with a Cox regression model (Table 2). The multivariate analysis showed that age (≧60 years) and tumor grade (G2/3) were the most important poor prognostic factors for both PFS and OS in this trial.

Recurrence sites

Table 3 presents data on sites of initial recurrence. Thirty recurrences (15.5%) occurred in the PRT group, and 33 recurrences (17.2%) occurred in the CAP group. The patterns of recurrence were similar in both treatment groups. Specifically, the incidence of intrapelvic recurrence sites, such as the pelvis or vagina, was 6.7% (13/193) in the PRT group and 7.3% (14/192) in the CAP group, while the incidence of extrapelvic recurrence sites, such as the peritoneal cavity, liver, lung, paraaortic lymph nodes, and others, was 13.5% (26/193) and 16.1% (31/192) respectively.

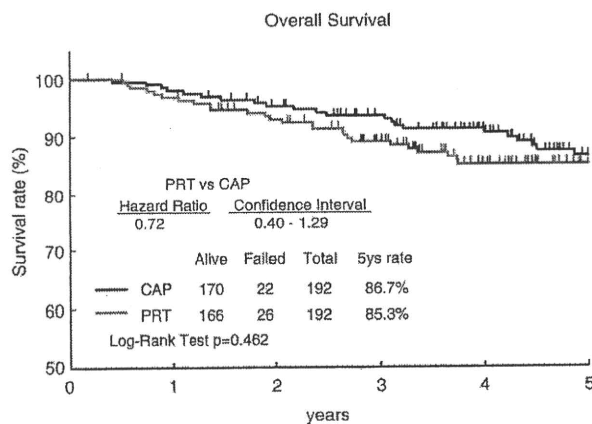

Fig. 3. Overall survival rates in the PRT (pelvic radiation treatment) group and CAP (cyclophosphamide, doxorubicin, and cisplatin) group. Kaplan–Meier analysis. Overall survival rates in both groups were also similar, with no statistical difference.

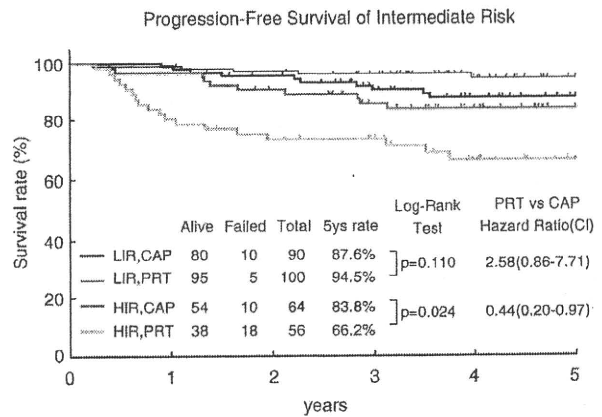

Fig. 4. Progression survival rates of intermediate risk in the PRT (pelvic radiation treatment) group and CAP (cyclophosphamide, doxorubicin, and cisplatin) group. Low–intermediate risk (LIR) was defined as stage IC patients under 70 years of age and with G1/2 endometrioid adenocarcinoma. High–intermediate risk (HIR) was defined as (1) stage IC patients over age 70 years or having G3 endometrioid adenocarcinoma or (2) stage II or IIIA (positive cytology) patients with deeper than 50% myometrial invasion in the corpus. Among LIR patients, PFS rates at 5 years in the PRT and CAP groups were not statistically different. However, among HIR patients, the CAP group had significantly higher PFS rate.

Outcome

Fig. 2 presents the PFS rates of all patients in both randomized treatment groups. Data for the two groups nearly overlap. PFS rate at 5 years was 83.5% in the PRT group and 81.8% in the CAP group. The hazard ratio was 1.07 (95% CI, 0.65–1.76; P=0.726).

Fig. 3 shows that the OS rates in both groups were also similar, with no statistical difference. The OS rate at 5 years was

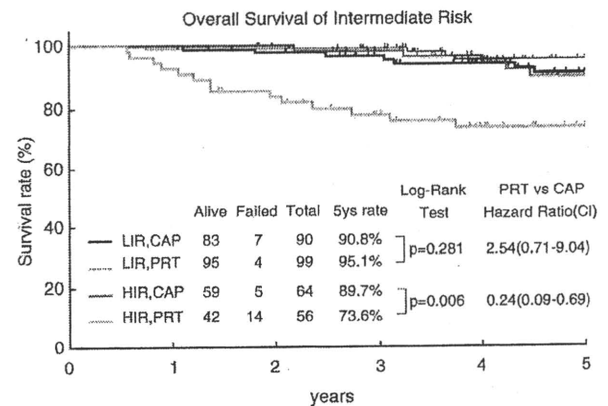

Fig. 5. Overall survival rates of intermediate risk in the PRT (pelvic radiation treatment) group and CAP (cyclophosphamide, doxorubicin, and cisplatin) group. Low–intermediate risk (LIR) was defined as stage IC patients under 70 years of age and with G1/2 endometrioid adenocarcinoma. High–intermediate risk (HIR) was defined as (1) stage IC patients over age 70 years or having G3 endometrioid adenocarcinoma or (2) stage II or IIIA (positive cytology) patients with deeper than 50% myometrial invasion in the corpus. Among LIR patients, OS rates at 5 years in the PRT and CAP groups were not statistically different. However, among HIR patients, the CAP group had significantly higher OS rate.

85.3% in the PRT group and 86.7% in the CAP group (log-rank test $P=0.462$). The hazard ratio was 0.72 (95% CI, 0.40–1.29; Cox proportion hazards model $P=0.268$).

Overall, 48 patients died, of whom 26 had been assigned to the PRT group and 22 to the CAP group. In the PRT group, 21 deaths were related to endometrial cancer, 1 death to another cancer, and 2 deaths to other diseases. In the CAP group, 13 deaths were related to endometrial cancer, 4 deaths to other cancers, and 4 deaths to other diseases.

We performed a subgroup analysis, defining the criteria for low- to intermediate-risk (LIR) and high- to intermediate-risk (HIR) subgroups. When LIR was defined as stage IC patients under 70 years of age and with G1/2 endometrioid adenocarcinoma, among 190 LIR patients, PFS rates at 5 years in the PRT and CAP groups were 94.5% and 87.6% respectively ($P=0.110$) (Fig. 4), and OS rates at 5 years in the PRT and CAP groups were 95.1% and 90.8% respectively ($P=0.281$) (Fig. 5). The HIR subgroup was defined as (1) stage IC patients over age 70 years or having G3 endometrioid adenocarcinoma or (2) stage II or IIIA (positive cytology) patients with deeper than 50% myometrial invasion in the corpus. Among these 120 patients, the CAP group had significantly higher PFS rate (83.8%) (hazard ratio 0.44, 95% CI, 0.20–0.97; $P=0.024$) (Fig. 4) and OS rate (89.7%) (hazard ratio 0.24, 95% CI, 0.09–0.69; $P=0.006$) (Fig. 5) versus the PRT group (66.2% and 73.6%, respectively).

We performed another analysis for high-risk group. For 75 cases in high-risk group, OS rates and PFS rates were not statistically different between PRT group and CAP group. The OS rate at 5 years was 75.8% in the PRT group and 71.1% in the CAP group (log-rank test $P=0.667$). The hazard ratio was 1.123 (95% CI, 0.42–3.04; $P=0.819$). The PFS rate at 5 years was 78.6% in the PRT group and 64.4% in the CAP group (log-rank test $P=0.169$). The hazard ratio was 1.847 (95% CI, 0.73–4.65; $P=0.193$).

Discussion

This study by the Japan Gynecologic Oncology Group is the first report of a randomized controlled study comparing adjuvant pelvic RT with chemotherapy for early-stage endometrial cancer with deeper than 50% myometrial invasion. We observed no statistically significant differences in survivals in the two regimens. We also found that adverse effects were not significantly increased in a platinum-based combined chemotherapy group, and we showed that chemotherapy significantly improved PFS and OS in HIR patients, versus pelvic radiation.

The eligibility criteria for this study were FIGO stage IC–IIIC endometrial carcinoma with deeper than 50% myometrial invasion. The majority (77.4%) of registered patients had stage IC or II lesions, and only 11.9% had stage IIIC lesions. We therefore believe that the efficacy of pelvic radiation and chemotherapy as adjuvant treatments for early-stage endometrial cancer was compared appropriately.

All patients had undergone a hysterectomy and bilateral adnexectomy, and pelvic lymphadenectomy and paraaortic lymphadenectomy were performed in 96.1% and 28.6% of patients respectively. Paraaortic lymphadenectomy was not performed when no paraaortic lymph nodes were palpable and no enlarged paraaortic lymph nodes were detected preoperatively by computed tomography. We therefore regard our surgical staging as appropriate. However, our eligibility criteria were somewhat heterogeneous for the inclusion of post-surgical stage IC, IIA, IIB, IIIA, IIIB, and IIIC lesions.

To verify the efficacy of chemotherapy in intermediate- and high-risk groups, a subgroup analysis is potentially important. Generally, prognostic risk factors have been classified as low, intermediate, or high risks using different criteria [2,3,6,8,14,15]. In these previous reports, stage IC was definitely classified as intermediate risk. Stage III and IV were usually classified as high-risk, locally advanced. The GOG defined stage IC and II, without inclusion of IIIA (positive cytology) as intermediate risk. GOG Study 99 [8] defined HIR as (1) G2/3 tumors with lymphovascular space invasion and outer-third myometrial invasion, (2) age of 50 years or greater in addition to any two factors listed above, or (3) age of at 70 years or greater with any risk factor listed above. FIGO stages IB, IC, and II (occult disease) were defined as LIR.

In our subgroup analysis an LIR group comprised stage IC patients under 70 years of age with G1/2 endometrioid adenocarcinoma. Our HIR group comprised (1) stage IC patients who were over 70 years of age or had G3 endometrioid adenocarcinoma and (2) stage II or IIIA (positive cytology) patients with deeper than 50% myometrial invasion in the corpus. Our high-risk group comprised other stage IIIA patients with factors other than a positive peritoneal cytology and stage IIIB and IIIC patients.

PFS and OS rates for the PRT and CAP groups were the same in the LIR subgroup. In the HIR subgroup, however, we found significantly higher PFS and OS rates in the CAP group versus the PRT group. Since patients with FIGO stage IIIA endometrial cancer only with positive washing cytology have a better prognosis [5,16], we included patients with positive washing cytology in the HIR group, along with stage II disease patients. However, we recognize that the validity of this subset analysis is limited. Demonstration of a true advantage of chemotherapy requires a large-scale randomized controlled trial with stratification for risk factors including age and tumor grade prior to randomization.

In the early 1990s, the CAP regimen was used as the standard chemotherapy for endometrial cancer and ovarian cancer in Japan. Most Japanese gynecologists adopted CAP as the standard adjuvant chemotherapy rather than AP. In our trial, the dosage of doxorubicin was lower than in other trials using AP, such as GOG study 107/122/177 (60 mg/m^2) and GOG study 184 (45 mg/m^2) [17–19]. Due to this relatively low dose, G3 and G4 adverse effects were rare (4.7%), and protocol compliance was very high (95.3%) in the CAP group. The number of CAP courses was relatively small (median: 3 courses). Thus, cisplatin-based chemotherapy may be a feasible alternative to adjuvant pelvic radiation therapy for patients with intermediate-risk endometrial cancers. However, validation of a true efficacy of adjuvant chemotherapy for early-stage endometrial cancer, especially for LIR patients, requires a randomized controlled trial of no-treatment versus chemotherapy.

In HIR patients, chemotherapy was superior to radiation therapy. In patients with low-risk and LIR endometrial cancer, most recurrence sites are vaginal or intrapelvic, making pelvic

radiation or vaginal vault brachytherapy effective for reducing the loco-regional recurrence rate [7,20,21]. The reason for the superiority of chemotherapy in HIR patients is partly that extrapelvic recurrence cannot be prevented by pelvic radiation, as reported by Creutzberg et al. [7,14] and other investigators [6,8,20–22]. In this study, the incidence of recurrences at vaginal wall was lower in PRT group compared with CAP group, however, there was no significant difference in the incidences of extrapelvic recurrence between the PRT and CAP groups. In Japan, different types of hysterectomy, such as simple hysterectomy, extended hysterectomy (type II modified radical hysterectomy), and radical hysterectomy (type III), were performed in each institution. However, radical hysterectomy is selected only for those patients with macroscopically apparent cervical involvement in most of JGOG institutions. In addition, in this study, we included simple hysterectomy with a small amount of removal of vaginal cuff into extended hysterectomy. For this reason, the percentage of radical hysterectomy and modified radical hysterectomy is not thought to be high, and the influence of surgical procedure over the incidence of vaginal recurrence may be limited in our study.

In our study, we performed pelvic lymphadenectomy in 96% cases. Local recurrence rate was 2.6% in the cases of LIR and HIR with pelvic radiation treatment. Local recurrence rate in the radiotherapy group was 3.9% in PORTEC study [2,7] with no pelvic lymphadenectomy and 1.6% at 2 years in GOG study 99 [8] with selective pelvic and paraaortic lymphadenectomy. It seems that there is a tendency of low local recurrence rate in the intermediate-risk patients with pelvic lymphadenectomy in pelvic radiation treatment, however, we cannot simply compare those data as there are differences in the definition of intermediate risk.

The superiority of chemotherapy in HIR patients must also be considered in relation to the conclusions of GOG study 122 on advanced-stage endometrial cancer [4]. In stage III/IV endometrial cancer, AP chemotherapy was superior to whole-abdominal radiation as a therapeutic modality. Further investigation of the use of chemotherapeutic agents in patients with HIR endometrial cancer or high-risk endometrial cancer is needed. The JGOG has just finished accruing for a comparative phase II trial comparing three combined chemotherapy regimens (paclitaxel and carboplatin vs. docetaxel and cisplatin vs. docetaxel and carboplatin). These results are forthcoming.

In patients with early-stage endometrial cancer and deeper than 50% myometrial invasion, adjuvant platinum-based combined chemotherapy and pelvic radiation therapy each led to a good prognosis. In patients with HIR endometrial cancers, the aforementioned chemotherapy improved the prognosis significantly compared to pelvic radiation. Additional phase III randomized controlled trials are required to establish a standard adjuvant chemotherapy regimen including anthracyclin, taxane or platinum for intermediate-risk or high-risk endometrial cancer.

Acknowledgments

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The participating institutions for all studies described in this report are listed in Appendix A.

Appendix A

The following member institutions participated in this study: Akita City Hospital, Aomori Prefectural Central Hospital, Asahi General Hospital, Asahikawa Medical College, Asahikawa Red Cross Hospital, Chiba Kaihin General Hospital, Chiba Social Insurance Hospital, Chiba University, Daiyukai General Hospital, Dokkyo University School of Medicine, Fujita Health University, Gifu Prefectural Tajimi Hospital, Gifu University, Hakodate Goryokaku Hospital, Hamamatsu Medical Center, Himeji Red Cross Hospital, Hiroshima University, Hyogo Medical Center for Adults, Hyogo Prefectural Awaji Hospital, Hyogo Prefectural Tsukaguchi Hospital, Iwate Medical University, Iwate Prefectural Kuji Hospital, JA Kochi Hospital, Japanese Red Cross Akita Hospital, Jiaikai Imamura Hospital, Juntendo University Urayasu Hospital, Kagawa University, Kanazawa Medical University, Kanazawa University, Kanebo Memorial Hospital, Kansai Medical University, Kanto Central Hospital of the Mutual Aid Association of Public School Teachers, Kawasaki Medical School, Keio University, Keiyu Hospital, Kinki University, Kitasato University, Kobe University, Kokura Memorial Hospital, Kumamoto City Hospital, Kumamoto University, Kurashiki Central Hospital, Kurume University, Kyosai Tachikawa Hospital, Kyoto Prefectural University of Medicine, Kyoto Second Red Cross Hospital, Kyoundo Hospital, Kyushu University (Medical Institute of Bioregulation), Miyazaki Prefectural Nichinan Hospital, Nagaoka Red Cross Hospital, Nagasaki University, Nagoya Daini Red Cross Hospital, Nantan General Hospital, Nara Medical University, Nara Prefectural Hospital, National Hospital Organization Hokkaido Cancer Center, National Hospital Organization Iwakuni Clinical Center, National Hospital Organization Matsumoto National Hospital, National Hospital Organization Saitama National Hospital, National Hospital Organization Sendai Medical Center, National Hospital Organization Tokyo Medical Center, Ogaki Municipal Hospital, Ohta General Hospital (Nishinouchi Hospital), Oita University, Okayama Red Cross General Hospital, Okayama Saiseikai General Hospital, Osaka City General Hospital, Osaka General Medical Center, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka Medical College, Osaka Police Hospital, Saga University, Saiseikai Central Hospital, Saiseikai Utsunomiya Hospital, Saitama Shakai-Hoken Hospital, Sapporo Medical University, Sapporo-Kosei General Hospital, Sasebo City General Hospital, Seirei Yokohama Hospital, Senboku Kumiai General Hospital, Shimane Prefectural Central Hospital, Shimane University, Shizuoka General Hospital, Shonai Hospital, Showa University, Showa University Fujigaoka Hospital, Social Insurance Tagawa Hospital, St. Marianna University School of Medicine, St. Marianna University School of Medicine Yokohama City Seibu Hospital, Takamatsu Red Cross Hospital, Teikyo University Ichihara Hospital, Tohoku University, Tokyo Medical and Dental University, Tokyo Medical University, Tokyo Women's Medical University, Tosei General Hospital, Tottori

Municipal Hospital, Tottori University, Toyama Medical and Pharmaceutical University, Toyama Prefectural Central Hospital, University of Tokushima, Yamagata University, Yamaguchi Grand Medical Center.

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Analysis of a correlation between the *BRAF* V600E mutation and abnormal DNA mismatch repair in patients with sporadic endometrial cancer

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Abstract. Point mutations of *KRAS* and *BRAF* genes are thought to be important in carcinogenesis of colon cancer. In particular, gene instability caused by decreased expression of the *hMLH1* gene, a DNA mismatch repair (MMR) gene, may be linked to the activating *BRAF* V600E point mutation in sporadic colon cancer. However, a consensus has not been established regarding the correlation between point mutations of *KRAS* or *BRAF* and carcinogenesis in patients with endometrial cancer, which is closely related to colon cancer. Therefore, we analyzed aberrant hypermethylation of the *hMLH1* gene, microsatellite instability (MSI), and point mutations of *KRAS* and *BRAF* in 44 samples of sporadic endometrial cancer, with the aim of examining the mechanism of carcinogenesis in patients with endometrial cancer. Aberrant *hMLH1* hypermethylation was found in 17 of the 44 cases (38.6%) and showed a significant positive correlation with MSI ($p=0.02$). This suggests that an abnormal MMR mechanism plays an important role in carcinogenesis of sporadic endometrial cancer. Point mutation of *KRAS* was found in 6 of the 44 cases (13.6%), but no *BRAF* V600E mutation was detected. These data suggest that the *BRAF* V600E mutation is not the target gene for abnormal MMR in carcinogenesis in patients with sporadic endometrial cancer, unlike in colon cancer. This is supported by the relatively few previous reports indicating a correlation between endometrial cancer and the *BRAF* V600E mutation. Identification of new candidates for the target gene for abnormal MMR in endometrial cancer requires further work.

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Key words: endometrial cancer, *BRAF*, *KRAS*, *hMLH1*, microsatellite instability

Introduction

A cancer may develop as a result of repeated mutation of genes involved in differentiation or proliferation. Such a multistep mechanism of carcinogenesis with mutation of multiple cancer-related genes is often observed in patients with colon cancer. The correlation between colon cancer carcinogenesis and point mutation of *RAS/RAF* genes in the *MAP* kinase pathway suggests that these genes have an important role at an early stage of malignant alteration of colon cancer (1).

Endometrial cancer has many similarities with colon cancer and is detected at high rates as a double cancer of hereditary non-polyposis colon cancer (HNPCC). Germline mutation of *hMLH1*, a DNA mismatch repair (MMR) gene, occurs at high rates in HNPCC patients (2), and decreased expression of *hMLH1* due to aberrant hypermethylation has also been found in patients with sporadic colon cancer and endometrial cancer (3). Decreased expression of *hMLH1* due to epigenetic changes may facilitate gene replication errors and cause gene instability, which can be detected as microsatellite instability (MSI) (4). Microsatellite DNA is a region with short repeated sequences of 1-2 bases, and PCR-based detection of replication errors in this region has been used widely as a clinical test to examine gene instability. Such instability may cause mutation of cancer-related genes, and a correlation between MSI due to decreased *hMLH1* expression and point mutations of *KRAS* and *BRAF* genes has been proposed in patients with colon cancer (5,6).

Mutation of the *BRAF* gene has been found in many human cancers, including colon cancer, malignant melanomas, thyroid carcinoma and ovarian carcinoma (7-9). *BRAF* is one of the 3 subtypes of *RAF* family genes and encodes a tyrosine kinase involved in mitogenic signaling in the *RAS-RAF-MEK-ERK-MAP* kinase pathway. The function of *RAF* is regulated by *RAS*, and an activating point mutation of *BRAF* causes unregulated constitutive activation of the tyrosine kinase activity and facilitates cell proliferation via the *MAP* kinase pathway. The V600E mutation in exon 15 of *BRAF* is of particular interest, since tyrosine kinase activity 10-fold that of wild-type has been found in tumor tissue with this mutation (10). The V600E mutation is found in about 15% of patients with sporadic colon cancer and can be used for clinical diagnosis of non-inheritive sporadic colon cancer (10). Furthermore, since *BRAF* V600E is observed in 32% of cases of MSI-positive

Table I. Primer sequences used in PCR and MSP analysis.

Gene	PCR method	Sense	Antisense	Size (bp)	Annealing (°C)
hMLH1	Methylated	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	112	60
	Unmethylated	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124	60
KRAS	Codons 12, 13	GCCTGCTGAAAATGACTGAAT	TTATCTGTATCAAAGAATGGTC	180	64
BRAF	Codon 600	TCATAATGCTTGCTCTGATAGGA	GGCCAAAAATTTAATCAGTGGA	150	60

sporadic colon cancer and 75% of cases with sporadic colon cancer with aberrant hypermethylation of *hMLH1*, *BRAF* has been proposed as the target gene of abnormal MMR (11).

In contrast to colon cancer, only a few reports have shown mutation of *BRAF* in patients with endometrial cancer. Feng *et al* found *BRAF* mutations in 21% of patients with endometrial cancer and suggested that the mutation correlated with decreased *hMLH1* expression (12). However, Salvesen *et al* found a *BRAF* mutation in only 2% of patients with endometrial cancer (13). Therefore, it is unclear whether mutation of *BRAF* is important in carcinogenesis of endometrial cancer and whether the mutation may be linked to abnormal expression of the *hMLH1* gene. In this study, we analyzed aberrant hypermethylation of *hMLH1*, MSI, and mutations of *KRAS* and *BRAF* in patients with sporadic endometrial cancer to examine correlations among point mutations in *RAS/RAF* family genes, abnormal MMR caused by aberrant *hMLH1* hypermethylation, and carcinogenesis of sporadic endometrial cancer.

Materials and methods

Cell lines. Eight cell strains were used in the study: HEC108, Ishikawa (a human endometrial cancer-derived cultured cell line supplied by Dr Hiroyuki Kuramoto); HOOUA and HHUA (supplied by Dr Isamu Ishiwata); and SNG-II, SNG-M, HEC-1B and KLE. KLE cells were cultured in a DMEM/F12 (1:1) medium (Gibco-BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Sanko Junyaku Co., Tokyo, Japan). All other cells were cultured in 10% FBS-supplemented F12 medium (Sigma, St. Louis, MO, USA). The cells were incubated in a dish of 10 cm in diameter under 5% CO_2 at 37°C.

Clinical specimens. The subjects were 44 patients with endometrial cancer (G1, 20; G2, 11; G3, 13) who gave informed consent to collection of cancer specimens. Of these patients, 37 had endometrioid adenocarcinoma and 7 had adenosquamous carcinoma. The grade of histological differentiation (G1-G3) and the cancer stage at surgery were determined based on the Guidelines for Endometrial Cancer published by the Japan Society of Obstetrics and Gynecology.

DNA extraction and methylation-specific PCR (MSP) in the hMLH1 promoter region. DNA was extracted from the 44 endometrial cancer specimens using liquid-based cytology with a Get Pure DNA Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Distilled water was added to 1 μg of the extracted DNA up to a volume of 50 μl and 5.5 μl of 3 N NaOH solution was added. After mixing, the solution was incubated at 37°C for 15 min, and then 520 μl of 3 M sodium bisulfite (Sigma) prepared at pH 5.5 with 30 μl of 10 mM hydroquinone (Sigma) and 10 N NaOH was added to the solution. After mixing in an upturned position to prevent vaporization, the solution was overlaid with mineral oil and incubated at 50°C overnight. Next, 1 ml of clean-up resin (Promega, Madison, WI, USA) was added to the lower layer and the resulting solution was mixed in an upturned position and then injected into a column. The column was rinsed with 2 ml of 80% isopropanol and then centrifuged at 15,000 rpm for 3 min to remove the isopropanol completely. Next, 50 μl of distilled water (70°C) was added directly to the column, which was then centrifuged at 15,000 rpm for 2 min to extract DNA adsorbed on the column. Then, 5.5 μl of 2 N NaOH was added to the resulting DNA solution. After mixing, the solution was incubated at 37°C for 20 min, after which 66 μl of 5 N ammonium acetate solution and 243 μl of 95% ethanol were added and the solution was incubated at 80°C for 1 h and centrifuged at 15,000 rpm for 30 min to precipitate DNA. Approximately 50 μl of the supernatant was left in the tube. The rest of the supernatant was collected, mixed with 1 ml of 70% ethanol, and then centrifuged at 15,000 rpm for 30 min to rinse the DNA. The precipitated DNA was air-dried and dissolved in 20 μl of distilled water; 2 μl of this solution was used as the MSP template solution. AmpliTaq Gold and 10X PCR buffer/$MgCl_2$ with dNTP (Applied Biosystems, Foster City, CA, USA) was used in PCR analysis and DNA was analyzed using a GeneAmp PCR System 9700 (Applied Biosystems). The PCR primer sequences are shown in Table I. DNA extracted from the cultured cell lines was also used in MSP analysis of *hMLH1* (14).

Microsatellite instability analysis. Genomic DNA extracted from normal and tumor tissue samples from the 44 patients with endometrial cancer was PCR amplified at the microsatellite repeat loci D2S123, D5S346, D17S250, BAT26, BAT25, MSH3, MSH6, TGF-ßRII, BAX, MBD4A10 and MBD4A6, which include 3 dinucleotide (CA) and 8 mononucleotide repeats as microsatellite markers. PCR reactions were performed in a total volume of 25 μl containing 10X buffer, 0.125 mM deoxynucleoside triphosphate, 0.2 μM of each primer, and 0.25 units of TaqDNA polymerase. The PCR

conditions were as follows: 94˚C for 10 min; 30 cycles at 94˚C for 45 sec, 58˚C for 45 sec, and 72˚C for 40 sec; followed by a final extension step at 72˚C for 10 min. After PCR, 1 μl of the product was mixed with 12 μl of loading buffer containing formamide and Rox size standards. This mixture was denatured at 95˚C for 2 min and cooled on ice before loading onto an ABI PRISM 310 sequencer (Applied Biosystems). The results were analyzed using GeneScan software (Applied Biosystems). Tumors were classified as MSI-H when ≥30% of these markers showed MSI, in accordance with the recent recommendation of the National Cancer Institute Workshop. Low-frequency MSI (<30% of 11 markers) was included in the category of MSI-L and alteration of even one microsatellite region led to definition of the patient as MSI-positive (15).

Determination of KRAS and BRAF mutations. DNA was extracted from the 8 endometrial cancer-derived cell lines and 44 endometrial cancer specimens using liquid-based cytology with a Get Pure DNA Kit (Dojindo Molecular Technologies). Individual point mutations of the *KRAS* and *BRAF* genes were documented using two gene-specific oligonucleotide primer pairs designed for PCR amplification of the region of the *KRAS* gene harboring codons 12 and 13 and the region of exon 15 of the *BRAF* gene encompassing codon 600, respectively. The oligonucleotide primers for sequencing of *KRAS* and *BRAF* are shown in Table I. Each exon was amplified by PCR using 0.5 Ag of template DNA, sense and antisense primers, and an AmpliTaq Gold PCR kit (Applied Biosystems). A total of 50 μl of reaction mixture was prepared according to the manufacturer's instructions and PCR was commenced at 94˚C for 3 min; followed by 35 cycles of 94˚C for 30 sec, 64˚C or 60˚C for 30 sec, and 72˚C for 1 min; with a final extension step for 5 min. The PCR products were purified using an UltraClean PCR Clean-up kit (Mobio Laboratories, Solana Beach, CA) and subjected to direct sequencing using purified products and the same sets of primers in a capillary automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). Sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) software located at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov) (12).

Statistical analysis. Correlation of *KRAS* mutations with the grade of histological differentiation and the cancer stage at surgery were analyzed using the χ^2 test and Mann-Whitney test, respectively. Correlation of *KRAS* mutations with patient age was also examined, after establishing that age had a normal distribution in the groups of patients with and without *KRAS* mutations. Mann-Whitney test was used to examine whether the population medians of the two independent groups differed significantly. Correlation of aberrant DNA hypermethylation of *hMLH1* with MSI was analyzed by the χ^2 test.

Results

MSP analysis of samples of endometrial cancer showed aberrant *hMLH1* hypermethylation in 17 of the 44 cases (38.6%) (Fig. 1, Table II). In MSI analysis, 31.8% (14 samples), 6.8% (3 samples), and 61.4% (27 samples) of the cases were categorized as MSI-H, MSI-L and MSS (microsatellite

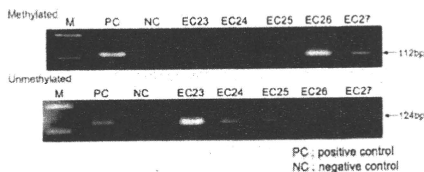

Figure 1. Detection of abnormal hypermethylation of the *hMLH1* gene in endometrial cancer using MSP analysis. The 112 bp band indicating abnormal hypermethylation was found in EC26 and EC27.

stability), respectively; that is, 38.6% were judged to be MSI-positive. Aberrant hypermethylation of the *hMLH1* gene was found in a higher number of MSI-positive cases, with a statistically significant positive correlation (p=0.02) between abnormal *hMLH1* methylation and MSI (Table III).

A point mutation at codon 12 of *KRAS* was found in 3 (HEC-1B, HHUA and SNG-M) of the 8 endometrial cancer-derived cell lines that were examined. These changes resulted in a G-D mutation in one cell line and G-V mutations in the other 2 cell lines. None of the cell lines had a point mutation at codon 13 of *KRAS* or at codon 600 of *BRAF* (Table IV). A point mutation at codon 12 of *KRAS* was observed in 6 of the 44 samples of endometrial cancer (13.6%) (Fig. 2, Table II), with a similar mutation to those in the cultured cell lines (G-D or V) in 5 of the 6 cases (83.3%). The point mutation at codon 12 of *KRAS* showed no correlation with clinicopathological characteristics of endometrial cancer or with age upon development of cancer, but tended to occur more frequently in well-differentiated adenocarcinoma (p=0.1, Table V). There were no correlations among aberrant *hMLH1* hypermethylation, MSI, and point mutation at codon 12 of *KRAS*. No point mutation at codon 13 of *KRAS* (Table II) or at codon 600 of *BRAF* (Table VI) was found in the 44 clinical samples of endometrial cancer.

Discussion

Carcinogenesis of colon cancer has been correlated with point mutation of the *RAS/RAF* family of genes in the MAP kinase pathway, suggesting the importance of mutation of these genes in an early stage of malignant change in colon cancer (1). Since mutations of *KRAS* and *BRAF* are observed in many MSI-positive cases of sporadic colon cancer with aberrant hypermethylation of the *hMLH1* gene, a correlation with MSI caused by decreased expression of hypermethylated *hMLH1* has been suggested (5). Similar decreased expression of *hMLH1* due to aberrant hypermethylation has been reported in endometrial cancer (14), but the correlation with point mutations of *KRAS* and *BRAF* remains unclear.

In the present study, aberrant hypermethylation of *hMLH1* was found in 38.7% of cases of sporadic endometrial cancer. Expression of *hMLH1* is significantly reduced by aberrant hypermethylation (14) and this may induce gene instability that can be detected as microsatellite instability (MSI). Previous studies have shown that about 13% of cases of sporadic colon cancer are MSI-positive (16) and that 84% of cases of MSI-

Table II. Results of MSI analysis, MSP analysis, and analysis of *BRAF* and *KRAS* gene mutations in cases of endometrial cancer.

No.	Age	Type	Stage	Grade	MSI	*hMLH1*	*BRAF* mutation Codon 600 GTG(V)	*KRAS* mutation Codon 12 GGT(G)	*KRAS* mutation Codon 13 GGC(G)
EC1	52	EM	Ib	G3	MSI-H	M	GTG	GGT	GGC
EC2	51	EM	IIIc	G1	MSI-H	U	GTG	GGT	GGC
EC3	54	AS	IIIc	G3	MSI-H	M	GTG	GGT	GGC
EC4	53	EM	Ib	G3	MSI-H	U	GTG	GGT	GGC
EC5	69	EM	IIIc	G2	MSI-H	M	GTG	GGT	GGC
EC6	55	EM	IIIc	G2	MSI-H	M	GTG	GGT	GGC
EC7	54	EM	Ia	G1	MSI-H	U	GTG	GGT	GGC
EC8	63	EM	Ia	G1	MSI-H	M	GTG	GGT	GGC
EC9	58	EM	Ib	G2	MSI-H	M	GTG	GGT	GGC
EC10	50	EM	IIIa	G3	MSI-H	U	GTG	GGT	GGC
EC11	61	EM	Ib	G1	MSI-H	M	GTG	GGT	GGC
EC12	55	AS	IVb	G2	MSI-H	U	GTG	GGT	GGC
EC13	78	EM	Ib	G3	MSI-H	U	GTG	GGT	GGC
EC14	65	EM	Ib	G2	MSI-H	M	GTG	GGT	GGC
EC15	61	EM	IIb	G1	MSI-L	U	GTG	GGT	GGC
EC16	57	EM	Ib	G3	MSI-L	U	GTG	GGT	GGC
EC17	41	EM	Ib	G1	MSI-L	M	GTG	GGT	GGC
EC18	50	EM	Ia	G1	MSS	U	GTG	GGT	GGC
EC19	61	EM	Ib	G1	MSS	M	GTG	GAT(D)	GGC
EC20	70	EM	IIIc	G2	MSS	U	GTG	GGT	GGC
EC21	62	AS	IIIa	G2	MSS	U	GTG	GCT(A)	GGC
EC22	40	EM	IIa	G1	MSS	U	GTG	GGT	GGC
EC23	59	EM	IIa	G3	MSS	U	GTG	GGT	GGC
EC24	80	EM	IIIc	G3	MSS	U	GTG	GGT	GGC
EC25	54	AS	Ib	G1	MSS	U	GTG	GGT	GGC
EC26	42	EM	IIb	G1	MSS	M	GTG	GGT	GGC
EC27	71	EM	IIIc	G3	MSS	M	GTG	GGT	GGC
EC28	60	EM	Ib	G1	MSS	U	GTG	GGT	GGC
EC29	57	EM	IIIa	G2	MSS	U	GTG	GGT	GGC
EC30	71	EM	IIa	G1	MSS	U	GTG	GTT(V)	GGC
EC31	37	EM	IIa	G2	MSS	M	GTG	GGT	GGC
EC32	47	EM	IIIb	G1	MSS	M	GTG	GAT(D)	GGC
EC33	67	EM	Ic	G2	MSS	U	GTG	GGT	GGC
EC34	53	EM	Ia	G1	MSS	M	GTG	GGT	GGC
EC35	62	AS	Ib	G1	MSS	M	GTG	GGT	GGC
EC36	56	EM	IIIc	G3	MSS	U	GTG	GGT	GGC
EC37	71	EM	Ib	G2	MSS	U	GTG	GAT(D)	GGC
EC38	53	EM	Ib	G3	MSS	U	GTG	GGT	GGC
EC39	42	AS	IIIc	G3	MSS	U	GTG	GGT	GGC
EC40	55	EM	Ic	G3	MSS	U	GTG	GGT	GGC
EC41	34	AS	IIIc	G1	MSS	U	GTG	GTT(V)	GGC
EC42	61	EM	Ic	G1	MSS	U	GTG	GGT	GGC
EC43	61	EM	Ic	G1	MSS	U	GTG	GGT	GGC
EC44	59	EM	Ib	G1	MSS	U	GTG	GGT	GGC

Table III. Correlation between MSI and abnormal hypermethylation of the *hMLH1* gene in cases of endometrial cancer.

	hMLH1		
	M	U	
MSI	10	7	
MSS	7	20	p=0.02

MSI, microsatellite instability; MSS, microsatellite stability; M, methylated; U, unmethylated.

Table IV. *KRAS* and *BRAF* gene mutations in human endometrial cancer-derived cell lines.

	KRAS		*BRAF*
	Codon 12 GCT(G)	Codon 13 GGC(G)	Codon 600 GTG(V)
Cell lines			
Hec108	GGT	GGC	GTG
SNG-II	GGT	GGC	GTG
Ishikawa	GGT	GGC	GTG
Hec-1B	GAT(D)	GGC	GTG
HHUA	GTT(V)	GGC	GTG
SNG-M	GTT(V)	GGC	GTG
HOOUA	GGT	GGC	GTG
KLE	GGT	GGC	GTG

Table V. Correlation of *KRAS* gene mutations with clinicopathological factors in cases of endometrial cancer.

	Grade		Stage		Age
	G1, 2	G3	I, II	III, IV	(average)
KRAS codon 12					
Mut	6	0	3	3	57.7
Wt	25	13	26	12	57
% Mut	19.4	0	10.3	20	
	p=0.1		p=0.32		p=0.88

Statistical analysis was performed with the χ^2 test and Mann-Whitney test. Mut, mutation; Wt, wild-type.

Table VI. Correlation of abnormal *BRAF* V600E genes with abnormal MMR and mutated *KRAS* genes.

	MSI		*hMLH1*		*KRAS* codon 12	
	Positive	Negative	M	U	Mut	Wt
BRAF						
Mut	0	0	0	0	0	0
Wt	17	27	17	27	6	38

MSI, microsatellite instability; M, methylated; U, unmethylated; Mut, mutation; Wt, wild-type.

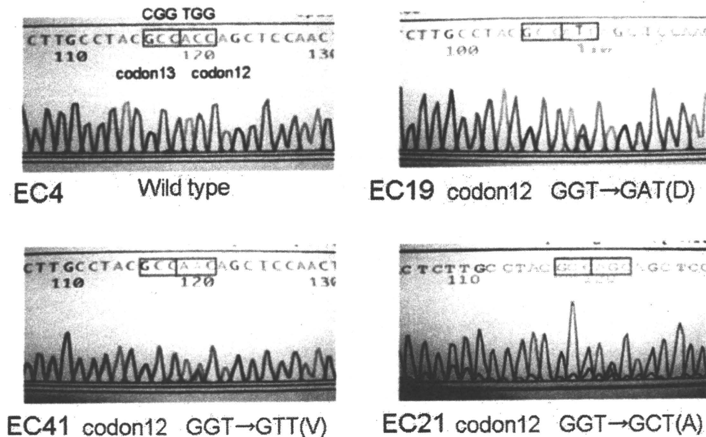

Figure 2. Analysis of point mutations of the *KRAS* gene in clinical samples of endometrial cancer. Three types of *KRAS* point mutation were detected at codon 12. No point mutation was observed at codon 13.

positive colon cancer have aberrant *hMLH1* hypermethylation (17). In our analysis, MSI-positive cases accounted for 38.6% of all cases of sporadic endometrial cancer. Mutch *et al* reported an incidence of MSI-positive cancer of 29% (18), with MSI occurring at higher rates in endometrial cancer than in colon cancer, suggesting that gene instability caused by an abnormal MMR gene is important in carcinogenesis of endometrial cancer. Our analysis showed aberrant *hMLH1* hypermethylation in 58.8% (10/17) of MSI-positive cases, with a significant positive correlation between aberrant *hMLH1* hypermethylation and MSI-positive cases of sporadic endometrial cancer (p=0.02). Based on this, we suggest that aberrant *hMLH1* hypermethylation causes MSI in endometrial cancer, as also seen in colon cancer.

Point mutations of the *KRAS* gene at codons 12 have been reported to occur in 0-46% of endometrial cancers and the most frequent codon 12 *KRAS* mutations are transitions from G to D, to V (19). Point mutations of the *KRAS* gene at codons 12 and 13 have been reported in 5.9% and 2.9% of patients with endometrial cancer, respectively, and the mutation showed a positive correlation with age upon development (20). Mutch *et al* found point mutations at codons 12, 13, and 61 of *KRAS* in 19.9%, 3.4% and 0.7% of cases of endometrial cancer, respectively, with a correlation with age upon development and a high rate of mutation in MSI-positive cases (18). In our analysis, point mutation at codon 12 was confirmed in 14% of cases, but none were observed at codon 13 and *KRAS* mutation showed no correlation with age. The incidence of well-differentiated adenocarcinoma tended to be high among cases with a mutation of *KRAS*, but the relationship was not significant, and there was no tendency for a higher rate of mutation of *KRAS* in MSI-positive cases. Point mutation of *KRAS* has been found in 51% of cases with colon cancer, and the rate in endometrial cancer is much lower (1). Mutation of *KRAS* may have some correlation with carcinogenesis in patients who develop sporadic endometrial cancer at an old age, but the current and previous results suggest that this mutation is not important for carcinogenesis in other cases of sporadic endometrial cancer.

Feng *et al* found mutation of the *BRAF* gene in 21% of cases of endometrial cancer, and proposed a correlation with decreased expression of the MMR gene (12). In contrast, Salvesen *et al* found the activating *BRAF* V600E mutation in only 2% of cases of endometrial cancer, and a consensus has not been obtained regarding the correlation between carcinogenesis of endometrial cancer and *BRAF* mutation (13). In our analysis, no *BRAF* V600E mutation was observed in cases of sporadic endometrial cancer. Collectively, these data suggested that the *BRAF* V600E mutation occurs at an extremely low rate in endometrial cancer, and thus may not be important for carcinogenesis of sporadic endometrial cancer. In contrast, the *BRAF* V600E mutation occurs at a high rate in sporadic colon cancer, and may be useful diagnostically to rule out the possibility of a hereditary tumor. However, this mutation is not useful in diagnosis of sporadic endometrial cancer.

Since we did not find a *BRAF* V600E mutation in our analysis, there was clearly no correlation between the *BRAF* V600E mutation and aberrant hypermethylation of *hMLH1* or MSI. Decreased expression of *hMLH1* due to aberrant hypermethylation could cause gene instability, with a high rate of mutation of a target gene such as *BRAF*. However, our results suggest that *BRAF* is not the target of abnormal MMR in sporadic endometrial cancer. On the other hand, since aberrant hypermethylation of *hMLH1* and MSI were detected at high rates in sporadic endometrial cancer patients, an abnormal MMR system is clearly associated with the mechanism of carcinogenesis in endometrial cancer. Identification of the new target gene for abnormal MMR will be extremely important for clarification of this mechanism.

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Analysis of candidate target genes for mononucleotide repeat mutation in microsatellite instability-high (MSI-H) endometrial cancer

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Abstract. Microsatellite instability (MSI) is an indicator of DNA instability and is caused by abnormalities in DNA mismatch repair (MMR) genes such as *hMLH1*, *hMSH2* and *hMSH6*. MSI occurs frequently in endometrial cancer (in approximately 30% of cases), and accumulation of gene mutations due to MSI may therefore have a major role in the mechanism of malignant transformation. However, a responsible target gene has not been identified in endometrial cancer. In this study, we analyzed mutations in 11 cancer-related genes with mononucleotide repeats susceptible to MSI in a coding region [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6 in exon 7), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] in 22 patients with MSI-H sporadic endometrial cancer. Mutations in *hMSH6* (C8) and *TGF-βRII* (A10) were found most frequently, at rates of 36.3% (8/22) each. Mutations of *BAX* (G8) and *TCF-4* (A9), which are common in MSI-positive colorectal cancer, occurred at rates of 22.7 and 0%, respectively, which suggests that the MSI target gene may differ between endometrial and colorectal cancers. Mutations in *hMSH6* (C8) were correlated with reduced protein expression (p=0.042) and patients with these mutations had significantly more mutations in mononucleotide repeats in other cancer-related genes compared to patients without *hMSH6* (C8) mutations (p=0.042). This suggests the possibility of a novel cascade in carcinogenesis of endometrial cancer in which MSI mutates *hMSH6* (C8), increases gene instability, and leads to accumulation of mutations in other cancer-related genes. To our knowledge, this is the first report to show that *hMSH6* (C8) has an important role as an MSI target gene in sporadic endometrial cancer.

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Introduction

Microsatellite instability (MSI) is an indicator of genetic instability at the DNA level (1,2). MSI can be evaluated by PCR-based detection of errors in replication of DNA sequences called microsatellites, which consist of repeating units of 1-2 base pairs. MSI has been found in many carcinomas and is particularly common in patients with hereditary non-polyposis colorectal cancer (HNPCC), a familial colon and endometrial cancer that is frequently MSI-positive (3). The mutated genes associated with HNPCC, *hMLH1* (4,5), *hMSH2* (6,7), *hMSH3* (8), *hMSH6* (9,10), *hPMS1* and *hPMS2* (11), are mismatch repair (MMR) genes that repair errors during DNA replication. In HNPCC patients, germline mutations in these genes cause abnormalities in the MMR system, which results in frequent errors in target genes. In addition, approximately 15% of patients with non-hereditary sporadic colon cancer are MSI-positive (3). This may be due to inactivation of the *hMLH1* gene promoter by aberrant hypermethylation, which causes abnormalities in the MMR system similar to that in HNPCC and results in unstable MSI-positive genes (12,13). About 30% of patients with sporadic endometrial cancer are also MSI-positive (14,15) and this may also be due to inactivation of hypermethylated *hMLH1* (16).

In somatic cells, replication errors are likely to occur in DNA regions including repeat sequences. MSI-based mutations accumulate in target genes with repeat sequences, resulting in malignant transformation of cells. In particular, mutation of tumor suppressor genes with a mononucleotide or dinucleotide repeats (repeating unit of one or two base pairs, respectively) may be strongly associated with malignant transformation of cells. Cancer-related genes including mononucleotide repeats (i.e., candidate MSI-target genes) include *TGF-βR II* (17) and *PTEN* (18), which are related to cell growth inhibition; apoptosis-related *BAX* (19) and *Caspase-5* (20); *TCF-4* (21), *EPHB2* (22) and *AXIN2* (23), which are components of the Wnt-signaling pathway; and *HDAC2* (24), which codes for a histone deacetylase. *hMSH3* (25) and *hMSH6* (26), which are MMR genes, and *MBD4* (27), which codes for the methyl-CpG binding protein, also have a mononucleotide repeat sequence and are also candidate MSI-target genes. In MSI-positive sporadic colorectal cancer, mutations of *TGF-βRII* (A10) and *BAX* (G8) have been found in 90% (28) and 45%

Table I. Primer sequences used in gene mutation analysis.

Gene	Repeat	Sense	Antisense
hMSH3	A8	AGATGTGAATCCCCTAATCAAGC	ACTCCCACAATGCCAATAAAAAT
hMSH6	C8	GGGTGATGGTCCTATGTGTC	CGTAATGCAAGGATGGCGT
TGF-βRII	A10	CTTTATTCTGGAAGATGCTGC	GAAGAAAGTCTCACCAGG
MBD4	A10	TGACCAGTGAAGAAAACAGCC	GTTTATGATGCCAGAAGTTTTTTG
BAX	G8	ATCCAGGATCGAGCAGGGCG	ACTCGCTCAGCTTCTTGGTG
PTEN	A6	CCTGTGAAATAATACTGGTATG	CTCCCAATGAAAGTAAAGTACA
HDAC2	A9	ACCTCCGATTCCGAGCTTT	CCGCTCACCGTCGTAGTAGT
EPHB2	A9	CACGAGACGTCACCAAGAAA	CGCAAGAACAGTCATTGCTTT
Caspase-5	A10	CAGAGTTATGTCTTAGGTGAAGG	ACCATGAAGAACATCTTTGCCCAG
TCF-4	A9	GCCTCTATTCACAGATAACTC	GTTCACCTTGTATGTAGCGAA
Axin2	G7	CCTACCCCTTGGAGTCTGC	CAGGGTCCTGGGTGAACA

(29) of cases, respectively, which suggests that MSI plays an important role in malignant transformation in this cancer. However, the mutation frequency of target genes varies between carcinoma types and a responsible MSI-target gene has not been identified in endometrial cancer. Mutation of the tumor suppressor gene *PTEN* has been found in MSI-positive endometrial cancer (18). However, genes including mononucleotide repeats have not been investigated in endometrial cancer.

In this study, we analyzed mutations of 11 cancer-related genes with mononucleotide repeat sequences [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6 in exon 7), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] in MSI-positive sporadic endometrial cancer, in order to identify MSI-target genes that contribute to the pathogenic mechanism of endometrial cancer.

Materials and methods

Clinical specimens. The subjects were 69 patients with endometrial cancer (G1, 32; G2, 17 and G3, 20) who gave informed consent to collection of tissue specimens. Of these patients, 59 had endometrioid adenocarcinoma and 10 had adenosquamous carcinoma. The grade of histological differentiation (G1-G3) and the cancer stage at surgery were determined based on the Guidelines for Endometrial Cancer published by the Japan Society of Obstetrics and Gynecology.

Microsatellite instability (MSI) analysis. Genomic DNA was extracted from normal and tumor tissue samples collected from the 69 patients with endometrial cancer using a Get Pure DNA kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The genomic DNA was PCR amplified at the microsatellite repeat loci D2S123, D5S346, D17S250, BAT26 and BAT25. PCR reactions were performed in a total volume of 25 μl containing 10X buffer, 0.125 mM deoxynucleoside triphosphate, 0.2 μM of each primer and 0.25 Units of TaqDNA polymerase. The PCR conditions were as follows: 94˚C for 10 min; 30 cycles at 94˚C for 45 sec, 58˚C for 45 sec, and 72˚C for 40 sec; followed by a final extension step at 72˚C for 10 min. After PCR, 1 μl of the product was mixed with 12 μl of loading buffer containing formamide and Rox size standards. This mixture was denatured at 95˚C for 2 min and cooled on ice before loading onto an ABI 310 Prism sequencer (Applied Biosystems, Foster City, CA). The results were analyzed using Genescan software (Applied Biosystems). Tumors were classified as MSI-H when ≥30% of the markers showed MSI in accordance with the recent recommendation of the National Cancer Institute Workshop. Tumors in which <30% of the markers showed MSI were included in the MSI-L category. Alteration of even one microsatellite region led to definition of the patient as MSI-positive.

Determination of frameshift mutations of mononucleotide repeats in 11 cancer-related genes. DNA was extracted from tumor tissue from patients with MSI-H endometrial cancer using a Get Pure DNA kit (Dojindo Molecular Technologies). Somatic frameshift mutations in 11 cancer-related genes [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] were determined using two gene-specific oligonucleotide primer pairs designed for PCR amplification of mononucleotide repeat regions. The oligonucleotide primers for sequencing of the 11 genes are shown in Table I. Each mononucleotide region was amplified by PCR using 0.5 μg of template DNA, sense and antisense primers, and an AmpliTaq Gold PCR kit (Applied Biosystems). A 50-μl reaction mixture was prepared according to the manufacturer's instructions and PCR was started at 94˚C for 3 min; followed by 35 cycles of 94˚C for 30 sec, 64˚C or 60˚C for 30 sec, and 72˚C for 1 min; with a final extension step for 5 min. The PCR products were purified using an UltraClean PCR Clean-up kit (Mobio Laboratories, Solana Beach, CA) and subjected to direct sequencing using purified products and the same sets of primers in a capillary automatic sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). Sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) software located at the National Center for Biotechnology Information web site (http:/ www.ncbi.nlm.nih.gov).

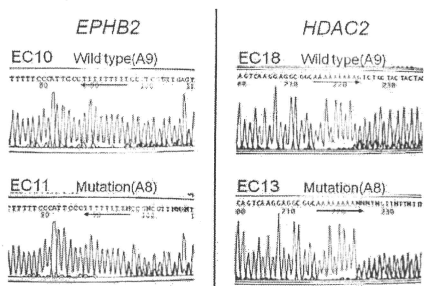

Figure 1. Analysis of mutations in MSI-H endometrial cancer. Frameshift mutations were observed in *EPHB2* (A9) in case EC11 and *HDAC2* (A9) in EC13.

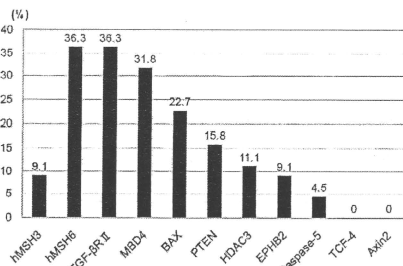

Figure 2. Frequency of mutations in mononucleotide repeats in cancer-related genes in tissue samples from patients with MSI-H endometrial cancer. Mutations in *hMSH6* and *TGF-βRII* were found most frequently (36.3%).

Immunohistochemistry. Immunohistochemical staining was performed on 2-μm sections of formalin-fixed, paraffin-embedded tissues using standard procedures. Slides were cleaned in xylene and dehydrated in graded alcohols. Antigen retrieval was performed with 10-min microwave treatment in 10 mM citrate buffer (pH 7.0). Endogenous peroxidase was blocked by dipping sections in 0.3% H_2O_2 in methanol for 10 min. Slides were incubated with mouse monoclonal antibody to hMSH6 (clone44; BD Transduction Laboratories, San Jose, CA) (1:500) for 90 min at room temperature. Immunostaining was performed by the avidin-biotin-peroxidase complex technique with an Elite ABC kit (Vector Laboratories, Burlingame, CA), using 3,3-diaminobenzidine as a chromogen and H_2O_2. Slides were counterstained with hematoxylin, dehydrated in graded alcohol, dried and cover-slipped. The normal staining pattern for hMSH6 is nuclear, and nuclei in stromal cells were used as internal positive controls. For the purpose of the study, staining of tumor nuclei for hMSH6 was evaluated as positive (+) or negative (-).

Statistical analysis. The association of frameshift mutations in the mononucleotide repeat region of *hMSH6* (C8) in MSI-H endometrial cancer specimens with mutations in the other 10 genes was analyzed using a Mann-Whitney test. The statistical association between mutations in *hMSH6* (C8) and hMSH6 protein expression was analyzed using a Fisher's exact test.

Results

MSI was determined by PCR in 69 patients with endometrial cancer and 22 cases (31.8%) were diagnosed as MSI-H. Mutations in mononucleotide repeats in 11 cancer-related genes [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] were examined in the 22 cases of MSI-H endometrial cancer. Mutations in *hMSH6* (C8) and *TGF-βRII* (A10) were found most frequently, each in 36.3% (8/22) of the cases. For the other genes, the percentages of cases with mutations were 9.1% (2/22) for *hMSH3* (A8), 31.8% (6/18) for *MBD4* (A10), 22.7% (5/22) for *BAX* (G8), 15.8% (3/19) for *PTEN* (A6), 11.1% (2/18) for *HDAC2* (A9), 9.1% (2/22) for *EPHB2* (A9), and 4.5% (1/22) for *Caspase-5* (A10). No mutation was found in the mononucleotide repeat regions of *TCF-4* (A9) or *Axin2* (G7) (Figs. 1 and 2, Table II).

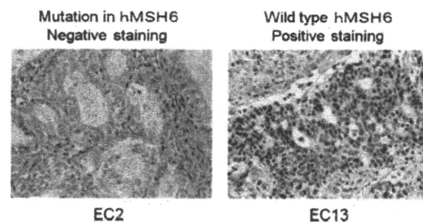

Figure 3. Immunohistochemical analysis of hMSH6 protein in endometrial cancer. Reduced expression of hMSH6 in tumor regions was found in case EC2, which had mutations in *hMSH6*. In contrast, hMSH6 showed clear staining in tumor cell nuclei in EC13, in which there were no mutations in *hMSH6*. In both specimens, normal nuclei surrounding the tumor are normally stained.

Mutations were found most frequently in mononucleotide repeats in *hMSH6* (C8) among the 11 genes that were analyzed. Further analysis in patients with mutations in *hMSH6* (C8) showed a statistically significant tendency for accumulation of mutations in mononucleotide repeats in one or more genes other than *hMSH6* (p=0.012, Tables II and III). Furthermore, tumors with mutations in *hMSH6* showed significant negative immunostaining for hMSH6 protein (p=0.042, Fig. 3, Table IV), indicating that mutations in hMSH6 correlated with reduced *hMSH6* protein expression.

Discussion

Microsatellite instability (MSI) is an indicator of genetic instability at the DNA level. MSI can be evaluated by detecting errors in replication of DNA regions referred to as

Table II. Analysis of mutations in mononucleotide repeats in 11 cancer-related genes in tissue samples from patients with MSI-H endometrial cancer.

Case	hMSH3 A8	hMSH6 C8	TGF-ßRII A10	MBD4 A10	BAX G8	PTEN A6	HDAC2 A9	EPHB2 A9	Caspase-5 A10	TCF-4 A9	Axin2 G7
EC1	-	+	-	+	-	ND	ND	-	-	-	-
EC2	-	+	+	+	-	ND	-	-	-	-	-
EC3	-	+	-	-	-	+	-	-	-	-	-
EC4	-	+	+	ND	-	-	-	+	-	-	-
EC5	-	+	+	ND	+	-	-	-	-	-	-
EC6	-	+	+	+	-	+	-	-	-	-	-
EC7	+	+	+	-	-	+	-	+	-	-	-
EC8	-	+	+	-	+	-	-	-	-	-	-
EC9	-	-	-	-	+	ND	+	-	-	-	-
EC10	-	-	+	-	-	-	-	-	-	-	-
EC11	-	-	+	-	-	-	ND	-	-	-	-
EC12	-	-	-	+		-	ND	-	-	-	-
EC13	-	-	-	-	-	-	+	-	-	-	-
EC14	-	-	-	-	-	-	-	-	-	-	-
EC15	-	-	-	-	-	-	-	-	+	-	-
EC16	-	-	-	-	-	-	-	-	-	-	-
EC17	+	-	-	-	-	-	-	-	-	-	-
EC18	-	-	-	ND	-	-	-	-	-	-	-
EC19	-	-	-	-	-	-	-	-	-	-	-
EC20	-	-	-	ND		-	ND	-	-	-	-
EC21	-	-	-	+	+	-	-	-	-	-	-
EC22	-	-	-	+	+	-	-	-	-	-	-

+, mutated; -, wild-type and ND, not done.

Table III. Association of mutations in *hMSH6* in MSI-H endometrial cancer with mutations in 10 other cancer-related genes (p=0.012, Mann-Whitney test).

	No. of mutations in 10 genes (other than hMSH6)				
	0	1	2	3	4
Mutation in hMSH6	0	2	4	0	2
No mutation in hMSH6	4	7	2	1	0

Statistical analysis was performed by Mann-Whitney test (p=0.012).

microsatellites, which consist of a sequence of repeating units of 1 or 2 base pairs. HNPCC is a familial tumor that is very frequently MSI positive and is probably caused by germline mutations in DNA mismatch repair (MMR) genes that cause abnormalities in the MMR system. This results in frequent replication errors of various target genes followed by malignant transformation. In MSI-positive colorectal cancer, mutations of *TGF-ßRII* and *BAX* tumor suppressor genes are frequently found and these genes are considered to be MSI target genes. *TGF-ßRII* and *BAX* include mononucleotide repeats susceptible to MSI and are likely to be mutated in MSI-positive tumors; therefore, these mutations are suspected to be involved in malignant transformation of cells.

Approximately 30% of MSI-positive endometrial cancer is defined as MSI-H, but a responsible MSI-target gene has not been identified in endometrial cancer. In this study, we analyzed MSI in 69 patients with endometrial cancer and 22 (31.8%) were diagnosed as MSI-H. This result is similar to those in previous studies. Mutations in mononucleotide repeats in 11 cancer-related genes were analyzed in the 22 cases of MSI-H endometrial cancer. Mutations in *hMSH6* (C8) and *TGF-ßRII* (A10) were found most frequently (36.3%), whereas no mutation was found in *TCF-4* (A9) or *Axin2* (G7), which are components of the Wnt-signaling pathway. Mutations in *PTEN* (A6), which has a high frequency of mutations in MSI-positive endometrial cancer, were found in 15.8% of the 22 cases.

TGF-ß inhibits growth of epithelial cells and TGF-ßRII transmits growth inhibitory signals; therefore, a loss of the function of these proteins may lead to malignant transformation of cells. In a previous study, mutations of *TGF-ßRII* (A10) were found in 90% of MSI-positive colorectal cancer, whereas no mutation was found in MSI-negative colorectal cancer, which suggests that *TGF-ßRII* plays an important role in malignant transformation as an MSI-target gene (28). Similarly, mutations of *BAX* (G8), which is involved in apoptosis, have been found in 45% of cases of MSI-positive colorectal cancer and *BAX* is thought to be related to malignant

Table IV. Association of mutations in *hMSH6* in MSI-H endometrial cancer with reduced hMSH6 protein expression.

	Positive	Negative	Total
Mutation in hMSH6	2	6	8
Wild-type in hMSH6	11	1	12
Total	13	7	20

Statistical analysis was performed by Fisher's exact test (p=0.042).

transformation in this cancer as an MSI target gene (29). The mutation rates of *TGF-βRII* (A10) and *BAX* (G8) in MSI-H endometrial cancer have been shown to be 12 and 33%, respectively (29), whereas we found rates of 36.3 and 22.7%, respectively, with these rates being the highest and third highest among the 11 genes analyzed. However, both rates are much lower than those found in MSI-positive colorectal cancer. Mutations in *TCF-4* (A9), a component of the Wnt-signaling pathway, were not found in our specimens, but occur at a frequency of 39% in MSI-positive colorectal cancer (29). This suggests that the frequency of mutations in mononucleotide repeats differs substantially between colorectal and endometrial cancers, and that MSI target genes and the mechanism of malignant transformation may also differ between these cancers.

Mutations in *PTEN* are found in about 60% of cases of MSI-positive endometrial cancer and about 30% of cases of MSI-negative endometrial cancer (30,31). The significantly higher rate in MSI-positive endometrial cancer suggests an association with MSI. Mutations in mononucleotide A repeats in exons 7 and 8 of *PTEN* are found in 27% of cases of MSI-positive endometrial cancer, which suggests that *PTEN* is an MSI-target gene (31), but PTEN mutation patterns vary and another study found mutations in the mononucleotide A repeats in only 3% of cases of endometrial cancers with microsatellite instability (32). The results of our study showed a 15.8% mutation rate for *PTEN* (A6), which was lower than those for *hMSH6* and *TGF-βRII*.

The function of hMSH6 is to detect deletion or insertion of a base pair in a mononucleotide repeat sequence and to initiate repair by forming a complex with hMSH2, hPMS2 and hMLH1. Reduced expression of hMSH6 due to mutation of *hMSH6* damages MMR function and induces MSI, which may result in malignant transformation of cells. Hendriks *et al* investigated families with germline mutations in *hMSH6* and showed that carriers of these mutations had a significantly higher risk of endometrial cancer than carriers of an *hMSH1* or *hMSH2* mutation (33). Furthermore, 69% of cases of endometrial cancer among *hMSH6* mutation carriers were MSI-H and immunohistochemistry showed that 97.5% were negative for hMSH6, indicating reduced expression of *hMSH6* (33). These results suggest that reduced expression of *hMSH6* caused by germline mutation induces MSI and is associated with development of hereditary endometrial cancer. Somatic mutations in *hMSH6* in MSI-positive sporadic endometrial cancer patients have been shown in several studies, but it is unclear if these mutations have an important role (34,35). Goodfellow *et al* found somatic mutations in *hMSH6* in 16 of 60 patients (26.6%) with MSI-H endometrial cancer and frameshift mutations in C8 in 12 of 16 patients with a somatic mutation, but no somatic mutation in *hMSH6* in MSI-negative patients. In the current study, frameshift mutations in *hMSH6* (C8) were found in 36.3% of patients, higher than the rate in Goodfellow *et al*, and immunohistochemical analysis showed that mutation of *hMSH6* correlated with reduced protein expression. These results suggest that mutation in *hMSH6* plays an important role in development of MSI-H sporadic endometrial cancer, similarly to hereditary endometrial cancer.

The current results also showed that patients with mutations in *hMSH6* (C8) had a tendency for accumulation of mutations in mononucleotide repeats in other genes. This tendency was found only in patients with mutations in *hMSH6* (C8). In MSI-positive colorectal cancer, Ikeda *et al* found that mutations in *E2F4* (CAG13), which codes for a transcriptional activator, were often associated with mutations in *hMSH3* (A8), an MMR gene that repairs dinucleotide and trinucleotide repeats, and proposed an interesting hypothesis in which mutations in trinucleotide repeats in *E2F4* are induced by mutations in *hMSH3*, with a subsequent reduction in expression (36). A cascade of malignant transformation with a similar mechanism to this hypothesis may also occur in MSI-H endometrial cancer, with mutations in *hMSH6*, a repair gene for mononucleotide repeats, inducing mutations in tumor suppressor genes that include mononucleotide repeats, such as *TGF-βRII* (A10), *BAX* (G8) and *β* (A6).

Collectively, the results of this study suggest the possibility of a novel cascade in endometrial cancer, in which MSI caused by reduced expression of *hMLH1* due to aberrant hypermethylation (epigenetic change) leads to mutation of *hMSH6* (C8), an MSI target gene, and reduced expression of hMSH6 subsequently increases gene instability and leads to accumulation of mutations in other cancer-related genes (genetic change), resulting in malignant transformation. This is the first study to show that *hMSH6* (C8) has an important role in the mechanism of malignant transformation in MSI-H sporadic endometrial cancer as a target gene, and further studies on the proposed cascade may provide new drugs and preventive approaches for endometrial cancer.

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Cyr61, a member of ccn (connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed) family, predicts survival of patients with endometrial cancer of endometrioid subtype☆

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Abstract

Objectives. It has been reported that expression of Cyr61 decreased in endometrial cancers and cancer cell lines compared with normal endometrium, and forced expression of Cyr61 could suppress the growth of human endometrial cancer cells. However, in another report, Cyr61 was immunostained in most of endometrial cancer tissues analyzed. Thus, the aim of this study was to examine the expression of Cyr61 in endometrial cancer and to correlate Cyr61 expression with cinicopathologic factors in a larger cohort.

Methods. We used immunohistochemistry and RT-PCR to examine the expression of Cyr61 in 92 endometrial carcinomas of endometrioid subtype. We correlated the expression of Cyr61 with various clinicopathologic factors in patients with endometrioid adenocarcinoma. Survival analyses were performed by the Kaplan Meier curves and the log-rank test. Independent prognostic factors were determined by multivariate Cox regression analysis.

Results. Cyr61 expression was high in 21 of 92 cases of endometrioid adenocarcinoma (22.8%). High expression of Cyr61 was related to poor survival of patients with endometrioid adenocarcinoma. Multivariate analysis including Cyr61 expression revealed that Cyr61 expression and positive lymph node metastasis (LNM) were independent prognostic factors for survival. Survival of patients with endometrioid adenocarcinoma could be stratified into three groups by combination of Cyr61 expression and positive LNM with an estimated 5-year survival rate of 96.5% for no LNM irrespective of Cyr61 expression (group A), 85.7% for positive LNM with low/moderate expression of Cyr61 (group B), and 0% for positive LNM with high expression of Cyr61 (group C)($p=0.18$ for group A vs group B, $p=0.008$ for group B vs group C, and $p<0.0001$ for group A vs group C).

Conclusions. Cyr61 is highly expressed in some endometrial cancer of endometrioid subtype. Cyr61 expression and positive LNM were independent prognostic factors for patients with endometrioid adenocarcinoma. Cyr61 might be a new molecular marker to predict the prognosis of patients with endometrioid adenocarcinoma.
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Keywords: Cyr61; Endometrial carcinoma; Immunohistochemistry; Survival; Prognostic factor

Introduction

Cyr61 (cysteine-rich 61/ccn1) belongs to the ccn (connective tissue growth factor/cysteine-rich 61/neuroblastoma overexpressed) family, which includes six members. The other five known members are connective tissue growth factor (CTGF/ccn2), nephroblastoma overexpressed (NOV/ccn3), Wnt-induced secreted protein-1, 2 and 3 (WISP-1/ccn4, WISP-2/ccn5, WISP-3/ccn6). The term "ccn family" was introduced by Bork in 1993, as Cyr61, CTGF and NOV were the three prototype members of this family [1]. Encoded by a growth factor-inducible immediate early gene, Cyr61 is a 40 kDa protein which is extremely cysteine-rich. This heparin-binding protein shares a 40 to 50% amino-acid homology with the other ccn family members. An important structural feature of ccn proteins is that they contain four conserved modules which exhibit similarities to the insulin-like growth factor-binding

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proteins (IGFBPs) , the von Willebrand factor type C (VWC), the thrombospondin type 1 (TSP1) and the carboxyl termini of several extra-cellular mosaic proteins (CT). However ccn5 is an exception, as it lacks the CT module.

The ccn proteins are involved in a variety of biological processes such as cell adhesion, proliferation, differentiation and migration; angiogenesis, chondrogenesis, wound-healing and tumorigenesis [2–4]. With respect to tumorigenesis, Cyr61 overexpression is associated with progression and formation of larger tumors in breast cancer [5,6]. Cyr61 can also stimulate the growth of gastric adenocarcinoma [7]. In non-small-cell lung cancer, prostate and papillary thyroid carcinoma, Cyr61 is found to be down-regulated [8–11]. These findings indicate that ccn proteins have variable biological functions which are dependent on the cellular contexts.

Difference of Cyr61 expression between endometrial cancer tissues and normal endometrium remains to be determined. Chien and colleagues reported that expression of Cyr61 decreased in endometrial cancers and cancer cell lines compared with normal endometrium, and forced expression of Cyr61 could suppress the growth of human endometrial cancer cells [12]. On the contrary, MacLaughlan et al. recently reported that Cyr61 was detected by immunohistochemistry and Western blot analysis in most of endometrial cancer tissues examined [13]. Thus, we used immunohistochemistry to examine the expression of Cyr61 in a larger cohort of endometrial carcinomas and to correlate Cyr61 expression with clinicopathological factors to explore the clinical significance of Cyr61 expression in endometrioid adenocarcinoma in this study.

Table 1
Cyr61 expression and clinicopathological factors of 92 endometrioid adenocarcinoma

Factor	Cyr61 expression		p-value
	Low/moderate	High	
Age			0.87
−49	19	6	
50-	52	15	
FIGO stage (1988)			0.87
I/II	46	14	
III/IV	25	7	
Architectural grade			0.38
1/2	60	16	
3	11	5	
Nuclear grade			0.16
1	25	11	
2/ 3	46	10	
Myometrial invasion			0.87
<=1/2	42	12	
>1/2	29	9	
Cervical invasion			0.58
negative	58	16	
positive	13	5	
Lymph-vascular space invasion			0.67
−/+	54	15	
++/+++	17	6	
Ovarian metastasis			0.88
Negative	65	19	
Positive	6	2	
Lymphnode metastasis			0.48
Negative	62	17	
Positive	9	4	

Materials and methods

Patients

A total of 92 endometrioid adenocarcinomas were obtained from archives of paraffin embedded tissues between January, 1994 and May, 2004 at the Department of Gynecology of Hokkaido University, Sapporo, Japan. We focused on endometrioid adenocarcinomas alone in this study because serous adenocarcinomas have different genetic abnormalities and biological properties from endometrioid adenocarcinomas. All subjects underwent modified radical hysterectomy, bilateral salpingo-oophorectomy and systematic retroperitoneal lymphadenectomy which consisted of complete dissection of pelvic and para-aortic lymph nodes from the femoral ring to the level of the renal vein. All lymphatic tissues that surrounded the arteries and veins were completely removed. Hematoxylin-Eosin (HE) sections were reviewed to confirm the pathological diagnosis of endometrioid carcinoma. The age of subjects ranged from 23 to 74 years with an average of 54.6 years. The following histopathologic prognostic factors were correlated with staining intensity of Cyr61 expression by immunohistochemistry : FIGO (1988) stage, architectural grade (AG), nuclear grade (NG), lymphvascular space invasion (LVSI), depth of myometrial invasion, cervical invasion (CI), ovarian metastasis, lymph node metastasis (LNM). All risk factors were determined as previously described [14]. Clinicopathological characteristics of endometrial cancer patients were shown in Table 1.

Immunohistochemistry

A standard streptavidin-biotin-peroxidase method was used for the immunohistochemistry (IHC) study. Briefly, 4 μm thick sections from each sample were deparaffinized in xylene and rehydrated through a sequence of alcohol. For antigen retrieval, the sections were boiled in 10 mM sodium citrate buffer (pH 6) for 6 min using autoclave. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 10 min. After phosphate buffered saline (PBS) wash for 5 min, the sections were treated with 10% normal goat serum at room temperature for 30 min to block the nonspecific binding. The sections were then incubated overnight in moist chamber at 4°C with a Cyr61 primary antibody, which was kindly provided by Dr Brahim Chaqour (State University of New York, Down state medical center, Brooklyn, New York, USA), at 1:100 working dilution. After washing away excess antibody with PBS (3 × 10 mins), the sections were incubated with biotinylated goat against anti-rabbit immunoglobulin for 30 min at 37°C. Following PBS washes(3 × 10 mins), the sections were then incubated with streptavidin-peroxidase conjugate for 30 min at room temperature and washed again with PBS (3 × 10 mins). 3′-3′-diaminobenzidine was used as a chromogen substrate. All sections were then washed in running tap water and