Table 1
Clinicopathologic characteristics of 55 patients with lymph node metastasis

	No.	%
FIGO stage (1988)		
IIIc	44	80.0
IV	11	20.0
Histologic subtype		
Endometrioid	43	78.2
Serous/Clear	12	21.8
Architectural grade		
1	16	29.1
2	27	49.1
3	12	21.8
Nuclear grade		
1	14	25.4
2	25	45.5
3	16	29.1
Depth of myometrial invasion		
< = 1/2	15	27.3
> 1/2	40	72.7
Lymph-vascular space invasion		
Nil/minimal	20	36.4
Moderate/prominent	35	63.6
Cervical invasion		
Negative	28	50.9
Positive	27	49.1
Ovarian metastasis		
Negative	37	67.3
Positive	18	32.7
Para-aortic lymph node metastasis		
Negative	26	47.3
Positive	29	52.7

included in right para-aortic nodes. PAN groups, therefore, consists of four groups.

Statistics

Correlation between the variables was analyzed using chi^2 test. Patients survival was calculated using Kaplan–Meier method. The significance of the survival difference was examined by the log-rank test. Univariate and multivariate survival analyses were performed using the Cox regression model with disease-specific overall survival as the outcome measure. Forward stepwise procedure was used to select the independent variable in multivariate analysis. $P < 0.05$ was considered statistically significant. Statistical analyses were performed with the Statview software package (SAS Institute, Inc, Cary, NC).

Results

Univariate and multivariate survival analysis for all node-positive patients

Age of patients ranged from 40 to 75 (median 58) years. The univariate analysis revealed that the FIGO (1988) stage (IIIc vs. IV, $P < 0.0001$), the histologic subtype (endometrioid vs. serous/clear, $P = 0.0216$), architectural grade (G1/2 vs. G3, $P = 0.0024$), nuclear grade (G1 vs. G2/3, $P = 0.0201$), depth of myometrial invasion (absence or presence of serosal invasion, $P < 0.0001$), LVSI (none/minimal vs. moderate/prominent, $P = 0.0026$), cervical invasion ($P = 0.0159$), and PAN metastasis ($P = 0.0078$) were shown to be related to poor survival. Ovarian metastasis was not related to survival ($P = 0.3306$) (Table 2).

Multivariate analysis, which included the prognostic factors determined by univariate analysis to have statistical significance, was performed using a forward stepwise procedure (Table 3). It was shown that FIGO (1988) stage ($P < 0.0001$) and LVSI ($P = 0.0002$) were independent prognostic factors. We could stratify the patients into three prognostic risk-groups by integrating those two histopathologic risk factors, that is, low risk group (group A: stage IIIc with nil/minimal LVSI, $n = 19$), intermediate risk group (group B: stage IIIc with moderate/prominent LVSI, $n = 25$) and high risk group (group C: stage IV with any LVSI, $n = 11$) with an estimated 5-year survival rate of 93.3%, 50.9%, and 20.0%, respectively (Fig. 1). There was statistically significant difference of survival rate between each group (A vs. B: $P = 0.0024$, B vs. C: $P < 0.0001$, A vs. C: $P < 0.0001$). Because prognostic impact of FIGO (1988) stage was extremely strong for the survival of node-positive patients, we performed further analysis on stage IIIc patients alone ($n = 44$).

Lymph node metastasis in stage IIIc patients

Incidences of pelvic lymph node (PLN) metastasis alone, PAN metastasis alone, and both PLN and PAN metastasis were 52.3% (23/44), 4.5% (2/44), and 43.2% (19/44),

Table 2
Univariate and multivariate Cox regression analysis of prognostic factors of node positive endometrial carcinoma

Univariate		Multivariate		
Prognostic factor	P value	Risk ratio	95% CI	P value
FIGO (1988) stage	< 0.0001	11.2	4.0–31.3	< 0.0001
Histologic subtype	0.0216	–	–	NS
Architectural grade	0.0024	–	–	NS
Nuclear grade	0.0201	–	–	NS
Lymph-vascular space invasion	0.0026	9.3	2.1–41.7	0.0033
Myometrial invasion	< 0.0001	–	–	NS
Cervical invasion	0.0159	–	–	NS
Ovarian metastasis	0.3306	–	–	S
Para-aortic node metastasis	0.0078	–	–	NS

NS: not significant.
FIGO (1988) stage: stage IIIc vs stage IV determined by the presence of peritoneal metastasis.
Tumor cell type: endometrioid vs serous/clear cell.
Architectural grade: G1/2 vs G3.
Nuclear grade: G1 vs G2/3.
Lymph-vascular space invasion: (−)/(+) vs (++)/(+++).
Myometrial invasion: serosal invasion (−) vs (+).
Cervical in vs. ion: (−) vs (+).
Ovarian metastasis: (−) vs (+).
Para-aortic lymph node metastasis: (−) vs (+).

Table 3
Univariate and multivariate Cox regression analysis of prognostic factors of stage IIIc endometrial carcinoma

Univariate		Multivariate		
Prognostic factor	*P* value	Risk ratio	95% CI	*P* value
Histologic subtype	0.7226	–	–	NS
Architectural grade	0.3911	–	–	NS
Nuclear grade	0.0605	–	–	NS
Lymph-vascular space invasion	0.0173	8.8	1.1–71.4	0.0413
Myometrial invasion	0.6036	–	–	NS
Cervical invasion	0.4577	–	–	NS
Ovarian metastasis	0.1815	–	–	NS
Number of positive PAN	0.0016	3.9	1.2–13.0	0.0260

NS: not significant.
Tumor cell type: endometrioid vs serous/clear cell.
Architectural grade: G1/2 vs G3.
Nuclear grade: G1 vs G2/3.
Lymph-vascular space invasion: (−)/(+) vs (++)/(+++).
Myometrial invasion: serosal invasion (−) vs (+).
Cervical invasion: (−) vs (+).
Ovarian metastasis: (−) vs (+).
Number of positive PAN: 0, 1 vs ≥ 2.

respectively. The estimated 5-year survival rate of patients without or with PAN metastasis was 86.4%, 48.1%, respectively (Fig. 2). The difference was statistically significant ($P = 0.0108$).

Prognostic impact of the number of positive PLN groups in stage IIIc patients

The estimated 5-year survival rate for patients with one positive PLN group was 79.3% and that for patients with ≥ 2 positive PLN groups was 60.8%. The difference of survival was not statistically significant. However, 21 of 22 patients (95.5%) with no or one positive PLN group had no or one positive PAN group and only one of 22 patients (4.5%) had two positive PAN groups, while 15 of 22 patients (68.2%) with ≥ 2 positive PLN groups had no or one positive PAN group and 7 of 22 patients (31.8%) had ≥

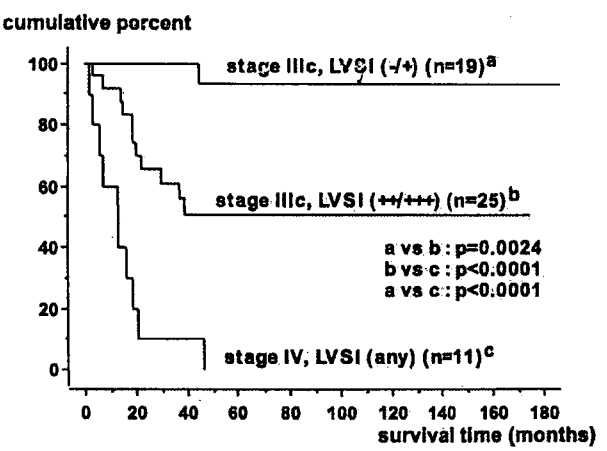

Fig. 1. Survival of node-positive patients with endometrial carcinoma by combination of FIGO (1988) stage and LVSI.

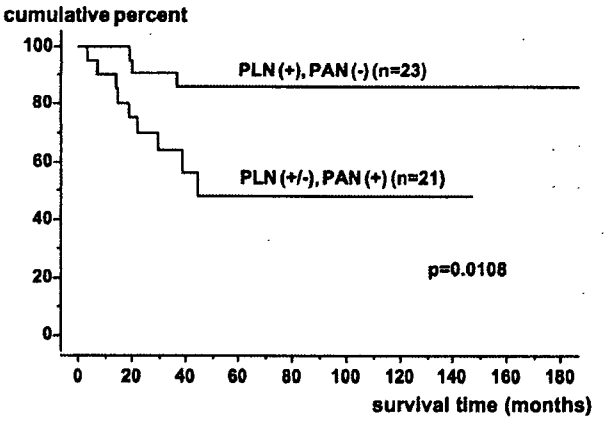

Fig. 2. Survival of patients with stage IIIc endometrial carcinoma by PAN metastasis.

2 positive PAN groups. There was a statistically significant difference in incidence of ≥ 2 positive PAN groups between patients who had no or one positive PLN group and those who had ≥ 2 positive PLN groups ($P = 0.023$). (Table 4).

Prognostic impact of the number of positive PAN groups in stage IIIc patients

Fig. 3 shows the survival of stage IIIc patients according to the number of positive PAN groups. The estimated 5-year survival rate was 86.4% for patients without positive PAN group ($n = 23$), 60.4% for those with one positive PAN group ($n = 13$), and 20.0% for those with ≥ 2 positive PAN groups ($n = 8$). There was statistically significant difference between no positive PAN group and ≥ 2 positive PAN groups ($P < 0.0007$), between one positive PAN group and ≥ 2 positive PAN groups ($P = 0.0319$). There was no statistically significant difference between no positive PAN group and one positive PAN group ($P = 0.1354$).

Univariate and multivariate survival analysis for stage IIIc patients

Since the number of positive PAN group was shown to have significant impact on the survival of stage IIIc patients,

Table 4
Incidence of para-aortic lymph node metastasis according to number of positive pelvic lymph node groups in stage IIIc endometrial cancer patients

	Positive PAN group		Total
	0, 1	≥ 2	
positive PLN group			
0, 1	21	1	22
> 2	15	7	22
Total	36	8	44
			$P = 0.0023$

PLN: pelvic lymph node, PAN: para-aortic lymph node.
There was a statistically significant difference in incidence of ≥ 2 positive PAN groups between patients who had no or one positive PLN group and those who had ≥ 2 positive PLN groups ($P = 0.023$).

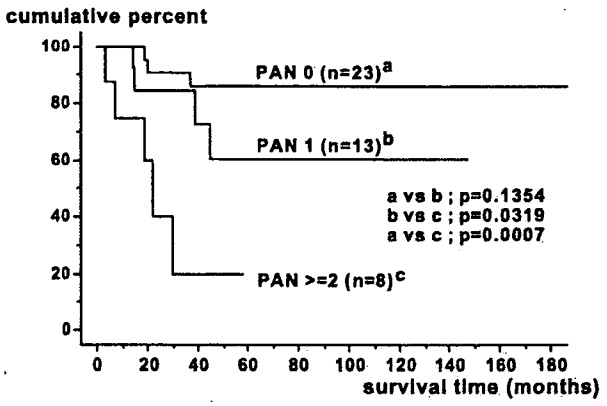

Fig. 3. Survival of patients with stage IIIc endometrial carcinoma by the number of positive PAN group.

we included number of positive PAN groups in the univariate analysis instead of presence or absence of PAN metastasis. The univariate analysis revealed that the LVSI ($P = 0.0173$), number of positive PAN groups ($P = 0.0016$) were shown to be related to poor survival. Histologic subtype ($P = 0.7226$), architectural grade ($P = 0.3911$), nuclear grade ($P = 0.0605$), depth of myometrial invasion ($P = 0.6036$), cervical invasion ($P = 0.4577$), ovarian metastasis ($P = 0.1815$) were not related to poor survival (Table 3, Fig. 4).

Multivariate analysis revealed that both LVSI ($P = 0.0413$) and number of positive PAN groups ($P = 0.026$) were independent prognostic factors. Survival of patients with stage IIIc disease could be stratified into three groups by combination of LVSI and number of positive PAN group with an estimated 5-year survival rate of 93.3% for no or one positive PAN group with nil or minimal LVSI (group D), 62.6% for no or one positive PAN group with intermediate or prominent LVSI (group E), and 20.0% for ≥ 2 positive PAN groups irrespective of LVSI (group F). The difference of survival rate between each group was statistically significant ($P = 0.0002$ for group D vs. group F, $P = 0.023$ for group D vs. group E, $P = 0.0388$ for group E vs. group F.

Discussion

Stages I and II endometrial carcinomas have shown a favorable prognosis by combination of surgery, radiotherapy, and/or chemotherapy. Some histopathologic factors have been found to be related to prognosis of endometrial carcinoma. Lymph node metastasis is one of the most important prognostic factors of endometrial carcinoma and advanced endometrial carcinoma with lymph node metastasis (IIIc/IV) has been shown to have poorer prognosis. In our series of patients treated in the same manner, the prognosis for patients with stage IIIa endometrial carcinoma was excellent with an estimated 5-year survival of over 90% (patients with stage IIIb disease were not found in our series). However, the prognosis for patients with stage IIIc endometrial carcinoma with an estimated 5-year survival rate of 79.6% was poorer than that of stage IIIa in spite of intensive treatment consisting of extended surgery including pelvic and para-aortic lymphadenectomy and systemic adjuvant chemotherapy. We, therefore, performed retrospective analysis on the prognostic factors for node-positive patients to determine appropriate therapeutic and follow-up modality to achieve their favorable prognosis.

Concerning the distribution of lymph node metastases, 95.5% (42/44) of patients with nodal disease had pelvic node metastases and 45.2% (19/42) of patients with PLN metastases had concomitant PAN metastases. McMeekin et al. [4] analyzed nodal distribution in 47 cases of stage IIIc endometrial cancer and found that an increasing number of positive PLN was associated with PAN metastasis. Our result on nodal distribution in stage IIIc patients is similar to McMeekin et al. [4], i.e., patients with single positive PLN group rarely have multiple positive PAN groups. We also found that positive aortic nodes were associated with poorer prognosis than were positive pelvic nodes alone (estimated 5-year survival 48.1% for positive PAN vs. 86.4% for negative PAN, $P = 0.0108$), suggesting that involvement of pelvic lymph nodes alone does not necessarily carry a poor prognosis as previously reported by Onda et al [12] who performed the same operative procedure as ours. This can be explained in part due to the therapeutic significance of our operative procedure including systematic pelvic and para-aortic lymphadenectomy. Para-aortic lymphadenectomy until just below the renal vein may contribute to the favorable survival of patients with multiple positive PLN groups.

In this study, we firstly reported that the survival of patients with stage IIIc endometrial carcinoma can be stratified by the number of positive PAN groups. The survival of patients without positive PAN (PLN metastasis

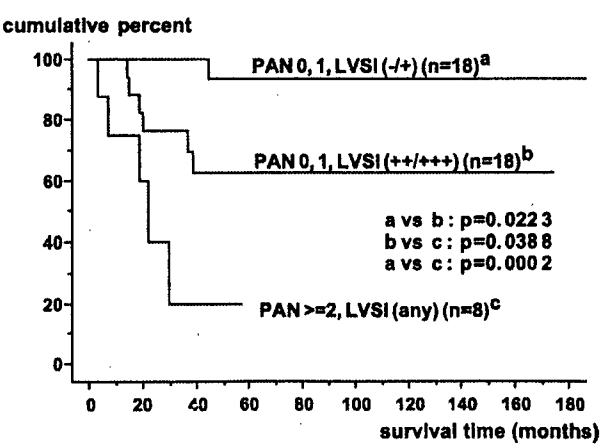

Fig. 4. Survival of patients with stage IIIc endometrial carcinoma by combination of LVSI and number of positive PAN group.

alone), with one positive PAN group, and with ≥ 2 positive PAN groups was 86.4%, 60.4%, 20.0%, respectively. The survival of patients with ≥ 2 positive PAN groups was much worse than others. Notably, there was no statistically significant difference between the patients without positive PAN group and those with one positive PAN group (P = 0.14), suggesting that single PAN metastasis is still a local disease that can be cured by complete lymphadenectomy and subsequent chemotherapy. When patients have multiple PAN metastasis, lymphadenectomy does not prolong survival.

Radiotherapy and chemotherapy have been employed as adjuvant therapies for endometrial cancer. Radiotherapy has been considered as a standard adjuvant therapy in Western countries. The result of GOG 122, however, clearly demonstrated that adjuvant chemotherapy (adriamycin and CDDP) significantly improved progression free survival and overall survival than adjuvant radiotherapy (whole abdominal radiotherapy) for stage III/IV patients [13], indicating that chemotherapy should be considered as a standard adjuvant therapy for endometrial cancer. Systemic chemotherapy has been widely accepted as a standard adjuvant therapy for endometrial cancer in Japan. Adriamycin has been used as a key drug for endometrial cancer. We have used CAP regimen for endometrial carcinoma with risk factors for recurrence. However, the poorer survival of stage IIIc patients with multiple positive PAN groups than single positive PAN group, who received adjuvant chemotherapy (CAP), clearly indicates that we should consider a new chemotherapeutic regimen to improve prognosis of node-positive patients. The most promising drug for endometrial cancer is taxane [14].

In this study, we found that LVSI and number of positive PAN group are independent prognostic factors by multivariate analysis, indicating that para-aortic lymphadenectomy should be routinely included in the surgical procedure for endometrial cancer to predict the survival of node-positive patients. We also conclude that we should investigate LVSI with more careful attention for node-positive patients. Careful investigation of LVSI, however, is time-consuming and it is impossible to evaluate LVSI preoperatively and during operation by frozen section. To individualize the therapeutic modality for each patient, we need to search for new molecular markers which can be easily assessed and reflect disease status in regard to LVSI preoperatively. There have been few reports on the useful molecular markers for survival of endometrial carcinoma. We reported that p53 overexpression by immunohistochemical staining was found to be an independent prognostic factor and the estimated 5-year survival rate of patients with stage III/IV disease without p53 overexpression was significantly better than that with p53 overexpression, indicating that p53 missense mutation, which is closely related to immunohistochemical p53 overexpression, have a significant prognostic impact on the survival of advanced endometrial carcinoma [15]. Kanamori et al. [16] reported that PTEN expression was found to be associated with prognosis for patients with advanced endometrial carcinoma undergoing postoperative chemotherapy. Yokoyama et al. [17] reported that high levels of immunoreactivity for vascular endothelial growth factor (VEGF)-D in stromal cells and its receptor, VEGF-R-3 in carcinoma cells were independent prognostic factors in endometrial carcinoma. We need to further investigate more useful prognostic factors for endometrial carcinoma using molecular biological techniques.

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Functional analysis of *p53* gene and the prognostic impact of dominant-negative *p53* mutation in endometrial cancer

Noriaki Sakuragi[1]*, Hidemichi Watari[1], Yasuhiko Ebina[1], Ritsu Yamamoto[1], Eric Steiner[2], Heinz Koelbl[2], Masahiro Yano[3], Mitsuhiro Tada[3] and Tetsuya Moriuchi[3]

[1]*Department of Gynecology, Hokkaido University Graduate School of Medicine and School of Medicine, Sapporo, Japan*
[2]*Department of Obstetrics and Gynecology, Mainz University, Mainz, Germany*
[3]*Division of Cancer-Related Genes, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan*

In addition to the loss of function, mutant p53 can possess a dominant-negative effect on wild-type p53 and may also exert gain-of-function activity. It is not clear whether the functional status of *p53* mutation contributes to differences in outcome in endometrial cancer. We collected a total of 92 RNA samples of high quality from endometrial cancer tissues, and the samples were subjected to yeast functional assay and sequencing for *p53* mutations. The detected mutant *p53* genes were further investigated for their dominant-negative activity using a yeast-based transdominance assay. *p53* mutation was found in 24 out of 92 (26.1%) tumors, of which 10 exhibited no dominant-negative activity (recessive mutation) and 14 showed dominant-negative activity. Dominant-negative *p53* mutation was related to advanced stages ($p = 0.01$), non-endometrioid type tumors ($p = 0.01$) and grade 3 tumors ($p = 0.04$). The patients with dominant-negative mutation had significantly shorter survival than patients with no mutation ($p < 0.0001$) and those with a recessive mutation ($p = 0.01$) in the *p53* gene. No difference in survival was found between the patients with tumors harboring a recessive *p53* mutation and those with tumors harboring a wild-type *p53*. Multivariate analysis revealed that dominant-negative *p53* mutation ($p = 0.019$), FIGO stage ($p = 0.0037$) and histologic subtype ($p = 0.014$) were independently related to patient survival. Dominant-negative *p53* mutation was the most important prognostic factor for stage III/IV endometrial cancer ($p = 0.0023$). In conclusion, dominant-negative *p53* mutation is often found in advanced stages and aggressive histologic subtypes of endometrial cancer and it is a strong predictor of survival of patients with advanced endometrial cancer. To elucidate further the role of *p53* mutation in endometrial cancer, it is necessary to investigate gain-of-function activity involving dominant-negative p53 mutant proteins.

Key words: endometrial cancer; *p53*; mutation; dominant negative; survival; serous adenocarcinoma

The *p53* tumor suppressor gene is mutated in about 50% of all tumors,[1–3] and more than 19,000 different somatic mutations have been identified.[4] Mutation of the *p53* gene plays a key role in the carcinogenesis and progression of many different malignancies, including endometrial cancer. p53 overexpression has been shown to predict patient survival in endometrial cancer.[5–7] p53 overexpression, as determined by immunohistochemistry (IHC), is a surrogate marker of missense mutation of p53 protein. Because MDM2 is a transcriptional target of p53, loss of p53 function reduces the production of MDM2. MDM2 degrades p53 protein through ubiquitination of the protein, and reduced MDM2 production will lead to an accumulation of p53 protein in the nucleus, which is detected as overexpression by IHC.[8] IHC is a convenient method for the investigation of p53 status. However, it can be affected by many factors, such as antibody used, antigen retrieval technique and subjectivity of criteria for p53 overexpression. p53 overexpression does not necessarily correspond to *p53 gene* mutation. One report showed that only 32% of tumors with exclusively nuclear staining were found to contain a *p53* gene mutation.[6] Dominant-negative *p53* mutation will lead to decreased MDM2 production, irrespective of the status of the second allele of *p53*. *p53* mutations abolishing the production of p53 protein will lead to loss of p53 protein when it is associated with loss of heterozygosity (LOH) of the second allele. Therefore, p53 overexpression can be related to both dominant-negative mutation and recessive missense mutation with LOH. It is therefore reasonable to expect that p53 overexpression does not necessarily correspond to a dominant-negative mutation of *p53*.

At least 2 distinct pathways have been proposed that contribute to cisplatin-induced apoptosis *in vitro*. One involves p53 tumor suppressor protein, and the other is mediated by the p53-related protein p73. Inhibition of p73 function by dominant-negative p73 proteins or by mutant p53 abrogates apoptosis and cytotoxicity induced by these agents.[9] Therefore, investigation into the status of p73 in endometrial cancer is expected to provide further information regarding the response to adjuvant chemotherapy in cases involving this type of cancer.

An understanding of the role played by *p53* mutation in endometrial cancer may lead to tailored treatment planning and more rational targeted approaches for treating this disease. *p53* gene mutation and LOH result in the loss of p53 function. In addition to the loss of function, mutant p53 can possess a dominant-negative effect that suppresses wild-type p53, and which may also exert gain-of-function activity.[10] There have been many studies of the prognostic significance of and/or therapeutic outcome related to the type of *p53* mutations. However, the results of such studies have been inconsistent,[11] and the significance of the dominant-negative function in terms of both prognosis and therapeutic success remains unclear at present.[12]

In order to elucidate the prognostic importance of dominant-negative activity in endometrial cancer, we surveyed the functional status of p53 protein in endometrial carcinoma using a yeast p53 functional assay and a transdominance assay. The yeast p53 functional assay tests the ability of p53 to activate transcription *in vivo* in yeast.[13,14] A modification of this method (transdominance assay) can identify the dominant-negative/recessive properties of the mutant p53.[15] This is the first report to compare directly the functional status of p53 (*i.e.*, the presence or absence of dominant-negative activity) to the survival of patients with endometrial cancer.

Material and methods

Tissue specimens

A total of 92 endometrial carcinoma tissue samples, which were obtained from the resected uterus of patients with endometrial carcinoma treated surgically at the Department of Obstetrics and Gynecology, Mainz University in Mainz, Germany, and at the

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***Correspondence to:** Department of Obstetrics and Gynecology, Hokkaido University School of Medicine, N-15, W-7, Kitaku, Sapporo 060-8638, Japan. Fax: +81-11-706-7711.
E-mail: sakuragi@med.hokudai.ac.jp
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TABLE I – CLINICOPATHOLOGIC CHARACTERISTICS OF PATIENTS WITH ENDOMETRIAL CARCINOMA TREATED IN MAINZ UNIVERSITY AND HOKKAIDO UNIVERSITY

	Mainz, Germany (n = 49)	Sapporo, Japan (n = 43)	p
Age			
< 60	13	26	
≥ 60	36	17	0.001
FIGO stage			
I/II	37	28	
III/IV	12	15	0.27
Histologic subtype			
Endometrioid	44	35	
Nonendometrioid	5	8	0.25
Grade			
1	17	14	
2	19	17	
3	13	12	0.98
Follow-up period (months)	1–130 (median, 58.5)	5–125 (median, 41.0)	0.11

Department of Gynecology, Hokkaido University Graduate School of Medicine and School of Medicine in Sapporo, Japan, were used for this study. Informed consent was obtained from all study participants. From among the 92 samples, 49 were obtained from German patients and 43 from Japanese patients. Surgical treatment was initiated during the period between April 1990 and January 2000 for the German cohort and between February 1990 and December 2002 for the Japanese cohort. The data from 23 patients of the 43 Japanese cohort participants were reported in a previous study.[16] The clinicopathologic variables, namely, FIGO stage, histologic subtype, grade of tumor and follow-up period, did not differ between the 2 cohorts, with the exception of patient age (Table I). The tissue samples included 79 cases of endometrioid-type adenocarcinoma and 13 cases of nonendometrioid adenocarcinoma. The group of nonendometrioid tumors included 11 serous adenocarcinomas, 1 clear cell adenocarcinoma and 1 squamous cell carcinoma. The treatment strategies employed in the 2 institutes were different. At Mainz University, the surgical procedure involved selective lymph node dissection rather than routine systematic lymphadenectomy. At Hokkaido University, routine systematic pelvic and paraaortic lymphadenectomy was carried out. The modality of adjuvant therapy employed at Mainz University was radiotherapy, whereas that used at Hokkaido University was chemotherapy.

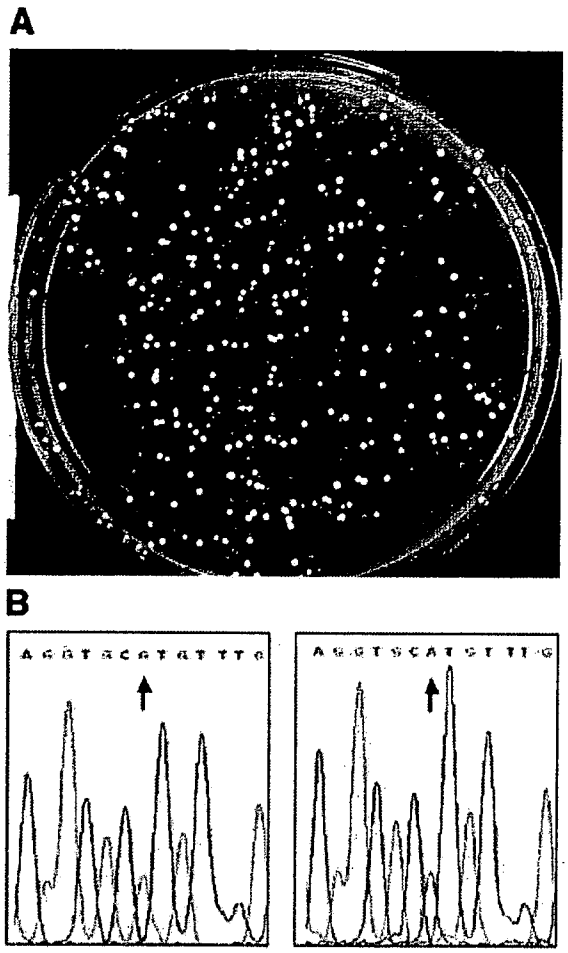

FIGURE 1 – An example of positive yeast p53 functional assay (*a*) and confirmation of p53 mutation by DNA sequencing of the reverse strand: (*b*) wild-type sequence; (*C*) missense mutation of CGT to CAT at codon 273.

RNA extraction and reverse transcription (RT)-PCR

Total RNA was extracted from 100–200 mg of each frozen tissue sample by the guanidinium/phenol/chloroform method (TRIzol reagent; Gibco-BRL, Gaithersburg, MD). RNA integrity was verified by electrophoresis on 1% agarose gel. *p53* cDNA was synthesized at 37°C for 1 hr with 200 units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL) from 1–3 μg of total RNA in 20 μl of RT buffer containing 25 pmol *p53*-specific primer RT-1 (5′-CGGGAGGTAGAC-3′), 7.5 mM dithiothreitol (DTT), 0.5 mM $MgCl_2$ and 0.5 mM of each dNTP. The *p53* cDNA was PCR-amplified in 20 μl of reaction mixture containing 2 μl of RT reaction product, 1.25 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA), 10% DMSO, 50 μM of each dNTP and 10 pmol of primers P3 [5′-ATTTGATGCTGTCCCCGGACGATATTGAA(s)C-3′, where (s) represents a phosphorothioate linkage] and P4 [5′-ACCCTTTTTGGACTTCAGGTGGCTGGAGT(s)G-3′]. PCR was run on a Thermal Cycler Model 2400 (Perkin-Elmer, Chiba, Japan) at 96°C for 1 min, then for 35 cycles of 95°C for 40 sec, 65°C for 70 sec and 78°C for 90 sec, followed by 78°C for 2 min. Satisfactory amplification was confirmed by examining the PCR product in a 1% agarose gel. Each crude PCR product was used for the transformation of yeast.

Plasmids

The yeast expression vector pSS16[17] was digested with excess amounts of *Hind*III and *Stu*I, dephosphorylated with calf intestinal alkaline phosphatase (Takara, Otsu, Japan) and electrophoresed on 1% low-melting-temperature agarose (Sea Plaque agarose; FMC, Rockland, ME). The linearized plasmids were recovered from the gel and purified with Wizard PCR prep kit (Promega, Madison, WI). A gap was created between codons 67 and 347.

Yeast p53 functional assay

The yeast functional assay was performed according to a method described previously.[14,18] The yeast reporter strain yIG397[15] was used throughout the study. The strain yIG397 contains an integrated plasmid with the ADE2 (phosphoribosylaminoimidazole carboxylase, EC 4.1.1.21) open reading frame under the control of a p53-responsive promoter. The genotype is *MAT*a *ade2-1 leu2-3 112trp1-1 his3-11 15can1-100 ura3-1*::[*URA3* 3xRGC-p*CYC1-ADE2*]. When a yeast cell is transformed with a plasmid encoding mutant p53, the cell fails to express ADE2 and forms a red colony due to the accumulation of an oxidized polymerized derivative of phosphoribosyl-aminoimidazole.[19] The yeast was cultured in 100 ml of YPD medium supplemented with 200 μg/ml of adenine until the OD600 value reached 0.8. The cells

TABLE II – DISCOVERED *p53* MUTATIONS AND ITS TRANSDOMINANCE IN 92 ENDOMETRIAL CARCINOMAS

Institute and case number	FIGO stage	Histologic subtype	Grade	Mutation in *p53* gene	Base change	Type of mutation	Transdominance
Hokkaido University							
1	I	Endometrioid adenocarcinoma	2	213 Arg→Stop	CGA→TGA	Nonsense	
2	I	Endometrioid adenocarcinoma	2	321bp deletion (nt 673–993)		Deletion	
3	I	Endometrioid adenocarcinoma	1	133 Met→Arg	ATG→AGG	Missense	
4	I	Endometrioid adenocarcinoma	1	244 Gly→Asp	GGC→GAC	Missense	DN
5	I	Endometrioid adenocarcinoma	2	363bp deletion (nt 385–747)		Deletion	
6	I	Endometrioid adenocarcinoma	1	108 Gly→Ser	GGT→AGT	Missense	
7	I	Endometrioid adenocarcinoma	1	241 Ser→Ala	TCC→GCC	Missense	DN
8	I	Serous adenocarcinoma	3	273 Arg→His	CGT→CAT	Missense	DN
9	II	Serous adenocarcinoma	1	240–243 in frame deletion	AGTTCCTGC ATG→GTG	Deletion	DN
10	III	Endometrioid adenocarcinoma	3	264 Leu→Arg	CTA→CGA	Missense	
11	III	Endometrioid adenocarcinoma	2	280 Arg→Ile	AGA→ATA	Missense	DN
12	III	Endometrioid adenocarcinoma	2	173 Val→Leu	GTG→TTG	Missense	
13	III	Serous adenocarcinoma	2	273 Arg→His	CGT→CAT	Missense	DN
14	IV	Endometrioid adenocarcinoma	3	280 Arg→Gly	AGA→GGA	Missense	DN
15	IV	Serous adenocarcinoma	3	248 Arg→Trp	CGG→TGG	Missense	DN
Mainz University							
1	I	Endometrioid adenocarcinoma	3	306 Arg→Stop	CGA→TGA	Nonsense	
2	I	Endometrioid adenocarcinoma	2	280 Arg→Ser	AGA→AGT	Missense	DN
3	I	Endometrioid adenocarcinoma	3	273 Arg→Cys	CGT→TGT	Missense	DN
4	I	Clear cell adenocarcinoma	3	245 Gly→Val	GGC→GTC	Missense	
5	II	Endometrioid adenocarcinoma	2	257 Leu→Pro	CTG→CCG	Missense	
6	III	Endometrioid adenocarcinoma	3	248 Arg→Gln	CGG→CAG	Missense	DN
7	III	Endometrioid adenocarcinoma	1	273 Arg→His	CGT→CAT	Missense	DN
8	IV	Endometrioid adenocarcinoma	3	175 Arg→His	CGC→CAC	Missense	DN
9	IV	Serous adenocarcinoma	3	273 Arg→His	CGT→CAT	Missense	DN

DN, dominant negative.

were pelleted, washed with LiOAc solution containing 0.1 M lithium acetate, 10 mM Tris-HCl, pH 8.0, and 1 mM $EDTANa_2$; the cells were then pelleted again and resuspended in 500 µl of LiOAc solution. For each transformation, 50 µl of yeast suspension were mixed with 1–5 µl of unpurified *p53* cDNA PCR product, 50–100 ng of linearized plasmid, 5 µl of sonicated single-stranded salmon sperm DNA (10 mg/ml) and 300 µl of LiOAc containing 40% polyethylene glycol 4000 (Kanto, Tokyo, Japan). The mixture was incubated at 30°C for 30 min and heat-shocked at 42°C for 15 min. The yeast was then plated on a synthetic dropout (SD) medium minus leucine plus adenine (5 µg/ml) and was incubated for 48 hr in a 30°C humidified chamber. More than 200 colonies were examined on each culture plate. In this assay system, 16% was the cutoff value for *p53* mutation.[20]

Recovery of p53 plasmids from yeast and DNA sequencing

The yeast was digested with Zymolase-100T (Seikagaku-Kogyo, Tokyo, Japan), and p53 expression plasmids were extracted by the alkaline lysis method (QIAprep plasmid kit; Qiagen, Hilden, Germany) and transfected into XL-1 blue *E. coli* by electroporation. The plasmids were recovered, purified and sequenced with a Dye-Deoxy Terminator Kit (Perkin-Elmer, Urayasu, Japan) on an ABI 377 automated sequencer (Applied Biosystems, Urayasu, Japan) as specified by the manufacturer's protocol and using the following primers: P3seq, 5′-ATTTGATGCTGTCCCCGGACGATATTGAAC-3′; P11seq, 5′-TACTCCCCTGCCCTCAACAAGATG-3′; P12seq, 5′-TTGCGTGTGGAGTATTTGGATGAC-3′; and P13seq, 5′-GCC-CATCCTCACCATCATCACACT-3′.

Transdominance assay

The dominant-negative potential of *p53* mutation was tested using a yeast-based transdominance assay as described previously.[15] Briefly, the yeast functional assay was performed using both a plasmid with wild-type *p53* and a plasmid with mutant *p53* that had been sequence-verified. For each transformation, 50 µl of yeast suspension were mixed with 100 ng of pTSHP53, 100 ng of mutant *p53*-containing pSS16, 50 µg of sonicated single-stranded salmon sperm DNA and 300 µl of LiOAc containing 40% polyethylene glycol 4000. The mixture was incubated at 30°C for 30 min and heat-shocked at 42°C for 15 min. Yeast were then plated on SD medium minus leucine and tryptophan, but which contained a limited amount of adenine (5 µg/ml). The samples were then incubated for 48 hr in a 30°C humidified atmosphere. Double-transformant clones (Leu^+, Trp^+) giving rise to white (Ade^+) or pink/red (Ade^-) colonies were interpreted as expressing recessive and dominant-negative mutations, respectively.

Statistical analysis

The statistical significance of differences between the categorical variables was examined by the chi-square test. Disease-specific survival curves were obtained by the Kaplan-Meier method and the differences between curves were examined by the log-rank test. Independence of prognostic significance was examined using a Cox regression analysis with forward stepwise selection of the variables. $p < 0.05$ was considered to be statistically significant. Statistical analyses were performed using the Statview 5.0 software package (SAS Institute, Cary, NC).

Results

p53 status

The wild-type *p53* gene was observed in 68 tumors. A *p53* mutation was found in 24 (26.1%) tumors. An example of a tumor in which the yeast functional assay gave a positive result (*i.e.*, the number of red colonies *vs.* white colonies = 46:54), and in which DNA sequencing analysis revealed a mutation in codon 273, is shown in Figure 1. The *p53* mutations and their respective transdominance property observed here are summarized in Table II. The mutations included 19 missense mutations, 2 nonsense mutations and 3 deletion mutations. Missense mutation accounted for 79% of all the mutations observed in this study. Codon 273 was most frequently mutated (4 273Arg→His and 1 273Arg→Cys),

TABLE III – RELATIONSHIP BETWEEN *p53* MUTATION AND CLINICAL FEATURES OF ENDOMETRIAL CANCER PATIENTS

	All mutations		Dominant-negative mutation	
	Number/total (%)	*p*	Number/total (%)	*p*
Age				
< 60	9/39 (23.1)		5/39 (12.8)	
≥ 60	15/53 (28.3)	0.64	9/53 (17.0)	0.58
FIGO Stage				
I/II	14/65 (21.5)		6/65 (9.2)	
III/IV	10/27 (37.0)	0.19	8/27 (29.6)	0.01
Histologic subtype				
Endometrioid	18/79 (22.8)		9/79 (11.4)	
Nonendometrioid	6/13 (46.2)	0.09	5/13 (38.5)	0.01
Grade				
1, 2	14/67 (20.9)		7/67 (10.4)	
3	10/25 (40.0)	0.06	7/25 (28.0)	0.04
Institute				
Mainz	9/49 (18.4)		6/49 (12.2)	
Sapporo	15/43 (34.9)	0.07	8/43 (18.6)	0.39

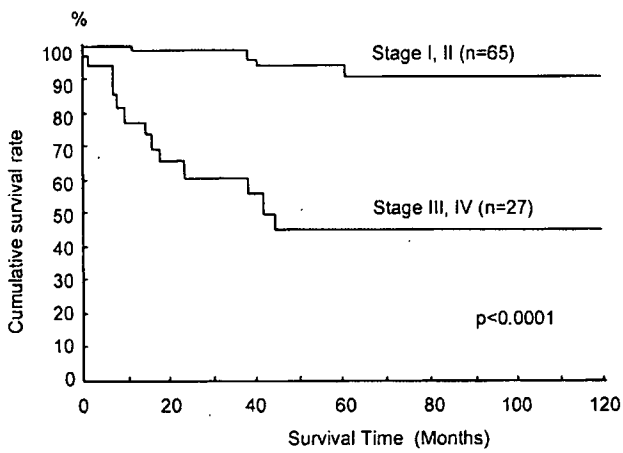

FIGURE 2 – Kaplan-Meier analysis and log-rank test for survival of patients according to FIGO stage.

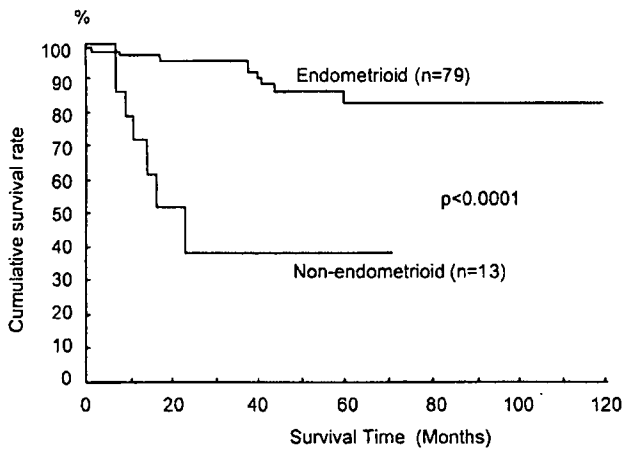

FIGURE 3 – Kaplan-Meier analysis and log-rank test for survival of patients according to histologic subtype of tumors.

followed by codon 280 (280Arg→Ile, 280Arg→Gly, 280Arg→Serand then codon 248 (248Arg→Trp and 248Arg→Gln). Regarding the transdominance of p53 mutation, 10 mutant p53 proteins (10.9%) exhibited recessive activity and 14 mutants

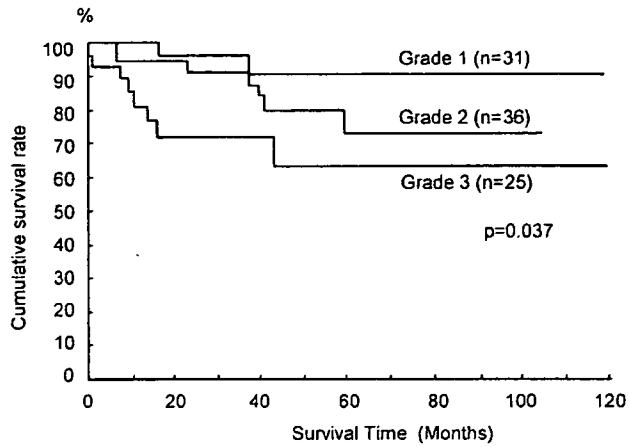

FIGURE 4 – Kaplan-Meier analysis and log-rank test for survival of patients according to grade of tumors.

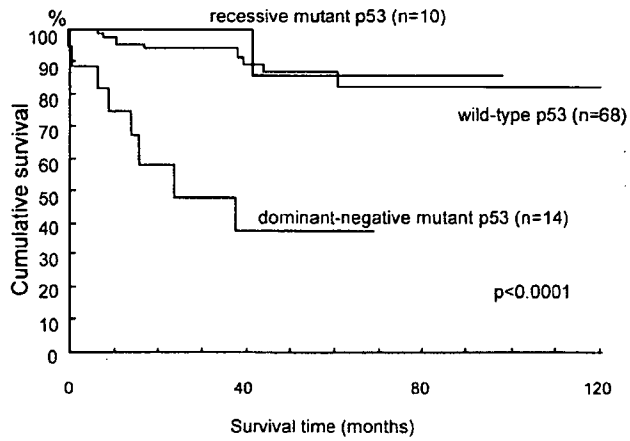

FIGURE 5 – Kaplan-Meier analysis and log-rank test for survival of patients with wild-type, recessive mutant and dominant-negative mutant p53.

(15.2%) showed dominant-negative activity. Not all of the missense mutations had a dominant-negative effect. Only 13 of 19 (68%) missense mutations of *p53* exhibited dominant-negative activity.

p53 mutation was compared to the clinical features (Table III). Total *p53* mutation tended to be related to the following features: nonendometrioid subtype ($p = 0.09$), grade 3 tumors ($p = 0.06$) and Japanese cohort ($p = 0.07$). Dominant-negative mutation was significantly related to advanced FIGO stage ($p = 0.01$), nonendometrioid subtype ($p = 0.01$) and grade 3 tumors ($p = 0.04$).

Survival analysis

The Kaplan-Meier analysis and log-rank test revealed that the survival of patients in this study was significantly related to conventional prognostic factors: FIGO stage ($p < 0.0001$; Fig. 2), histologic subtype ($p < 0.0001$; Fig. 3) and grade of tumor ($p = 0.037$; Fig. 4). As regards *p53* mutation, dominant-negative *p53* mutation was found to be related to poor patient survival ($p < 0.0001$; Fig. 5). Missense *p53* mutation was also significantly related to patient survival ($p = 0.0001$). The age and institute were not related to survival ($p = 0.17$ and 0.52, respectively). When we further examined the functional status of *p53* mutation in relation to patients' survival, the estimated 5-year

TABLE IV – COX REGRESSION ANALYSES FOR PATIENTS WITH ENDOMETRIAL CARCINOMA

Variable	Univariate Cox analysis	Multivariate Cox analysis			
	p	coefficient	Standard error	Hazard ratio	*p*
Dominant negative *p53* mutation	< 0.0001	1.37	0.58	3.9	0.019
FIGO stage	0.0002	1.81	0.62	6.1	0.0037
Histologic subtype	0.0004	1.69	0.58	5.4	0.014
Grade	0.048				NS
Age	0.19				NS
Institute	0.48				NS

survival rate for patients with wild-type p53 (n = 68), recessive *p53* mutation (n = 10) and dominant-negative *p53* mutation (n = 14) was 84.9%, 85.7% and 35.1%, respectively. There was a statistically significant difference in survival between the patients with recessive *p53* mutation and those with dominant-negative *p53* mutation (p = 0.01), as well as between the patients with wild-type *p53* and those with dominant-negative *p53* mutation (p < 0.0001). No difference in survival was found between the patients with recessive *p53* mutation and those with wild-type *p53* (Fig. 5). Furthermore, the survival of patients with missense *p53* mutation tended to be related to the transdominance property of mutant *p53*. The 5-year survival rate was 75.0% for patients with recessive missense mutations (n = 10) and 34.6% for patients with dominant-negative missense mutations (n = 14; p = 0.09).

Using multivariate Cox regression analysis, we found that dominant-negative *p53* mutation (p = 0.019), FIGO stage (p = 0.0037) and histologic subtype (p = 0.014) were independent prognostic factor for the patients in this study (Table IV). When only advanced-stage tumors were taken into consideration, dominant-negative *p53* mutation (p = 0.0023) was the most significant predictor of patient survival.

Discussion

The functional activities of mutant p53 proteins have been grouped into 5 categories: retained wild-type activity, loss of function, gain of function, dominant-negative effect and temperature sensitivity.[4] The dominant-negative activity of *p53* mutation corresponds to the capacity of the mutant protein to complex with the product of the remaining wild-type allele to inactivate its function. Thus, dominant-negative *p53* mutation results in the total abrogation of p53 protein function, even if there is still wild-type protein expressed in the cell. Although the importance of analysis of the functional types of *p53* mutation, *i.e.*, the dominant-negative effect and gain of function, in terms of researching carcinogenesis and searching for novel human cancer therapies has been repeatedly emphasized,[10,12,21] it remains unclear whether or not the dominant-negative activity of mutant p53 proteins has a detrimental effect on the survival of cancer patients. Recessive *p53* mutation accompanied by loss of the second allele may be equal to dominant-negative *p53* mutation in terms of loss of function of the gene. Although we did not investigate LOH in this study, the present results did suggest that dominant-negative *p53* mutation is closely related to poor survival of patients with endometrial cancer, even after adjusting for established prognostic factors, that is, tumor stage, grade and histologic type. Future studies including the LOH status of the second allele will be of interest in this context. It is important to investigate whether or not the dominant-negative *p53* mutation exerts an influence on patient survival, not only through a loss of function, but also by other mechanisms attributable to mutant p53 protein such as certain gain-of-function activities.

Also of interest in this context is that dominant-negative *p53* mutation was found to be closely related to the survival of patients with advanced-stage endometrial cancer. Because the prognosis of patients with early-stage endometrial cancer is generally excellent, gynecologic oncologists need to focus increasingly on the survival of patients with advanced endometrial cancer. The histopathologic prognostic factors for endometrial cancer include depth of myometrial invasion, cervical involvement, serosal invasion, adnexal metastasis, positive peritoneal cytology, vaginal metastasis, lymph node metastasis, peritoneal metastasis and bladder/rectal involvement, which are incorporated in the FIGO surgical staging system.[22] The grade of tumor, histologic subtype and lymph-vascular space invasion, which represent the aggressiveness of a tumor, are also important histopathologic prognostic factors that should be taken into consideration in planning treatment for patients with endometrial cancer.[23] The histologic subtypes of serous adenocarcinoma and clear cell adenocarcinoma exhibit more aggressive biologic behavior than common endometrioid adenocarcinoma and has been shown to lead to disproportionate mortality.[24–26] Serous adenocarcinoma is frequently associated with p53 overexpression or *p53* mutation. Our current study has shown that dominant-negative *p53* mutation is an important prognostic factor, in addition to the established predictors of survival, namely, FIGO stage and histologic subtype, in cases of endometrial cancer. This suggests that dominant-negative *p53* mutation may be a reasonable target for a novel therapy for cases of endometrial cancer with a poor prognosis.

In the present study, only 68% of the missense mutations in the cases of endometrial cancer studied here exhibited dominant-negative activity. This finding suggests that determining the dominant-negative activity of a *p53* mutation is more important than merely determining the presence or absence of a *p53* mutation as part of a tailored treatment or rational targeted treatment for endometrial cancer. Because of the high frequency of *p53* mutations in human cancers, and due to the pivotal role of p53 in regulating growth, apoptosis and DNA repair, the introduction of the wild-type p53 gene has been regarded as a reasonable strategy for a gene therapy designed to restore the lost activity of p53.[27] However, this approach has achieved substantial effectiveness to date.[28] A possible reason for the unsatisfactory results may be the accumulation of dominant-negative mutant p53, which results in a high amount of mutant protein, which would override the effects of the wild-type protein introduced by gene therapy. This explanation may in part account for the recently reported failure of gene therapy in the study of ovarian cancer.[29] In addition to overriding wild-type p53, some dominant-negative mutants are known to cause a gain of function related to tumor progression.[10,12,30] Such mutants include 175 Arg→His, 273 Arg→His and 248 Arg→Trp, which accounted for 6 of the 14 dominant-negative mutants identified in the present study. The gain-of-function property of the p53 mutants is considered to lend further malignant phenotypes to the tumor cells, such as enhancement of tumorigenicity, metastatic potential and therapy resistance. These properties may account for the extremely poor survival of endometrial cancer patients with dominant-negative p53 mutations. The 6 cases with gain-of-function mutations in this study included 1 case of stage I serous adenocarcinoma (case 8 in the Japanese cohort), 1 case of stage III endometrioid adenocarcinoma (cases 7 in the German cohort), 1 case of stage III serous adenocarcinoma (case 13 in the Japanese cohort), 1 case of stage IV endometrioid adenocarcinoma (case 9 in the German cohort) and 2 cases of stage IV serous adenocarcinoma (case 15 in the Japanese cohort and case 9 in the German cohort). The gain-of-function mutation appears to be related to

advanced-stage tumors, although the number of cases studied was not large enough to draw any conclusions.

In summary, this study indicates that dominant-negative mutation of *p53* gene is often found in the advanced stages and aggressive histologic subtypes of endometrial cancer. Moreover, such mutation is a strong predictor of the survival of patients with advanced endometrial cancer. Further investigation will be needed in order to clarify whether or not identification of the dominant-negative property of *p53* mutation may be useful for tailoring the treatment of endometrial cancer.

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HER-2 codon 655 polymorphism in cervical carcinogenesis

M. UEDA*, Y.-C. HUNG†, Y. TERAI*, J. SAITO‡, O. NUNOBIKI‡, S. NODA‡ & M. UEKI*
**Department of Obstetrics and Gynecology, Osaka Medical College, Osaka, Japan; †Department of Obstetrics and Gynecology, China Medical College, Taichung, Taiwan; and ‡Osaka Cancer Prevention Center, Osaka, Japan*

Abstract. Ueda M, Hung Y-C, Terai Y, Saito J, Nunobiki O, Noda S, Ueki M. HER-2 codon 655 polymorphism in cervical carcinogenesis. *Int J Gynecol Cancer* 2006;**16**:325–328.

HER-2 codon 655 polymorphism together with human papillomavirus (HPV) types were examined in a total of 279 cervical smear samples. Forty-nine patients with high-grade squamous intraepithelial lesion had higher frequency of high-risk HPV than 167 patients with low-grade squamous intraepithelial lesion and 63 controls. There was no statistical difference in the frequencies of HER-2 Ile/Ile, Ile/Val, and Val/Val genotypes between squamous intraepithelial lesions (SILs) and controls. When the Ile/Ile genotype was compared to the Ile/Val + Val/Val genotypes, there was also no statistical difference in the genotype prevalence between SILs and controls either in 91 or 188 patients with or without high-risk HPV, respectively. These results suggest that the HER-2 polymorphism at codon 655 in cervical cell samples is unlikely to be associated with HPV status and the onset of cervical cancer in a Japanese population.

KEYWORDS: cervical carcinogenesis, HER-2, polymorphism, SIL.

Cervical cancer is the second most common cancer in women worldwide and is both a preventable and a curable disease especially if identified at an early stage. It is widely accepted that specific human papillomavirus (HPV) types are the central etiologic agent of cervical carcinogenesis. Recently, several candidate markers for cervical cancer risk, such as glutathione-S-transferase and p53, have been described[1–5]. Despite extensive studies on germ line polymorphisms of these cancer susceptibility genes in the patients with premalignant and malignant cervical lesions, no correlation has been reported between their genotype prevalence and increased risk of cervical cancer[1–3,5–7].

HER-2 (also known as c-erbB-2 or neu), the second member of the epidermal growth factor receptor family, encodes a transmembrane glycoprotein (p185) with tyrosine kinase activity[8–10]. Altered expression of the HER-2 gene has been implicated in the carcinogenesis and progression of breast, ovarian, gastric, prostate, and bladder cancer[11–15]. Previous studies have also demonstrated that repression of the HER-2 function suppresses the malignant phenotype of HER-2 overexpressing cancer cells[16,17]. However, there have been only a few reports on the possible correlation between HER-2 expression and the development of cervical cancer[18–21].

Sequence analysis of the human HER-2 gene identified a single nucleotide polymorphism at codon 655, resulting in a G to A transition (Ile 655 Val) in the transmembrane domain-coding region of this gene[22]. Recently, Xie *et al.*[23] reported that the variant Val allele was associated with an elevated risk of breast cancer in Chinese women, whereas another study in a Caucasian population failed to find such a significant association[24]. Although the biologic significance of the amino acid substitution at HER-2 codon 655 is still unclear, it would be of interest to investigate the relationship between the HER-2 polymorphism and pathogenesis in various kinds of human malignant tumors.

In this study, we investigated the HER-2 codon 655 polymorphism together with HPV status in exfoliated cervical cell samples from the patients with squamous intraepithelial lesion (SIL) of the cervix and evaluated the genotype prevalence of this gene in cervical carcinogenesis.

Address correspondence and reprint requests to: Masatsugu Ueda, MD, Department of Obstetrics and Gynecology, Osaka Medical College, 2-7 Daigakumachi, Takatsuki, Osaka 569-8686, Japan. Email: gyn017@poh.osaka-med.ac.jp

Materials and methods

Cell sample

We conducted HER-2 genotype analysis together with HPV typing in a total of 279 cervical smear samples from the patients who received cervical cancer screening. They consist of 63 normal, 167 low-grade SIL (LSIL), and 49 high-grade SIL (HSIL). All the 279 patients were Japanese women. Final histologic diagnosis was confirmed by colposcopy-directed biopsy.

DNA preparation

The exfoliated cervical cells were disrupted with lysis buffer (20 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM ethylenediaminetetraacetic acid [pH 8.0], 0.5% sodium dodecyl sulfate (SDS), 50 μg/mL proteinase K), and genomic DNA was extracted with phenol-chloroform and precipitated with ethanol using standard techniques. Purified DNA samples from the cells were stored at −20°C until use.

Genotyping of HER-2 codon 655

Polymerase chain reaction–restriction fragment length polymorphism analysis of codon 655 of the HER-2 gene, modified from a technique described by Wang *et al.*[25], was conducted to identify HER-2 genotypes using specific primers: sense 5′-AGAGAGCCAGCC-CTCTGACGTCCAT-3′ and antisense 5′-TCCGTTT-CCTGCAGCAGTCTCCGCA-3′. One microliter of the DNA template from each cell sample was amplified by polymerase chain reaction (PCR) in a final volume of 50 μL reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM deoxynucleioside triphosphate, 0.5 μM of each primer, and 1.25 units *Taq* polymerase (Applied Biosystems, Branchburg, NJ) as previously described[26]. After an initial denaturation at 96°C for 3 min, 40 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 2 min) were carried out on a Perkin-Elmer GeneAmp PCR System 9700. The final extension was performed at 72°C for 10 min. After confirmation of an amplified fragment of the expected size (148 bp) by 1.5% agarose gel electrophoresis with ethidium bromide staining, 13 μL of each PCR product was digested with 25 units of restriction enzyme BsmAI (New England Biolabs Inc., Beverly, MA) at 55°C for 3 h, and DNA fragments were visualized on a 3.0% agarose gel. Restriction fragments were 130 and 26 bp for the Ile allele and 94, 36, and 26 bp for the Val allele. The heterozygote contains the following four bands: 130, 94, 36, and 26 bp.

HPV typing

The presence of various HPV types was examined using L1-PCR according to the method reported by Nagano *et al.*[27] Briefly, 100 ng of cellular DNA was subjected to PCR in the presence of published consensus primers (L1C1 and L1C2)[28]. Amplified HPV fragments were typed on the basis of the restriction fragment length polymorphism among HPVs[28]. L1-PCR can detect 22 registered low-risk (6, 11, 34, 40, 42, 43, 44) and high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 69) HPV types.

Statistical analysis

The HPV status and polymorphic features of HER-2 gene in 279 cases were compared between normal, LSIL, and HSIL and checked by the Mann–Whitney and Chi-square tests. A level of $P < 0.05$ was accepted as statistically significant.

Results

The polymorphic site in codon 655 of the HER-2 gene was achieved by polymerase chain reaction–restriction fragment length polymorphism. As shown in Figure 1, fragments of 130 and 26 bp resulted from BsmAI digestion of the Ile allele. The fragments of 94, 36, and 26 bp indicated the digested PCR products from the Val allele. The heterozygote contains the following four bands: 130, 94, 36, and 26 bp. Although the 36 and 26 bp fragments were not clearly visualized, each genotype was judged by the presence or absence of the 130 and 94 bp fragments.

Table 1 shows HPV status and polymorphic frequency of HER-2 codon 655 in 279 samples examined.

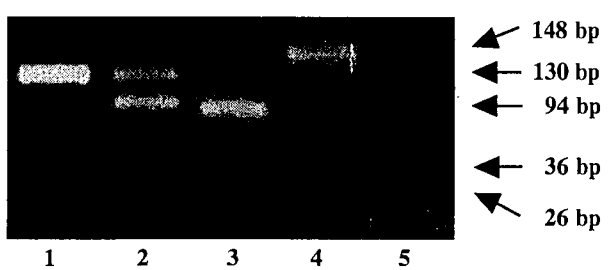

Figure 1. Polymerase chain reaction–restriction fragment length polymorphism analysis on HER-2 codon 655 in DNA samples purified from exfoliated cervical cells. Restriction fragments treated by BsmAI are 130 and 26 bp for the Ile homozygote (lane 1) and 94, 36, and 26 bp for the Val homozygote (lane 3). The heterozygote is in lane 2. An undigested PCR fragment (148 bp) is shown in lane 4. Although the 36 and 26 bp fragments are not clearly visualized, each genotype is judged by the presence or absence of the 130 and 94 bp fragments. Lane 5 is negative control.

Table 1. HPV status and HER-2 codon 655 polymorphism

Lesions	*n*	Number with high-risk HPV	Amino acid at HER2 codon 655		
			Ile/Ile	Ile/Val	Val/Val
Normal	63	10 (15.9%)*,***	46 (73.0%)	16 (25.4%)	1 (1.6%)
LSIL	167	46 (27.5%)***	123 (73.7%)	38 (22.8%)	6 (3.6%)
HSIL	49	35 (71.4%)*,***	35 (71.4%)	14 (28.6%)	0 (0%)
All SIL	216	81 (37.5%)**	158 (73.1%)	52 (24.1%)	6 (2.8%)

*$P < 0.0001$ χ^2 vs normal.
**$P = 0.0013$ χ^2 vs normal.
***$P < 0.0001$ χ^2 vs LSIL.

The patients with HSIL had significantly higher frequency of high-risk HPV than those with LSIL and controls. There was also statistical difference in the frequency of high-risk HPV between all SIL and controls. However, the differences in the polymorphic frequency of HER-2 Ile/Ile, Ile/Val, and Val/Val genotypes between SILs and controls were statistically not significant.

When the Ile/Ile genotype was compared to the Ile/Val + Val/Val genotypes, there was no statistical difference in the genotype prevalence between SILs and controls in 188 patients without high-risk HPV, as shown in Table 2. The relative risks of the combined Ile/Val + Val/Val genotypes for SILs in 91 patients with high-risk HPV showed slightly increased odds ratios (2.345 for LSIL, 1.833 for HSIL, and 2.113 for all SIL); however, there was again no statistical difference between SILs and controls.

Discussion

The genes that encode growth factor receptors can be activated through mutations in their coding sequences. A point mutation in the transmembrane region of the neu gene, a rat homologue of human HER-2 gene, increased the transforming capacity of this gene[29]. Transgenic mice that carry a mutationally activated HER-2 gene or an overexpressed normal HER-2 gene frequently develop mammary adenocarcinoma[30]. Although mutations in the human HER-2 gene have not been identified, sequence analysis revealed a single nucleotide polymorphism at codon 655[22]. Since the HER-2 polymorphism at codon 655 is located in the transmembrane coding region, it may be also associated with the structural and functional alteration of the HER-2 protein. Several previous studies have reported the germ line polymorphism and ethnic variation of the HER-2 gene in breast, bladder, and gastric cancer[23–25,31,32]. However, there has been no report on the correlation between the HER-2 polymorphism and development of malignant tumors of gynecological origin.

In our investigation using exfoliated cervical cell samples from a Japanese population, the differences in the polymorphic frequency of HER-2 Ile/Ile, Ile/Val, and Val/Val genotypes between 216 SILs and 63 controls were statistically not significant. There was also no statistical difference in the genotype prevalence between SILs and controls in 188 patients without high-risk HPV. Although Val allele slightly increased the relative risk of LSIL or HSIL compared to controls in 91 patients with high-risk HPVs, the differences were

Table 2. Relationships between HER-2 codon 655 polymorphism and SIL grade

Study group	*n*	Aminio acid at HER2 codon 655		OR	95% CI	*P* value
		Ile/Ile	Ile/Val + Val/Val			
High-risk HPV−						
Normal	53	38 (71.7%)	15 (28.3%)	1		
LSIL	121	94 (77.7%)	27 (22.3%)	0.728	0.350–1.514	0.396
HSIL	14	11 (78.6%)	3 (21.4%)	0.691	0.170–2.814	0.606
All SIL	135	105 (77.8%)	30 (22.2%)	0.724	0.352–1.487	0.379
High-risk HPV+						
Normal	10	8 (80.0%)	2 (20.0%)	1		
LSIL	46	29 (63.0%)	17 (37.0%)	2.345	0.461–11.930	0.305
HSIL	35	24 (68.6%)	11 (31.4%)	1.833	0.339–9.911	0.482
All SIL	81	53 (65.4%)	28 (34.6%)	2.113	0.433–10.315	0.355

again statistically not significant. To the best of our knowledge, this is the first study to examine the role of HER-2 codon 655 polymorphism in cytologic materials from women with premalignant cervical disease. Our present results suggest that Val655Ile of the HER-2 gene in cervical cell samples is unlikely to be associated with HPV status and the onset of cervical cancer in a Japanese population.

Acknowledgments

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Fas gene promoter −670 polymorphism in gynecological cancer

M. UEDA, Y. TERAI, K. KANDA, M. KANEMURA, M. TAKEHARA, H. YAMAGUCHI, K. NISHIYAMA, M. YASUDA & M. UEKI
Department of Obstetrics and Gynecology, Osaka Medical College, Takatsuki Osaka, Japan

Abstract. Ueda M, Terai Y, Kanda K, Kanemura M, Takehara M, Yamaguchi H, Nishiyama K, Yasuda M, Ueki M. Fas gene promoter −670 polymorphism in gynecological cancer. *Int J Gynecol Cancer* 2006;**16(Suppl. 1)**: 179–182.

Single-nucleotide polymorphism at −670 of Fas gene promoter (A/G) was examined in a total of 354 blood samples from normal healthy women and gynecological cancer patients. They consisted of 95 normal, 83 cervical, 108 endometrial, and 68 ovarian cancer cases. Eighty-three patients with cervical cancer had statistically higher frequency of GG genotype and G allele than 95 controls (P = 0.0353 and 0.0278, respectively). There was no significant difference in the genotype or allele prevalence between control subjects and endometrial or ovarian cancer patients. The Fas −670 GG genotype was associated with an increased risk for the development of cervical cancer (OR = 2.56, 95% CI = 1.08–6.10) compared with the AA genotype. The G allele also increased the risk of cervical cancer (OR = 1.60, 95% CI = 1.05–2.43) compared with the A allele. Germ-line polymorphism of Fas gene promoter −670 may be associated with the risk of cervical cancer in a Japanese population.

KEYWORDS: gynecological cancer, Fas, polymorphism.

Apoptosis is a physiologic process for the elimination of specific types of cells, occurring extensively in embryonic development, metamorphosis, and differentiation. This process also plays an important role in eliminating unwanted or potentially dangerous cells throughout life. Abnormal regulation of apoptosis is likely to contribute to the pathogenesis of autoimmune diseases and malignant tumors. The acquired ability to resist apoptotic stimuli is shared by many types of malignant diseases, and genetic alteration in the components of apoptotic pathways is a pivotal mechanism in the development of cancer[1,2].

Fas, also known as CD95 or APO-1, is a transmembrane receptor that plays a central role in apoptotic signaling in many cell types[3]. This receptor interacts with its natural ligand FASL, a member of the tumor necrosis factor superfamily, to initiate the death signal cascade, which results in apoptotic cell death[3,4]. Down-regulation of Fas with resultant resistance to death signals has been reported in many cancers. The transcriptional expression of Fas gene is regulated by a number of genetic elements located in the 5′-upstream region of the gene. Single-nucleotide polymorphism (SNP) at −670 of Fas gene promoter (A/G) has recently been identified[5,6]. This SNP has been found with potentially different transcriptional efficiency[5–7]. Recent studies have demonstrated that the A/G SNP at Fas promoter −670 is closely associated with the pathogenesis of autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus[7,8]. However, the correlation between this SNP and cancer susceptibility including the risk of gynecological malignancies has not been extensively studied, and previous experimental results are controversial.

Recently, Lai *et al.*[9] reported that SNP at position −670 in the Fas gene promoter is associated with the development of cervical cancer in a Chinese population. Engelmark *et al.*[10] also conducted Fas promoter −670 polymorphism analysis and suggested that this SNP does not have a major impact on the susceptibility to cervical cancer in Swedish patients. In this study, we examined germ-line polymorphism of Fas gene promoter −670 in a total of 354 blood samples from normal healthy women and gynecological cancer patients to reevaluate the possible association between this SNP and the risk of cervical cancer in a Japanese population.

Address correspondence and reprint requests to: Masatsugu Ueda, MD, Department of Obstetrics and Gynecology, Osaka Medical College, 2-7 Daigakumachi, Takatsuki, Osaka 569-8686, Japan. Email: gyn017@poh.osaka-med.ac.jp

Materials and methods

Blood sample

We conducted genotype analysis of Fas gene promoter −670 using blood samples from 95 normal healthy women and 83 cervical, 108 endometrial, and 68 ovarian cancer patients with invasive diseases. Table 1 shows the clinical characteristics of cancer patients. All subjects were Japanese women. No statistically significant differences were found between control subjects and cancer patients in each group in terms of age distribution, smoking, and menstrual status. This study was approved by our institutional review board, and all samples were obtained with consent.

DNA preparation

Peripheral blood lymphocytes were disrupted with lysis buffer (20 mM NaCl, 10 mM Tris–HCl [pH 8.0], 10 mM ethylenediaminetetraacetic acid [pH 8.0], 0.5% sodium dodecyl sulfate, 50 μg/mL proteinase K), and genomic DNA was extracted with phenol–chloroform and precipitated with ethanol using standard techniques. Purified DNA samples from the cells were stored at −20°C until use.

Genotyping of Fas gene promoter −670

Polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis of the Fas gene promoter −670, modified from a technique described by Lee *et al.*[11], was conducted with the primers 5′-CTACCTAAGAGCTATCTACCGTTC-3′ and 5′-GGCTGTCCATGTTGTGGCTGC-3′. Hundred nanograms of the DNA template from each sample was amplified by PCR in a final volume of the 50-μL reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM deoxynucleoside triphosphate, 0.5 μM each primer, and 1.25 U *Taq* polymerase (Applied Biosystems, Branchburg, NJ) as previously described[12]. After an initial denaturation at 96°C for 3 min, 40 cycles of denaturation (94°C for 1 min), annealing (58°C for 1 min), and extension (72°C for 2 min) were carried out on a Perkin-Elmer GeneAmp PCR System 9700. The final extension was performed at 72°C for 10 min. After digestion of PCR products with restriction enzyme *Mva*I (Roche Applied Science, Penzberg, Germany) under recommended conditions, DNA fragments were visualized on a 3.0% agarose gel electrophoresis with ethidium bromide staining, and gel images were obtained using the ATTO densitograph UV-image analyzer (ATTO Corp, Tokyo, Japan).

Statistical analysis

To compare the polymorphic features of Fas gene promoter −670 between control subjects and cancer patients, Pearson's Chi-square test was used. A level of $P < 0.05$ was accepted as statistically significant.

Results

The *Mva*I site at −670 position is polymorphic, whereas the *Mva*I site at −858 position is a constant restriction site. After digestion with *Mva*I enzyme, two fragments, 233 and 98 bp, were produced if A nucleotide was present at −670 position. In the presence of G

Table 1. Clinical characteristics of gynecological cancer patients

Variable	Cervical cancer ($n = 83$)	Endometrial cancer ($n = 108$)	Ovarian cancer ($n = 68$)
Age distribution (years)			
≤30	4 (4.8%)	3 (2.8%)	3 (4.4%)
31–50	39 (47.0%)	26 (24.1%)	20 (29.4%)
51–70	35 (42.2%)	68 (62.9%)	41 (60.3%)
>70	5 (6.0%)	11 (10.2%)	4 (5.9%)
Menstrual status			
Premenopause	45 (54.2%)	34 (31.5%)	25 (36.8%)
Postmenopause	38 (45.8%)	74 (68.5%)	43 (63.2%)
FIGO stage			
I	37 (44.6%)	84 (77.8%)	26 (38.2%)
II	31 (37.3%)	4 (3.7%)	7 (10.3%)
III	15 (18.1%)	19 (17.6%)	32 (47.1%)
IV	0 (0%)	1 (0.9%)	3 (4.4%)
Histologic type	Squamous	Endometrioid	Serous
	59 (71.1%)	97 (89.8%)	34 (50.0%)
	Nonsquamous	Nonendometrioid	Nonserous
	24 (28.9%)	11 (10.2%)	34 (50.0%)

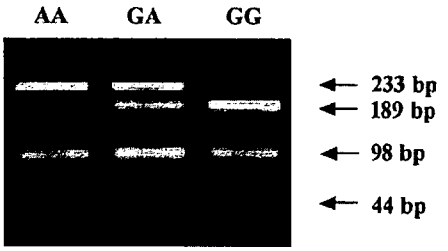

Figure 1. Genotyping of Fas gene promoter −670 in DNA samples from peripheral blood lymphocytes by PCR–restriction fragment length polymorphism. The genotypes AA (232 bp), GA (188, 233 bp), and GG (189 bp) are shown.

substitution at this position, the resultant 233-bp fragment was further cleaved into two fragments, 189 and 44 bp, because of the new *Mva*I restriction site. Figure 1 shows an example for genotyping of Fas gene promoter −670 in DNA samples purified from peripheral blood lymphocytes. The fragments of 233 and 189 bp indicated the AA and GG genotypes, respectively. The GA genotype contained these two bands.

Table 2 shows the genotype and allele frequencies of Fas promoter −670 in the 354 samples examined. Hardy–Weinberg equilibrium of the A/G allele was maintained in our population. Eighty-three patients with cervical cancer had a statistically higher frequency of GG genotype and G allele than 95 controls (P = 0.0353 and 0.0278, respectively). There was no significant difference in the genotype or allele prevalence between control subjects and endometrial or ovarian cancer patients. As shown in Table 3, the Fas −670 GG genotype was associated with an increased risk for the development of cervical cancer (OR = 2.56, 95% CI = 1.08–6.10) compared with the AA genotype. There also appeared to be a trend toward increased frequency from AA to GG genotype in cancer patients (P test for trend <0.05). The G allele also increased the risk of cervical cancer (OR = 1.60, 95% CI = 1.05–2.43) compared with the A allele.

Discussion

There is an expanding body of literature suggesting that host factors, including genetic polymorphisms, may explain some of the individual differences in cancer occurrence. A large number of previous studies have been conducted on the correlation between germ-line polymorphisms of cancer susceptibility genes and the higher risk of human malignant tumors. Polymorphisms in the promoter region or 5′-flanking region of genes can lead to different levels of gene expression and have been implicated in a number of diseases. The *Mva*I polymorphism at −670 position of the Fas gene within the enhancer region occurs at the consensus sequence of nuclear transcription element gamma interferon activation signal (GAS) binding site and could conceivably influence the expression of Fas gene[7,8]. Homozygosity for G allele could result in a complete deletion of the binding sequence of transcription element GAS, which is responsible for the signal emanated through STAT1, and in a significant alteration in the gene expression.

Several previous studies addressed the association of this SNP with autoimmune diseases[5,7,8,11]. Lai *et al.*[9] conducted Fas promoter −670 polymorphism analysis using surgical and biopsy tissue specimens of cervical neoplasm and reported that the frequency of AA genotype and A allele increased in accordance with the multistep carcinogenesis from cervical intraepithelial lesion to invasive squamous cell cancer. They stated that AA genotype, conferring more efficient Fas expression, could be one of the mechanisms that cells use to avoid carcinogenesis. In contrast, our present results on germ-line polymorphism of Fas promoter −670 demonstrated that the frequency of GG genotype or G allele increased the risk of cervical cancer. Very recently, Engelmark *et al.*[10] and Dybikowska *et al.*[13] have demonstrated that AA genotype in Fas gene promoter at −670 position may not be engaged in the development of cervical neoplasia in a Swedish and a Polish population, respectively. Allelic differences of Fas promoter −670 polymorphisms were

Table 2. Genotypic and allelic frequencies of Fas promoter −670 in control subjects and cancer patients

	Genotype frequency			Allele frequency	
Samples	AA	GA	GG	A	G
Normal (n = 95)	23 (24.2%)	54 (56.8%)	18 (18.9%)[a]	100 (52.6%)	90 (47.4%)[b]
Cervical cancer (n = 83)	15 (18.1%)	38 (45.8%)	30 (36.1%)[a]	68 (41.0%)	98 (59.0%)[b]
Endometrial cancer (n = 108)	39 (36.1%)	50 (46.3%)	19 (17.6%)	128 (59.3%)	88 (40.7%)
Ovarian cancer (n = 68)	18 (26.4%)	37 (54.4%)	13 (19.1%)	73 (53.7%)	63 (46.3%)

[a] P = 0.0353 χ^2 vs normal.
[b] P = 0.0278 χ^2 vs normal.

Table 3. Risk of cervical cancer associated with Fas promoter −670 genotypes and alleles

Fas promoter −670 polymorphism	Control subjects	Cancer patients	OR (95% CI)
Genotype			
AA	23 (24.2%)	15 (18.1%)	1.00 (referent)
GA	54 (56.8%)	38 (45.8%)	1.08 (0.49–2.37)
GG	18 (18.9%)	30 (36.1%)	2.56 (1.08–6.10)
Allele			
A	100 (52.6%)	68 (41.0%)	1.00 (referent)
G	90 (47.4%)	98 (59.0%)	1.60 (1.05–2.43)

observed in various ethnic groups[13]. The frequency of −670 A/G alleles in healthy individuals differed among American, Korean, and Japanese populations. The genotype distribution in normal controls of the Japanese population obtained in our study was consistent with previous reports[13,14]. The differences of materials analyzed and the ethnic variation of genotype frequency of Fas gene promoter in different geographical regions may affect the discrepancy in the results between our present study and other reports.

In cervical cancer tissues and cell lines, significant decrease in the expression levels of Fas has been reported[15,16]. The higher frequency of GG genotype or G allele in our series may result in a significant decrease in Fas gene expression and subsequent escape from apoptosis of the cells in cervical carcinogenesis. Sun *et al.*[17] also observed an increased risk of esophageal squamous cell carcinoma associated with Fas −670 GG genotype. Moreover, our recent study[18] using 279 cervical smear samples has demonstrated that the frequency of G allele increased from low-grade squamous intraepithelial lesion to high-grade squamous intraepithelial lesion (HSIL) and that there was an increased odds ratio for G allele in HSIL cases compared to controls among the patients with high-risk human papilloma virus. Here we demonstrated that the germ-line polymorphism of Fas gene promoter −670 is closely associated with the risk of cervical cancer in a Japanese population. It might be of interest to evaluate further whether this SNP could be used as a disease marker for the natural history of cervical neoplasias in a setting of a longitudinal cohort study.

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II. 子宮体がん

ハイリスク症例に対する後療法には adjuvant radiotherapy か adjuvant chemotherapy か

—adjuvant radiotherapy の立場から

寒河江　悟*　杉村　政樹*　江坂　嘉昭*

要　旨

子宮体癌の術後療法として子宮に病巣が限局している進行期 I/II 期の場合，放射線療法が標準治療として汎用され有効である．しかし，近年その治療効果は骨盤内再発予防としての局所制御には有効であるが，長期的な予後に関しては生存率が向上するというエビデンスに乏しいとも言われる．最近の本邦の無作為化比較試験 JGOG 2033 では IC 期，II 期の早期癌では放射線療法と化学療法がともに有効であることが示唆された．今後，放射線療法と並んで化学療法の有用性が注目される可能性がある．

Sagae Satoru
* 札幌医科大学産婦人科　助教授
(〒060-0061 北海道札幌市中央区南 1 条西 16 丁目)

はじめに

子宮体癌は早期症例が多くを占め，手術療法で完全摘出可能であれば比較的予後良好な疾患である．しかし，手術療法で完全に腫瘍が摘出できなかった場合や摘出物の病理所見で種々の再発危険因子を認めた場合，術後の追加療法の選択には苦慮することが多い．そこで術後の追加治療である放射線療法を中心に述べ，特に最近報告された化学療法との無作為化比較試験の成績と今後の展開を考察してみた．

1．子宮体癌治療成績と治療法の現状

欧米を中心とした FIGO annual report vol. 25 の報告[1]では，進行期 Ic (n=1,219)，IIa (n=364)，IIb (n=426)，IIIa (n=484)，IIIb (n=73)，IIIc (n=293) 症例，約 2,900 例の 5 年生