of p53 protein to degradation by E6 protein derived from oncogenic HPV [4].

Despite extensive studies on germline polymorphisms of GSTM1, GSTT1 and p53 genes in the patients with premalignant and malignant cervical lesions, no correlation has been reported so far between genetic polymorphisms of these genes and increased risk of cervical cancer [1–3,5–7]. In this study, we investigated GSTM1, GSTT1 and p53 codon 72 polymorphisms in exfoliated cervical cell samples from the patients with squamous intraepithelial lesion (SIL) of the cervix and evaluated the clinical significance of polymorphic frequency of these genes in cervical carcinogenesis.

Materials and methods

Cell sample

We conducted GST and p53 genotype analysis together with HPV typing in a total of 198 cervical smear samples obtained from the patients with consent who received cervical cancer screening. They consist of 54 normal, 102 low-grade SIL (LSIL) and 42 high-grade SIL (HSIL). All of 198 patients were Japanese women who visited Osaka Medical College, Kansai Medical College or Osaka Cancer Prevention Center in the past 5 years. Final histologic diagnosis was confirmed by colposcopy-directed biopsy for the patients with abnormal cytology.

DNA preparation

The exfoliated cervical cells were disrupted with lysis buffer [20 mM NaCl, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.5% 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 GSTM1, GSTT1 and p53 codon 72

The GSTM1 and GSTT1 genetic polymorphisms were evaluated using multiplex polymerase chain reaction (PCR) techniques according to the method reported by Chen et al. [8] with some modifications. For GSTM1, the primers 5′-GAACTCCCTGAAAAGCTAAAGC-3′ and 5′-GTTGGGCTCAAATATACGGTGG-3′; for GSTT1, the primers 5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGATCATGGCCAGCA-3′; for β-globin as a positive control, the primers 5′-CAACTTCATCCACGTTCACC-3′ and 5′-GAAGAGCCAAGGACAGGTAC-3′ were used. One hundred nanograms of the DNA template from each cell sample was amplified by PCR in a final volume of 50μl reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM dNTP, 0.5μM each primer and 1.25 units Taq polymerase (Applied Biosystems, Branchburg, NJ) as previously described [9]. 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 visualization of the PCR products by 2.0% agarose gel electrophoresis with ethidium bromide staining, gel images were obtained using the ATTO densitograph UV-image analyzer (ATTO Corp, Tokyo), and the presence or absence of the products was determined using ATTO's densitometry software version 2 (ATTO). The absence of amplified GSTM1 or GSTT1 product indicated the respective null genotype for each.

PCR restriction fragment length polymorphism (RFLP) analysis of codon 72 of the p53 gene, modified from a technique described by Ara et al. [10], was conducted to identify p53 genotypes with the primers 5′-TTGCC-GTCCCAAGCAATGGATGA-3′ and 5′-TCTGGGAAGG-GACAGAAGATGAC-3′. One hundred nanograms of the DNA template from each cell sample was amplified by PCR in a 50 μl reaction as described above with an annealing temperature at 60°C. After confirmation of an amplified fragment of the expected size (199 bp) on a 1.5% agarose gel, 17 μl of each PCR product was digested with 10 units of restriction enzyme *Bst*UI (New England Biolabs, ME) at 60°C for 3 h. DNA fragments were visualized on a 3.0% agarose gel with ethidium bromide as described above. The Arg allele is cleaved by *Bst*UI and yields two small fragments (113 and 86 bp). The Pro allele is not cleaved by *Bst*UI and has a single 199-bp band. The heterozygote contains three bands (199, 113 and 86 bp).

Sequence analysis

Polymorphisms in the p53 codon 72 were sequenced. Amplified DNA fragments were purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and directly sequenced using ABI PRISM 3100 sequencer (Applied Biosystems). Each single nucleotide polymorphism was verified in both the sense and antisense directions.

HPV typing

The presence of various HPV types was examined using L1-PCR according to the method reported by Nagano et al. [11]. Briefly, 100 ng of cellular DNA was subjected to PCR in the presence of published consensus primers (L1C1 and L1C2) [12]. Amplified HPV fragments were typed on the basis of the RFLP among HPVs. Initial typing of amplified HPV fragments was performed by digestions with *Rsa*I, *Dde*I, and then confirmed by digestions with several additional restriction enzymes as described previously [11,12]. HPV-negative or -positive cervical cancer cell line was used as a negative or positive control for HPV typing, respectively. 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.

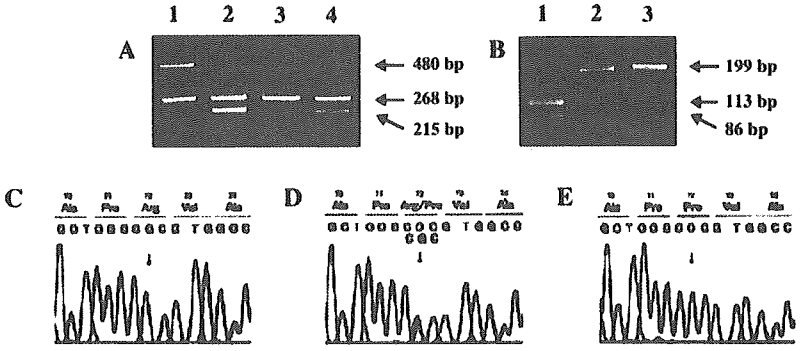

Fig. 1. Genotyping of GSTM1, GSTT1 and p53 codon 72 by multiplex PCR or PCR-RFLP and direct sequence. (A) Case 1, null GSTM1 genotype (absence of 215-bp fragment). Case 2, null GSTT1 genotype (absence of 480-bp fragment). Case 3, null GSTM1 and GSTT1 genotypes (absence of 215- and 480-bp fragments). Case 4, present GSTM1 and GSTT1 genotypes. β-globin as a positive control is detected as 268-bp fragment. (B) Case 1, Arg/Arg homozygotes. Case 2, Arg/Pro heterozygotes. Case 3, Pro/Pro homozygotes. The fragment of 199 bp is the nondigested PCR product from the Pro allele. Fragments of 113 and 86 bp result from *Bst*UI digestion of the Arg allele. The amplified fragments of the p53 codon 72 (cases 1–3 in B) were gel-purified and sequenced. Electropherograms of p53 codon 72 forward sequence indicate Arg (CGC) in case 1 (C), Arg/Pro (CGC/CCC) in case 2 (D), and Pro (CCC) in case 3 (E), respectively.

Statistical analysis

The HPV status and polymorphic features of GSTM1, GSTT1 and p53 genes in 198 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

Fig. 1A shows an example for genotyping of GSTM1 and GSTT1. The polymorphic deletion of the GSTM1 and GSTT1 genes was determined by multiplex PCR. The absence of 215- or 480-bp fragment indicated null GSTM1 or GSTT1 genotype, respectively. The polymorphic site in exon 4 (codon 72) of the p53 gene was achieved by PCR-RFLP. As shown in Fig. 1B, the fragment of 199 bp indicated the nondigested PCR product from the Pro allele. Fragments of 113 and 86 bp resulted from *Bst*UI digestion of the Arg allele. The Arg/Pro heterozygote contained these three bands (199, 113 and 86 bp). Polymorphisms of p53 codon 72 detected by PCR-RFLP were also confirmed by sequence analyses. As can be seen in Figs. 1C–E, sequencing of the gel-purified PCR product indicated CGC for Arg, CGC/CCC for Arg/Pro, and CCC for Pro genotype at codon 72, respectively.

Table 1 shows HPV status and polymorphic frequency of GSTM1, GSTT1 and p53 codon 72 in 198 samples examined. The 42 patients with HSIL had significantly higher frequency of high-risk HPV than 102 with LSIL and 54 controls. There was no significant difference in the frequency of null GSTM1 genotype between SILs and controls, whereas the 42 patients with HSIL had statistically higher frequency of null GSTT1 genotype than 102 with LSIL and 54 controls. In contrast, the differences in the polymorphic frequency of p53 Arg, Arg/Pro and Pro genotypes between SILs and controls were statistically not significant.

As shown in Table 2, the 31 patients with HSIL had also statistically higher frequency of null GSTT1 genotype than 28 with LSIL among the 69 patients with high-risk HPV. When the Arg genotype was compared to the Arg/Pro + Pro genotypes, there was again no statistical difference in the

Table 1
Frequency of GSTM1, GSTT1 and p53 codon 72 polymorphisms in exfoliated cervical cell samples

Lesions	Number with high-risk HPV	GSTM1 null	GSTT1 null	Amino acid at p53 codon 72		
				Arg	Arg/Pro	Pro
Normal (n = 54)	10 (18.5%)[a,b]	28 (51.9%)	24 (44.4%)[c]	24 (44.4%)	23 (42.6%)	7 (13.0%)
LSIL (n = 102)	28 (27.5%)[d]	55 (53.9%)	40 (99.2%)[c]	38 (37.3%)	40 (39.2%)	24 (23.5%)
HSIL (n = 42)	31 (73.8%)[a,d]	20 (47.6%)	29 (69.0%)[c,e]	18 (42.9%)	16 (38.1%)	8 (19.0%)
All SIL (n = 144)	59 (41.0%)[b]	75 (52.1%)	69 (47.9%)	56 (38.9%)	56 (38.9%)	32 (22.2%)

[a] $P < 0.0001$, OR = 12.4 x^2 vs. normal.
[b] $P = 0.0031$, OR = 3.1 x^2 vs. normal.
[c] $P = 0.0162$, OR = 2.8 x^2 vs. normal.
[d] $P < 0.0001$, OR = 7.4 x^2 vs. LSIL.
[e] $P = 0.0011$, OR = 3.5 x^2 vs. LSIL.

Table 2
HPV status and frequency of GSTT1 and p53 codon 72 polymorphisms in exfoliated cervical cell samples

Study group	*n*	GSTT1 null	Amino acid at p53 codon 72	
			Arg	Arg/Pro + Pro
High-risk HPV−				
Normal	44	20 (45.5%)	20 (45.5%)	24 (54.5%)
LSIL	74	31 (41.9%)	26 (35.1%)	48 (64.9%)
HSIL	11	8 (72.7%)	4 (36.4%)	7 (63.6%)
All SIL	85	39 (45.9%)	30 (35.3%)	55 (64.7%)
High-risk HPV+				
Normal	10	4 (40.0%)	4 (40.0%)	6 (60.0%)
LSIL	28	9 (32.1%)[a]	12 (42.9%)	16 (57.1%)
HSIL	31	21 (67.7%)[a]	14 (45.2%)	17 (54.8%)
All SIL	59	30 (50.8%)	26 (44.1%)	33 (55.9%)

[a] $P = 0.0063$, OR = 4.4 x^2 vs. LSIL.

genotype prevalence between SILs and controls among the 129 and 69 patients without and with high-risk HPV, respectively.

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 germline polymorphisms of cancer susceptibility genes and the higher risk of human malignant tumors.

The GSTM1 and GSTT1 gene products are thought to protect against somatic mutation in DNA by facilitating the conjugation and elimination of a variety of electrophilic species [13]. Previous epidemiological studies of GST and cervical neoplasia found no significant differences in the frequency of GSTM1 or GSTT1 in women with cervical SIL or cancer compared to controls with normal cervical pathology [1–3]. In our investigation using exfoliated cervical cell samples from a Japanese population, the GSTT1 null genotype was more common among HSIL cases than LSIL cases and controls. Moreover, the patients with HSIL also had higher frequency of null GSTT1 genotype than those with LSIL among high-risk HPV group. GSTT1 differs from other classes of GSTs in its lack of activity towards the GST model substrate 1-chloro-2, 4-dinitrobenzene and its failure to bind to S-hexyl-glutathione affinity matrices [14]. The gene defect of GSTT1 was reported to be associated with an increased risk of myelodysplastic syndromes [15], astrocytoma and meningioma [16]. However, there have been no other reports on the correlation between GSTT1 gene defects and cervical carcinogenesis. Recently, we have examined GSTM1 and GSTT1 genotypes in 104 cell lines originating from a variety of human malignant tumors and found that GSTT1 null genotype was more common in cervical cancer cells [17]. Further studies on the differential gene expression profiles between normal cervical keratinocytes and cervical cancer cell lines with or without GSTT1 deletion may provide the better understanding for the effect of this abnormal genotype in the sequence of cervical carcinogenesis. Moreover, it might be of interest to further examine the difference in the polymorphic frequency of the null GSTT1 genotype between SILs and invasive cervical cancer to clarify whether this genotype alteration occurs prior to the development of malignant phenotype cells or late in the development of neoplastic cells.

Tobacco smoking has been associated with the risk of cervical malignancy and SIL [18]. DNA adducts of bulky aromatic compounds have been found with increased frequency in the cervical epithelium of smokers compared to nonsmokers [19], providing biochemical evidence that smoking may act as a confounder in the etiology of cervical cancer. Molecular studies have identified polymorphic gene products that are associated with the metabolism of tobacco smoke procarcinogens and possibly with susceptibility to cancer. Lack of GST activity, caused by an inherited deletion of the GST gene, has also been reported to increase the risk of lung and other tobacco-related cancers [13]. It would be of interest to further examine the relationship of the development of SIL, smoking and GSTT1 null genotype in the group of patients we examined.

Initially, we evaluated p53 genotypes using the technique reported by Ara et al. [10]. Because these results may be affected by incomplete *Bst*UI digestion, we further confirmed p53 genotypes by sequence analyses. We found that incubation of PCR products with 10 units of *Bst*UI at 60°C for 3 h resulted in complete digestion and PCR-RFLP profiles exactly matched sequence data. Our present results revealed that the differences in the polymorphic frequency of p53 Arg, Arg/Pro and Pro genotypes between SILs and controls were statistically not significant. Moreover, neither Arg nor Pro allele affected the increased risk of SILs with or without high-risk HPVs compared to controls. Some previous studies have reported no correlation between germline polymorphisms of the p53 codon 72 and increased risk of cervical cancer [5–7]. The recent study reported by Nishikawa et al. [20] using cervical condyloma, dysplasia and cancer tissue samples also demonstrated that no statistically significant differences in the distribution of p53 genotypes were found among the patients with these diseases, regardless of HPV status. The other two reports examining for Japanese population supported their results [21,22]. These data suggest that p53 codon 72 polymorphism does not correlate with the development of HPV-associated cervical neoplasms.

To the best of our knowledge, this is the first study to examine the role of GST and p53 codon 72 polymorphisms in cytologic materials from women with premalignant cervical disease. GSTT1 null genotype in cervical cell samples may be associated with more severe precancerous lesions of the cervix in a Japanese population. However, the

p53 codon 72 polymorphism is unlikely to be associated with HPV status and the onset of cervical cancer. These observations are potentially important in managing SIL patients by cervical screening and in understanding the pathogenesis of cervical cancer.

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Fas gene promoter −670 polymorphism (A/G) is associated with cervical carcinogenesis

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Abstract

Objective. To investigate the biological significance of single nucleotide polymorphism (SNP) at Fas gene promoter in cervical carcinogenesis.

Methods. SNP at −670 of Fas gene promoter (A/G) together with human papillomavirus (HPV) types were examined in a total of 279 cervical smear samples and 8 human cervical squamous carcinoma cell lines using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) techniques.

Results. 49 patients with high-grade squamous intraepithelial lesion (HSIL) had higher frequency of high-risk HPV and GA + GG genotype than 167 with low-grade SIL (LSIL) and 63 controls. G allele frequency was also higher in HSIL than in LSIL and controls. There was an increased OR (6.00; CI, 1.32–27.37; $P = 0.021$) for GA + GG genotype in HSIL cases compared to controls among 96 patients with high-risk HPV. 7 of 8 cervical carcinoma cell lines also showed GA or GG genotype.

Conclusion. Fas gene promoter −670 polymorphism (A/G) may be closely associated with cervical carcinogenesis in a Japanese population.

Keywords: Fas; Polymorphism; SIL; Cervical carcinogenesis

Introduction

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. Other environmental and host factors also play decisive roles in the persistence of HPV infection and further malignant conversion of cervical epithelium [1]. Although many previous reports have focused on HPV and environmental factors, the role of host susceptibility to cervical carcinogenesis is largely unknown.

Apoptosis is a physiological process that regulates normal homeostasis and alterations of apoptosis-related genes are likely to contribute to the pathogenesis of autoimmune diseases [2] and malignant tumors [3]. Among various cell surface death receptors, Fas/CD95, a transmembrane receptor, is known as a member of tumor necrosis factor (TNF) receptors superfamily [4]. Downregulation of Fas with resultant resistance to death signals has been reported in many cancers [5–7]. The transcriptional expression of Fas gene is regulated by a number of genetic elements located in the 5′ upstream region of the gene. The promoter region of Fas gene consists of basal promoter, enhancer, and silencer regions [8]. Single nucleotide poly-

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morphism (SNP) at −670 in the enhancer region (A/G) situates at a binding element of gamma interferon activation signal (GAS). G allele results in an abolishment of the GAS element and a significant decrease in Fas gene expression in response to interferon (INF)-γ stimuli [9,10]. Recent studies have demonstrated that the A/G SNP at −670 of Fas gene promoter is closely associated with the pathogenesis of autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus [10,11]. However, there have been very few reports on the correlation between SNP of Fas gene and cancer susceptibility [12,13]. In this study, we investigated Fas gene promoter −670 polymorphism together with HPV types in exfoliated cervical cell samples from the patients with squamous intraepithelial lesion (SIL) of the cervix or human cervical squamous carcinoma cell lines, and evaluated the biological significance of this genotype in cervical carcinogenesis.

Materials and methods

Cell sample

We conducted genotype analysis of Fas gene promoter −670 together with HPV typing in a total of 279 cervical smear samples obtained from the patients with consent who received cervical cancer screening. They consist of 63 normal, 167 low-grade SIL (LSIL), and 49 high-grade SIL (HSIL). All of 279 patients were Japanese women who visited Osaka Medical College, Kansai Medical College or Osaka Cancer Prevention Center in the past 5 years. Cervical cell samples from these patients were collected from the uterine ectocervix and the endocervical canal by cotton swabs, placed in phosphate-buffered saline, and stored at −20°C until use. Final histologic diagnosis was confirmed by colposcopy-directed biopsy for the patients with abnormal cytology.

Cell line

Eight human cervical squamous carcinoma cell lines (SKG-I, SKG-II, SKG-IIIa, SKG-IIIb, OMC-1, YUMOTO, QG-U, and QG-H) were also used for genotype analysis of Fas gene promoter −670 together with HPV typing. All cell lines were originating from Japanese women. The OMC-1 cell line [14] was established in our laboratory. The SKG-I [15], SKG-II [16], SKG-IIIa, and SKG-IIIb [17] cell lines were kindly provided by Dr. Shiro Nozawa, Keio University, Tokyo. The YUMOTO [18], QG-U, and QG-H [19] cell lines were kindly provided by Dr. Naotake Tanaka, Chiba University, Chiba. The SKG-I, SKG-II, SKG-IIIa, SKG-IIIb, and OMC-1 cell lines were maintained as monolayer cultures in Ham's F-12 medium (Flow Laboratories Inc., Irvine, Scotland) supplemented with 10% fetal bovine serum (Mitsubishi Chemical Co., Tokyo) at 37°C in a humidified incubator with 5% CO_2 in air. The YUMOTO, QG-U, and QG-H cell lines were cultured in RPMI-1640 medium (GIBCO BRL, Bethesda, MD) supplemented with 10% fetal bovine serum. The cells were grown in 75-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) and cell viability was determined by trypan blue dye exclusion prior to use.

DNA preparation

The exfoliated cervical cells or cell lines were disrupted with lysis buffer [20 mM NaCl, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.5% SDS, 50 μg/ml proteinase K], and genomic DNA was extracted with phenol-chloroform and precipitated with ethanol using standard techniques.

Genotyping of Fas gene promoter −670

Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) 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′-GGCTG-TCCATGTTGTGGCTGC-3′. 100 ng of the DNA template from each cell sample or cell line was amplified by PCR in a final volume of 50 μl reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM dNTP, 0.5 μM each primer, and 1.25 units Taq polymerase (Applied Biosystems, Branchburg, NJ) as previously described [20]. 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). The genotype was determined with A allele fragment length of 232 bp and G allele of 188 bp.

HPV typing

The presence of various HPV types was examined using L1-PCR according to the method reported by Nagano et al. [21]. Briefly, 100 ng of cellular DNA was subjected to PCR in the presence of published consensus primers (L1C1 and L1C2) [22]. Amplified HPV fragments were typed on the basis of the RFLP among HPVs. Initial typing of amplified HPV fragments was performed by digestions with *Rsa*I, *Dde*I, and then confirmed by digestions with several additional restriction enzymes as described previously [21,22]. L1-PCR can detect 22 registered low-risk (6, 11,

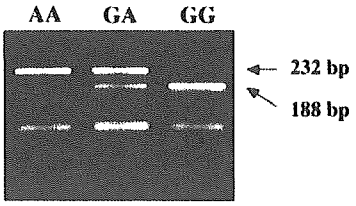

Fig. 1. Genotyping of Fas gene promoter −670 in exfoliated cervical cell samples by PCR-RFLP. The genotypes AA (232 bp), GA (188, 232 bp), and GG (188 bp) are shown.

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

To compare the HPV status and polymorphic features of Fas gene promoter −670 between normal, LSIL, and HSIL groups, chi-square test and Fisher's exact test were used. A level of $P < 0.05$ was accepted as statistically significant.

Results

Fig. 1 shows an example for genotyping of Fas gene promoter −670 in exfoliated cervical cell samples. The fragments of 232 and 188 bp indicated the AA and GG genotypes, respectively. The GA genotype contained these two bands.

Table 1 shows the frequency of high-risk HPV and Fas promoter −670 polymorphism in 279 samples examined. When AA genotype was compared to GA + GG genotype, 49 patients with HSIL had significantly higher frequency of high-risk HPV and GA + GG genotype than 167 with LSIL and 63 controls. G allele frequency was also higher in HSIL than in LSIL and controls. There was no statistical difference in the GA + GG genotype prevalence between SILs and controls among 183 patients without high-risk HPV as shown in Table 2. However, there was an increased OR (6.00; CI, 1.32–27.37; $P = 0.021$) for GA + GG genotype in HSIL cases compared to controls among 96 patients with high-risk HPV. There also appeared to be a trend toward decreased AA genotype from LSIL to HSIL in both groups (P test for trend <0.05).

Table 2
HPV status and frequency of Fas promoter −670 polymorphism in exfoliated cervical cell samples

Study group	*n*	Genotype at Fas promoter −670		OR	95% CI	*P* value
		AA	GA + GG			
High-risk HPV−						
Normal	53	15 (28.3%)	38 (71.7%)	1		
LSIL	121	36 (29.8%)	85 (70.2%)	0.93	0.44–1.95	0.847
HSIL	9	1 (11.1%)	8 (88.9%)	3.16	0.40–25.04	0.276
High-risk HPV+						
Normal	10	4 (40.0%)	6 (60.0%)	1		
LSIL	46	15 (32.6%)	31 (67.4%)	1.38	0.34–5.66	0.655
HSIL	40	4 (10.0%)	36 (90.0%)	6.00	1.32–27.37	0.021

As shown in Fig. 2, genotyping of Fas gene promoter −670 in 8 cervical squamous carcinoma cell lines revealed that AA genotype was detected only in the QG-U cell line, whereas the other 7 of 8 (87.5%) cell lines had GA or GG genotype. In addition, 7 of 8 cell lines except for YUMOTO were positive for high-risk HPV.

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 germline 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 also implicated in a number of diseases. SNP at −670 of Fas gene promoter (A/G) has been found with potentially different transcriptional efficiency [9,23]. Several studies addressed the association of this SNP with autoimmune diseases [9–11,23]. Recently, Lai et al. [12] conducted Fas promoter −670 polymorphism analysis using surgical and biopsy tissue specimens of cervical

Table 1
Frequency of high-risk HPV and Fas promoter −670 polymorphism in exfoliated cervical cell samples

Lesions	Number with high-risk HPV	Genotype frequency		Allele frequency	
		AA	GA + GG	A	G
Normal ($n = 63$)	10 (15.9%)[a]	19 (30.2%)	44 (69.8%)[b]	67 (53.2%)	59 (46.8%)[c]
LSIL ($n = 167$)	46 (27.5%)[d]	51 (30.5%)	116 (69.5%)[e]	165 (49.4%)	169 (50.6%)[f]
HSIL ($n = 49$)	40 (81.6%)[a,d]	5 (10.2%)	44 (89.8%)[b,e]	37 (37.8%)	61 (62.2%)[c,f]

[a] $P < 0.0001$ χ^2 vs. normal.
[b] $P = 0.0107$ χ^2 vs. normal.
[c] $P = 0.0217$ χ^2 vs. normal.
[d] $P < 0.0001$ χ^2 vs. LSIL.
[e] $P = 0.0043$ χ^2 vs. LSIL.
[f] $P = 0.0422$ χ^2 vs. LSIL.

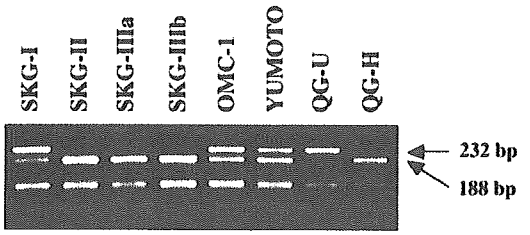

Fig. 2. Genotyping of Fas gene promoter −670 in 8 cervical squamous carcinoma cell lines by PCR-RFLP. The AA genotype was detected only for QG-U, whereas the GA genotype for SKG-I, OMC-1 and YUMOTO, and the GG genotype for SKG-II, SKG-IIIa, SKG-IIIb and QG-H cell lines, respectively.

neoplasm and reported that the frequency of A allele and AA genotype increased in accordance with the multi-step carcinogenesis from LSIL, HSIL to invasive squamous cell cancer. They stated that A allele and AA genotype, conferring an intact GAS element and more efficient Fas expression could be one of the mechanism that cells use to avoid carcinogenesis. In contrast, our present results using exfoliated cervical cell samples demonstrated that the frequency of GA + GG genotype or G allele increased from LSIL to HSIL. Moreover, there was an increased OR for GA + GG genotype in HSIL cases compared to controls among the patients with high-risk HPV. We also observed the opposite trend that AA genotype decreased in HSIL compared to LSIL and controls among the patients with or without high-risk HPV. Lai et al. [12] reported that HPV types 16 and 18, the most prevalent and aggressive types worldwide, are predominant in cases with GA or GG genotypes, whereas HPV type 58, prevalent in Southeast Asia, favors AA genotype. Very recently, Engelmark et al. [24] and Dybikowska et al. [25] have demonstrated that AA genotype in Fas gene promoter at −670 position may not be engaged in the development of cervical neoplasia in Swedish and Polish population, respectively. These discrepancies may be due to the ethnic variation of HPV prevalence and genotype frequency of Fas gene promoter in different geographical regions.

Previous studies [26,27] have demonstrated that high-risk HPV infection is inversely correlated with apoptosis of cervical epithelial cells and that a decrease of apoptosis is closely associated with higher histologic grade of SIL. In cervical cancer tissues and cell lines, significant decrease in the expression levels of Fas has been also reported [27,28]. The higher frequency of GA or GG genotype in HSIL cases in our series may result in a significant decrease in Fas gene expression and subsequent escape from apoptosis of the cells in high-risk HPV-related cervical carcinogenesis. Interestingly, 7 of 8 human cervical squamous carcinoma cell lines that possess high-risk HPV except for YUMOTO also showed GA or GG genotype. Further studies on the differential gene expression profiles between normal cervical keratinocytes and cervical cancer cell lines with or without G allele at −670 of Fas gene promoter may provide the better understanding for the effect of this SNP in the sequence of cervical carcinogenesis. Moreover, it might be of interest to further examine whether cultured cervical cancer cells with GA or GG genotype could escape from apoptosis in response to INF-γ stimuli through an abolishment of the GAS element and a decrease in the expression levels of Fas.

In the present study, we demonstrated the role of Fas gene promoter −670 polymorphism in cytologic materials or cell lines from women with premalignant or malignant cervical disease. Fas polymorphism may be closely associated with cervical carcinogenesis in a Japanese population particularly in high-risk HPV group. These observations are potentially important in managing SIL patients by cytologic examination and in understanding the pathogenesis of cervical cancer. It would be of interest to further evaluate whether this polymorphism could be used as a disease marker for the natural history of cervical neoplasias in a setting of longitudinal cohort study and for the determination of appropriate screening interval in patients with or without high-risk HPV.

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Lymph node pathway in the spread of endometrial carcinoma

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Summary

Objective: To elucidate the sentinel nodes of endometrial carcinoma, the spread pathway was clarified. The correlation between lymph node spread and other clinicopathological variables was also analyzed.

Methods: Dissected lymph node samples in 342 patients who underwent pelvic and selective paraaortic lymphadenectomy were reviewed. Pelvic and paraaortic node (PLN and PAN) status was compared with clinicopathological parameters.

Results: Lymph node metastasis was demonstrated in 52 patients, including 46 cases with PLN metastasis and six patients with independent PAN metastasis. The metastatic sites were most frequent in the obturator and internal iliac nodes. Eleven of 49 patients who underwent PAN dissection were positive for metastasis. Sixteen of 23 cases with parametrial metastasis also metastasized in the retroperitoneal lymph node.

Conclusion: The lymph node spread pathway in endometrial carcinoma consists of a major route via the obturator node or internal iliac node with or without parametrial involvement, and rarely a direct PAN pathway.

Key words: Endometrial carcinoma; Lymph node metastasis; Spread pattern; Prognostic factor; Staging laparotomy.

Introduction

The International Federation of Gynecology and Obstetrics (FIGO) has adopted surgical and pathological staging of endometrial carcinoma since 1988 [1]. In this classification, metastasis to the pelvic or paraaortic lymph node (PLN or PAN) should be staged as IIIc. This is based on the results of the Gynecologic Oncology Group (GOG) study, which reported PLN and PAN involvement in endometrial carcinoma in 9% and 5%, respectively [2]. Therefore, it is necessary to investigate lymph node status. Metastasis in PAN in endometrial carcinoma is reportedly more frequent than that in cervical carcinoma [2-5]. Recently, McMeekin *et al.* [6, 7] reported retroperitoneal lymph node metastasis, including PLN and PAN, in 8% of 607 patients of which 17% showed metastasis in PAN only, whereas Mariani *et al.* [8] reported that it accounted for 17.4% in 65 patients of which 7.7% was in PAN only. Many investigators have reported that patients with endometrial carcinoma had a poor prognosis if they revealed metastasis on PLN [9]. It is not, however, a reasonable method to dissect both PLN and PAN in all patients with endometrial carcinoma. Patients who should undergo lymphadenectomy of either PLN or PAN can be identified if spread patterns and pathways of lymph node metastasis in endometrial carcinoma are clarified. Holub *et al.* [10] tried to identify the sentinel node in endometrial carcinoma by using pre- or intraoperative dye and/or lymphoscintigraphy.

In this paper, we aimed to clarify the spread patterns of lymph node metastasis in endometrial carcinoma and analyze the correlation of lymph node metastasis with clinicopathological prognostic factors in a series of cases at Kitasato University Hospital.

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Patients and Methods

There were 342 patients with endometrial carcinoma who underwent complete surgical therapy, including systemic lymphadenectomy, treated during the period between 1971 and 1998. Radical hysterectomy in addition to bilateral salpingo-oophorectomy was basically performed for patients with clinical Stage II and III, and modified radical hysterectomy was done for those with Stage I. Pelvic lymphadenectomy was performed in all cases and PAN dissection was selectively performed for those who met the criteria such as enlarged PLN and PAN, myometrial invasion of more than one-third in the excised uterine specimen, adnexal metastasis, specific histological types including serous adenocarcinoma and clear cell adenocarcinoma, and positive peritoneal cytology.

Lymph node metastasis was carefully investigated and multiple metastatic lesions found in the same node region were classed as one metastasis. To investigate the spread pathway, the left or right pelvic nodes in each case were separately analyzed and expressed as a region calculated as a side. PAN was defined as one node. Metastasized nodes were mapped and the relationship among individual positive nodes was analyzed. In addition, relationships among lymph node status and various clinicopathological variables, including clinical stage, histopathological findings, myometrial invasion, lymphovascular space invasion (LVSI), cervical invasion, adnexal metastasis, parametrial involvement and peritoneal cytology were evaluated.

Statistical analysis was performed using the chi-square test and $p < 0.05$ was considered statistically significant.

Results

Incidence of lymph node metastasis

Among the 342 patients, 165 and 177 cases underwent modified radical hysterectomy and radical hysterectomy, respectively, and 46 were positive for PLN metastasis; Eleven of 49 who underwent both PLN and PAN dissection appeared positive, including six cases with positive PAN metastasis independently, without PLN metastasis. Consequently, 52 (15.2%) of 342 patients showed positive lymph node metastasis in PLN and/or PAN.

Analysis of positive node lesions

Ninety-nine nodes were positive in 52 patients, including 11 in PAN, 13 in the common iliac node, 19 in the external iliac node, 29 in the internal iliac node, 22 in the obturator node, four in the suprainguinal node and one in the sacral node. Single metastasis in unilateral PLN or PAN regions was found in 47 patients (55 sides), of which nine cases developed multiple node metastasis in the contralateral node regions. Multiple metastases were found in the bilateral sides in four cases and in the unilateral side in one case. As a result, multiple node metastasis was found in 14 cases (18 sides).

In 55 sides with single metastasis in unilateral PLN or PAN, lymph node metastasis was most frequent in the internal iliac and obturator nodes revealing 30.9%, followed by the external iliac node in 18.2% (Table 1).

Table 1. — *Single lymph node metastasis in 55 sides (47 cases) with endometrial carcinoma.*

	Metastatic nodes (%)
Paraaortic*	6 (10.9)
Common iliac	2 (3.6)
External iliac	10 (18.2)
Internal iliac	17 (30.9)
Suprainguinal	2 (3.6)
Obturator	17 (30.9)
Sacral	1 (1.8)
Total (sides)	55 (100.0)

*: without distinction of the side.

Forty-four nodes were positive in 14 cases that suffered multiple metastases in 18 sides of PLN and PAN. Metastasis in the internal iliac node and/or obturator node was found in 16 sides (88.9%). Metastasis in the internal iliac node was found on 12 sides, of which five and seven cases were also metastasized in the external and common iliac nodes, respectively. Of these, four cases metastasized into PAN with or without common iliac node metastasis. Metastasis in the obturator node, where single metastasis was frequently found, occurred on five sides, of which additional metastases were found in one of the internal iliac nodes and two in PAN. Additional metastasis, both in the external and the common iliac nodes was seen on two sides, one of which also metastasized in to PAN. Five (22.7%) of 22 sides with positive obturator nodes, 11 of 29 with positive internal iliac nodes and six of 19 with positive external iliac nodes had more metastasis in the distant cranial nodes, whereas only two with both negative obturator or internal iliac nodes were positive in the external iliac and/or suprainguinal node.

In 49 patients who underwent PLN and PAN dissection, 11 were positive for PAN metastasis. Thirteen cases were found to have metastasis in PLN, whereas 36 were not. The incidence of metastasis in PAN was 38.5% and 16.7%, respectively. Six cases that developed PAN metastasis without metastasis in PLN are listed in Table 2. Cancer lesions occupied the whole endometrium; there was also deeper myometrial invasion and frequent LVSI. No additional adnexal metastasis nor positive peritoneal cytology was determined.

Table 2. — *Cases with paraaortic node metastasis and without pelvic node metastasis.*

Case	Clin. Stage	Histology	Myometrial invasion	Cervical involvement	LVSI	Peritoneal cytology	Adnexal metastasis	Pm. metastasis
1	III	adenosq	outer 1/3	+	+	negative	–	–
2	II	clear cell	inner 1/3	+	–	negative	–	–
3	II	clear cell	middle 1/3	+	–	negative	–	–
4	II	G2 em	outer 1/3	+	+	negative	–	–
5	II	carcinoma	serosa	–	+	negative	–	+
6	III	G3 em	serosa	+	+	negative	–	–

Clin: clinical; LVSI: lymph vascular space invasion in the myometrium; Pm: parametrium; adenosq: adenosquamous cell carcinoma; clear cell: clear cell carcinoma; em: endometrioid adenocarcinoma; carcinosa: carcinosarcoma.

Parametrial metastasis was found in 23 of 342 cases (29 sides). Metastasis both in the parametrium and the lymph nodes was found in 16 cases (Table 3) and one of those 16 cases had a single metastasis in PAN. Ipsilateral PLN involvement was found in 13 cases or in 17 of 28 sides and contralateral node metastasis was also found in five sides. In two cases (2 sides), metastasis was found in only the contralateral lymph node. Among 17 sides with ipsilateral involvement, 14 were found to have metastasis either in the internal iliac node or obturator node. Single metastasis in PLN was recorded in nine sides with ipsilateral parametrial involvement, and seven were involved either in the internal iliac node or obturator node.

Table 3. — *Lymph node state of 15 patients with both parametrial and pelvic lymph node metastasis.*

	Number	Sacral	Suprainguinal	Obturator	Internal iliac	External iliac	Common iliac
Ipsilateral	17 sides*	1	2	3 (2)	11 (5)	4 (1)	6 (1)
Contralateral	7 sides**	–	1	2 (2)	4 (2)	3	2

( ): case number with single node metastasis; *: three sides had both pelvic and paraaortic node metastasis; **: one side had both pelvic and paraaortic node metastasis.

Correlation with clinicopathological parameters

Lymph node metastasis was statistically higher in patients with advanced clinical stage, unusual histologic type including adenosquamous cell carcinoma, higher grade of endometrioid carcinoma, deeper myometrial invasion, LVSI, cervical invasion, adnexal metastasis, parametrial involvement and positive peritoneal cytology (Table 4).

Discussion

In this study series, 15.2% of patients with endometrial carcinoma developed retroperitoneal lymph node metastasis. The most frequent metastatic single region was the internal iliac node and obturator node in 61.8%, and multiple metastasis also involved either of these nodes in 88.9%. Frequent lymph node metastasis in the internal and external iliac nodes has been reported in the literature [11]. However, we speculate from our results that metastasis originates either in the internal iliac or obturator node region and spreads further to the cranial and distant lymph nodes (Table 2). Therefore, the internal iliac and obturator nodes could be sentinel nodes of endometrial carcinoma.

Parametrial metastasis in endometrial carcinoma has a poorer prognosis [12]. Metastasis either in the internal

Table 4. — *Frequency of lymph node metastasis in 342 patients with endometrial carcinoma.*

		Overall	Positive (%)		p value
Clinical stage	I	176	13 (7.4)		< 0.01
	II	157	33 (21.0)		
	III	9	6 (66.7)		
Histological type	Endometrioid	276	32 (11.6)	*, **	
	Adenocanthoma	24	4 (16.7)		
	Adenosquamous	9	5 (55.6)	*	
	Serous	9	2 (22.2)	**	
	Mucinous	7	1 (14.3)		
	Clear	6	3 (50.0)		
	Carcinosarcoma	11	5 (45.4)		
Grade (Endometrioid)	G1	139	11 (7.9)		n.s
	G2	109	17 (15.6)		
	G3	28	4 (14.3)		
Myometrial invasion	Intra-endometrial	69	0		< 0.01
	Inner 1/3	142	8 (5.6)		
	Middle 1/3	63	9 (14.3)		
	Outer 1/3	56	26 (46.4)		
	Serosa	12	9 (75.0)		
LVSI	Positive	106	42 (39.6)		< 0.01
	Negative	236	10 (4.2)		
Cervical invasion	Positive	82	30 (36.6)		< 0.01
	Negative	260	22 (8.5)		
Adnexal metastasis	Positive	19	9 (47.4)		< 0.01
	Negative	323	43 (13.3)		
Parametrial metastasis	Positive	23	16 (69.6)		< 0.01
	Negative	319	35 (11.0)		
Peritoneal cytology	Positive	45	11 (24.4)		< 0.05
	Negative	212	26 (12.3)		

LVSI: lymph vascular space invasion in the myometrium; *, **: $p < 0.01$.

iliac or obturator node was highly correlated with that in the parametrium in 61.1%. Consequently, there may be a pathway via lymphatic lesions in the parametrium in addition to direct spread to PLN.

Direct spread to PAN was observed in 16.7% as well as the pathway via the pelvic lymph node in 38.5 in this study. When 293 patients who were not indicated to undergo PAN dissection were calculated as negative, the incidence of PAN metastasis was 1.8%, similar to that reported by Mariani *et al.* [8]. The incidence of metastasis both in PLN and PAN was reported to be 3% to 16% [2, 5], and that in PAN without coexisting pelvic node metastasis was from 0% to 2% [2, 5]. Thus, there may be two spread patterns to PAN, including a major pathway via PLN, and a rare direct pathway.

Lymph node metastasis in endometrial carcinoma is correlated with clinical stage, histological type of the carcinoma, grade of endometrioid adenocarcinoma, myometrial invasion, LVSI, cervical invasion, adnexal metastasis, parametrial involvement and positive peritoneal cytology. Correlations with these clinicopathological variables have been reported in the literature [2, 5, 13, 14]. However, Creasman *et al.* [2] and Girardi *et al.* [13] reported that lymph node metastasis was not correlated with histological type. Four cases (4%) of endometrial carcinoma with pelvic lymph node metastasis and without myometrial invasion were reported by Takeshima *et al.* [15]. Boronow *et al.* [3] also denied a link between the grade of endometrioid adenocarcinoma. Creasman *et al.* [2] demonstrated that positive peritoneal cytology in endometrioid carcinoma was correlated with metastasis both at PLN and PAN, whereas in our series PAN metastasis was not correlated with positive cytology (data not shown). Lymph node metastasis was found in 69.9% of the cases with parametrial involvement in our series, which was significantly higher than the 11.0% of parametrial negative patients (Table 4). Tammusino *et al.* [16] reported supporting data with a limited number of 24 cases.

In cases of clinical Stage I, well-differentiated adenocarcinoma and shallow myometrial invasion, and sentinel node dissection of the internal iliac and obturator nodes could be substituted for total systemic PLN and PAN dissection.

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Small-cell carcinoma of the uterine cervix: a clinicopathologic study of 11 cases

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Abstract. Tsunoda S, Jobo T, Arai M, Imai M, Kanai T, Tamura T, Watanabe J, Obokata A, Kuramoto H. Small-cell carcinoma of the uterine cervix: a clinicopathologic study of 11 cases. *Int J Gynecol Cancer* 2005; 15:295–300.

We report the clinical profiles and immunohistochemical features of small-cell carcinoma of the uterine cervix. Eleven cases that we have encountered at the Department of Gynecology, Kitasato University Hospital, between 1971 and 2003 are presented. Of 1370 invasive carcinomas of the uterine cervix, the incidence of small-cell carcinoma was 0.8%. Patient ages ranged between 32 and 65 years, with a mean age of 46.3 years. The clinical stages at diagnosis were Ib in four patients, IIb in three, IIIb in three, and IVb in one. All patients presented with abnormal vaginal bleeding. Two patients who are alive with no evidence of disease for 12 years and 3 years 6 months, while eight patients died of primary carcinoma between 4 and 25 months after treatment. Histopathologic findings showed solid nests with marked peripheral palisading pattern and rosette formation. Small tumor cells with scant cytoplasm demonstrated a very high nuclear/cytoplasm ratio and indistinct cell borders. The nuclei were round to oval and demonstrated increased but fine granular chromatin. Nucleoli were indistinct in all cases. Immunohistochemical findings were positive in 81.8% each for neuron-specific enolase and protein gene product 9.5, 72.7% for synaptophysin, 63.6% for chromogranin A, and 54.5% for neural cell adhesion molecule. All specimens were positive for at least one of the above. In conclusion, small-cell carcinoma of the uterine cervix revealed poor prognosis. Making an accurate diagnosis of small-cell carcinoma before performing treatment is of great significance but often difficult. Immunohistochemical analysis using several kinds of neuroendocrine markers is helpful in establishing the correct diagnosis in addition to focusing on characteristic histo- and cytopathologic features.

KEYWORDS: immunohistochemical study, neuroendocrine feature, small-cell carcinoma, uterine cervix.

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Small-cell carcinoma is a tumor commonly seen in the lung, with characteristic histologic findings, but it has been reported to develop in many other organs, including the stomach, rectum, breast, and ovary[1–4]. Rarely, it develops in the uterine cervix[5–10] and is known to metastasize to the lymph nodes within the early stage, promoting a poor prognosis. The term small-cell carcinoma was not used in the General Rules of Clinical and Pathological Management of the Uterine Cervix[11] published in 1982, and the disease was classified as undifferentiated carcinoma. However, because of the poor prognosis due to rapid clinical progression as well as characteristic histopathologic findings, it has been classified as an independent disease under the name small-cell carcinoma in the General Rules of Clinical and Pathological Management of the Uterine Cervix revised in 1997[12]. However, clinical and pathologic features of small-cell carcinoma of the uterine cervix have not yet been fully elucidated due to its low incidence. Therefore, we report small-cell carcinoma of the uterine cervix in a series of 11 cases, focusing on the clinicopathologic, histopathologic, and cytopathologic characteristics, including immunohistochemical features.

Materials and methods

Between July 1971 and December 2003 at the Department of Gynecology, Kitasato University Hospital, the tumor registry recorded 1370 patients with invasive carcinoma of the uterine cervix, including 11 cases of small-cell carcinoma, indicating an incidence of 0.8%. The clinical profiles of these cases, including age, symptoms, stage, treatment methods, and prognosis, were investigated. Patients were clinically staged according to the FIGO classification. The follow-up period ended in December 2003 or when patients died. One case was transferred to other hospital and was lost to follow-up.

The diagnosis was made based on histologic criteria for small-cell carcinoma in the General Rules of Clinical and Pathological Management of the Uterine Cervix[12], using pathologic specimens prepared by hematoxylin–eosin stain. Paraffin-block embedded specimens after formalin fixation were sectioned 3 μm thick. Additional specimens were placed for immunohistochemical search, with informed consent. Immunohistochemical stainings were performed using the labeled streptavidin–biotin method with the following antibodies: mouse anti-human neuron-specific enolase (NSE) monoclonal antibody (DAKO, Glostrup, Denmark; 1:1000), rabbit anti-human chromogranin A polyclonal antibody (DAKO; 1:200), rabbit anti-human synaptophysin polyclonal antibody (DAKO; 1:100), mouse anti-cd-56 (neural cell adhesion molecule [NCAM]) monoclonal antibody (Nihonkayaku, Tokyo, Japan; 1:400), and rabbit anti-human protein gene product 9.5 (PGP9.5) polyclonal antibody (Ultraclone, Cambridge, UK; 1:100).

The positivity criteria were as follows: −, when less than 5% of cells were stained; +, when 5–25% of cells were stained; ++, when 25–50% of cells were stained; and +++, when more than 50% of cells were stained.

Results

Patient ages ranged widely between 34 and 65 years, with a mean age of 46.3 years. All patients presented with abnormal vaginal bleeding. Presumptive diagnosis of small-cell carcinoma was made by cytology in only two cases (18.1%), although findings were positive for malignant cells in all cases. Likewise, preoperative histologic examination accurately diagnosed small-cell carcinoma in only four cases (36.4%) that we encountered recently. Three patients were diagnosed as having adenocarcinoma and three as having small-cell nonkeratinizing squamous cell carcinoma (SCC). Four patients were clinically staged in Ib, three in stage IIb, three in IIIb, and one in IVb with liver and bone metastases (Table 1). None of the patients demonstrated any clinical evidence of abnormal hormone production. Four patients with stage Ib and two with stage IIb underwent radical hysterectomy and bilateral salpingo-oophorectomy with pelvic lymphadenectomy. Postoperative chemotherapy (four to six courses of cisplatin 50 mg/m^2 and etoposide 100 mg/m^2) was given to two patients in stage Ib. Radiation therapy (Linac 50Gy) was given postoperatively to

Table 1. Clinical features of the patients with small-cell carcinoma of the uterine cervix

Case	Age	Stage	Therapy	Prognosis
1	34	Ib	RH	DOD at 12 months
2	47	Ib	RH + PE	A & W at 144 months
3	55	Ib	RH + PE	A & W at 44 months
4	32	Ib	RH	Lost to follow-up[a]
5	50	IIb	RH + radiotherapy	DOD at 14 months
6	52	IIb	RH + radiotherapy	DOD at 16 months
7	48	IIb	SIP[b] + SH + PE	DOD at 11 months
8	65	IIIb	Radiotherapy + PP	DOD at 9 months
9	36	IIIb	PE + RH	DOD at 13 months
10	42	IIIb	PE + radiotherapy	DOD at 7.6 months
11	48	IVb	Radiotherapy + PP	DOD at 4 months

RH, radical hysterectomy; SH, simple hysterectomy; PE, cisplatin + etoposide; PP, cisplatin + peplomycin; SIP, nedaplatin + ifosfamide + peplomycin; DOD, died of disease; A & W, alive and well.
[a]Transferred to other hospital.
[b]Treated with two courses of chemotherapy preoperatively.

two patients in stage IIb. One patient in stage IIb with coexisting SCC underwent simple hysterectomy and bilateral salpingo-oophorectomy, followed by chemotherapy (two courses of nedaplatin 80 mg/m^2/day 1, ifosfamide 1.5 g/body/days 1–5, and peplomycin 5 mg/body/days 1–6). Two patients in stage IIIb and one in IVb received radiation therapy (Linac 50Gy and ^{60}CO-RALS 30Gy in the vagina) in combination with chemotherapy (two to three courses of cisplatin 50 mg/m^2 and peplomycin 5 mg/body/day). One patient with stage IIIb underwent radical hysterectomy and right salpingo-oophorectomy, followed by chemotherapy (three courses of cisplatin 50 mg/m^2 and etoposide 100 mg/m^2). Of the 11 patients, including one who was lost to follow-up, only two with stage Ib are currently alive without any symptoms of recurrence, and both had received adjunctive chemotherapy postoperatively. These two patients have survived for 12 years and 3 years 6 months. Eight patients died of primary cancer between 4 and 25 months, with a mean surviving interval of 13.5 months. Four of seven patients who had undergone surgery developed recurrence, and the relapsing sites included the pelvic cavity in one patient (case 1), brain in two (cases 6 and 9), and multiple organs, including liver, kidney, and lung, in one (case 5). The interval before recurrence was 0.5–12 months. Recurrences were treated with radiation therapy and chemotherapy, including cisplatin and etoposide, none of which was effective and patients proceeded rapidly to death due to primary tumor. Four patients in stage III or IV who received combined chemoradiation therapy responded poorly and died between 4.4 and 13 months without a disease-free period.

Tumors in the extirpated uterus ranged from 3 to 4 cm in diameter. Histopathologic findings demonstrated marked lymph vascular space invasion in six of seven patients (Table 2). Metastasis to the obturator node was observed in two cases in stage IIb and that to the para-aortic node in one patient in stage IIb. Lymph node metastasis was not found in any patients in stage Ib.

Hematoxylin-eosin–stained specimens showed small tumor cells forming solid nests of various sizes with diffuse infiltrative proliferation (Fig. 1). A peripheral palisading pattern around the border of the nests was a typical feature (Fig. 1). In 9 of the 11 cases, rosette formation was observed (Fig. 1). The tumor cells were small with scant cytoplasm, showing a high nuclear/cytoplasm ratio and indistinct cell borders. The nuclei were round to angulated, with increased fine granular chromatin (Fig. 2). Nucleoli were inconspicuous in all cases. Mitosis is frequently observed, demonstrating more than 10 mitotic figures per 10 high-power fields. In addition to small-cell carcinoma,

Table 2. Pathological findings of the extirpated uterus

Case	Coexisting lesion	Lymphovascular space invasion	Metastasized lymph node
1	Adenocarcinoma	–	–
2	Squamous cell carcinoma	+	–
3	—	+	–
4	—	+	–
5	—	+	Right obturator
6	Squamous cell carcinoma	+	Bilateral obturator
7	Adenocarcinoma, squamous cell carcinoma	+	Para-aortic

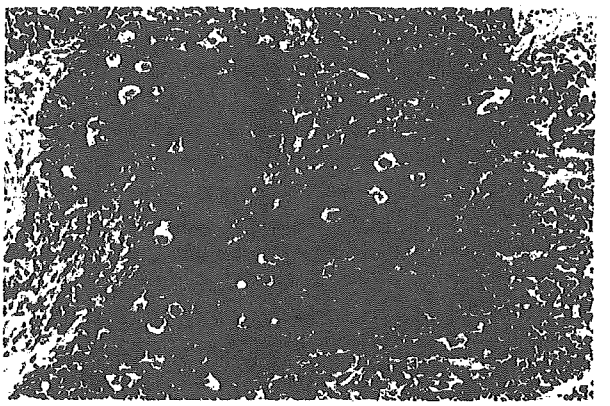

Figure 1. Small and large cancer nests with rosettes formation and peripheral palisade arrangement (H&E, 5 × 4).

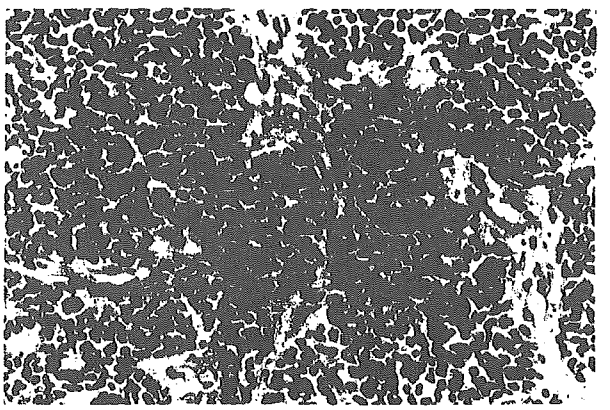

Figure 2. Tumor cells arranging in solid sheets with round, hyperchromatic nuclei and scanty cytoplasm (H&E, 5 × 20).

Table 3. Immunohistochemical results

Case	NSE	NCAM	PGP9.5	Chromogranin A	Synaptophysin
1	+++	+++	+++	+++	++
2	+	−	++	++	−
3	++	+	++	−	++
4	−	++	++	++	+++
5	++	-	−	++	++
6	++	−	++	++	−
7	+	++	+	−	++
8	++	++	++	++	+
9	−	++	++	−	++
10	+++		++	++	++
11	+++	−	−	−	

NCAM, NCC-Lu-243.

SCC coexisted in two cases, adenocarcinoma in one, and SCCand adenocarcinoma in one.

Immunohistochemical findings are shown in Table 3. Nine cases (81.8%) showed immunoreactions for NSE and PGP9.5, eight (72.7%) for synaptophysin, seven (63.6%) for chromogranin A, and six (54.5%) for NCAM. All specimens were positive for either of these neuroendocrine markers (Fig. 3).

Discussion

Small-cell carcinoma of the uterine cervix was first reported under the name carcinoid by Albores-Saavedra *et al.*[13] in 1972. Thereafter, various authors have reported on this entity as a tumor-expressing neuroendocrine differentiation[5–10,14] and numerous names besides carcinoid, eg, small-cell carcinoma, argyrophil cell carcinoma, small-cell tumor with neuroendocrine feature, neuroendocrine carcinoma, small-cell undifferentiated carcinoma, and small-cell neuroendocrine carcinoma. In Japan, it has been classified as small-cell carcinoma and distinguished from undifferentiated carcinoma in the General Rules of Clinical and Pathological Management of the Uterine Cervix in the 1997 revision[12].

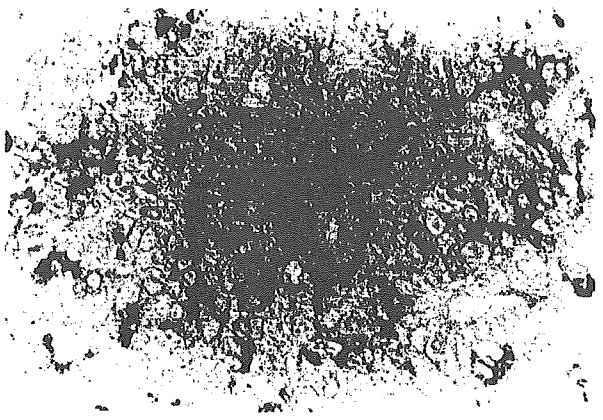

Figure 3. Immunohistochemically positive for chromagranin A (× 160).

The incidence of small-cell carcinoma is very low in 0.31–2% of the invasive carcinoma of the uterine cervix[5–10,15]. In our experience of 1 370 cases of invasive carcinoma, only 11 were small-ell carcinomas, showing an incidence of 0.80%. According to the literature, patient ages range widely from the 2nd to the 10th decade of life[5–8,10,15–22], with mean ages mostly in the forties. A mean age of 46.3 years in our study is consistent with previous reports. Ambros *et al.*[23] and Sheridan *et al.*[21] have reported that small-cell carcinoma is characterized by a number of cases showing juvenile onset. Sixty percent of patients in our series were in their thirties or forties.

Abnormal vaginal bleeding is the most common symptom, as being reported to comprise all cases[10,17,19–21]. Asymptomatic cases are very rare, 5.5%[17] and 6.7%[19]. The prognosis of patients with small-cell carcinoma is considered unfavorable[9,18–22] because of its high rate of lymph node metastasis[21] and early systemic metastasis, including to the lung[18,20]. In our study, only two patients in stage Ib currently remain alive without evidence of disease. Sevin *et al.*[24] reported that the overall 5-year disease-free survival rate of 12 patients, including 1 in stage Ia, 10 in stage Ib, and 1 in stage IIa, was 36.4%, which was significantly lower than that of 71.6% for other histologic types of carcinoma of the cervix.

Albores-Saavedra *et al.*[25] have classified neuroendocrine tumors of the uterine cervix into four categories: the typi3cal carcinoid, atypical carcinoid, large-cell neuroendocrine tumor, and small-cell carcinoma. The criteria for small-cell carcinoma are as follows. (1) Tumor cells are small round or fusiform, with scanty cytoplasm. (2) Nuclear chromatin is hyperchromatic and finely granular. Nucleoli are inconspicuous with nuclear molding. (3) The neoplastic cells may grow in a diffuse manner or may be arranged in nests, trabeculae, or cords. Peripheral palisading and a prominent perivascular concentration of cells are often seen. (4) Necrosis is a constant feature. The coexistence of adenocarcinoma or SCC is reported in 21–77% of the cases[9,17–20,26].

Immunohistochemically, tumor cells are positive for neuroendocrine markers. We performed an immunohistochemical study using NSE, NCAM, PGP9.5, chromogranin A, and synaptophysin. Although there were differences in positivity rates of antibodies, all cases reacted to at least one of the five neuroendocrine

markers. Thus, we can immunohistochemically verify the presence of cells with neuroendocrine differentiation. Similar results were reported in the literature[5,9,17–19]. However, Albores-Saavedra *et al.*[25] indicated that not all of the neuroendocrine markers need to be present to make the diagnosis because 60% of small-cell carcinomas are negative for chromogranin A and synaptophysin and 30% for NSE. Yamawaki *et al.*[9] have also reported that small-cell carcinoma can be diagnosed when tumor cells are positive for two or more of the neuroendocrine markers such as chromogranin A, NSE, and grimelius. In addition, Ambros *et al.*[23] indicated that small-cell carcinoma may be diagnosed when tumor cells are positive for neuroendocrine markers such as NSE, chromogranin A, and synaptophysin, and negative for keratin. Likewise, immunohistochemistry may be considered highly useful in diagnosing such confusing cases[16].

It is often difficult[21] to make a preoperative diagnosis of small-cell carcinoma. In our study, only 2 of the 11 patients had an accurate diagnosis of small-cell carcinoma by cytology and four by preoperative histology. Kim *et al.*[17] reported that histologic type could be presumed by cytology in 79% of 18 cases of small-cell carcinoma based on the findings, including nuclear molding, nuclear smearing effect, salt and pepper chromatin pattern with minimal cytoplasm, and cell clusters without a typical architectural pattern. Furthermore, inconspicuous nucleoli and isolated or loose cell aggregates are also described as cytologic features of small-cell carcinoma[9,27]. As Zhou *et al.*[28] have pointed out, it is often important in clinical practice to consider the differential diagnosis from follicular cervicitis, endometrial cells, adenocarcinoma of the uterine cervix, small-cell type SCC, and lymphoma.

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Clinical Studies

Phase II trial of docetaxel in advanced or metastatic endometrial cancer: a Japanese Cooperative Study

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The purpose of this study was to determine whether docetaxel has antitumour activity in patients with advanced or recurrent endometrial carcinoma. Chemotherapy-naïve or previously treated patients (one regimen) with histopathologically documented endometrial carcinoma and Eastern Cooperative Oncology Group performance status ≤2 entered the study. Docetaxel 70 mg m^{-2} was administered intravenously on day 1 of a 3-week cycle up to a maximum of six cycles. If patients responded well to docetaxel, additional cycles were administered until progressive disease or unacceptable toxicity occurred. Of 33 patients with a median age of 59 years (range, 39–74 years) who entered the study, 14 patients (42%) had received one prior chemotherapy regimen. In all, 32 patients were evaluable for efficacy, yielding an overall response rate of 31% (95% confidence interval, 16.1–50.0%); complete response and partial response (PR) were 3 and 28%, respectively. Of 13 pretreated patients, three (23%) had a PR. The median duration of response was 1.8 months. The median time to progression was 3.9 months. The predominant toxicity was grade 3–4 neutropenia, occurring in 94% of the patients, although febrile neutropenia arose in 9% of the patients. Oedema was mild and infrequent. Docetaxel has antitumour activity in patients with advanced or recurrent endometrial carcinoma, including those previously treated with chemotherapy; however, the effect was transient and accompanied by pronounced neutropenia in most patients.

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Most patients with endometrial cancer are diagnosed at an early stage when surgery alone may result in cure. However, the outcome for women with advanced stage or recurrent disease is poor and rarely curable. Both single-agent and combination regimens of chemotherapy have been studied in women with advanced endometrial carcinoma. Currently, no standard chemotherapy regimen for endometrial cancer exists, but single-agent doxorubicin is active, with responses observed in up to one-third of previously untreated patients (Moore *et al*, 1991). Other single agents with modest activity include cisplatin (Thigpen *et al*, 1984a, 1989) and carboplatin (van Wijk *et al*, 2003). Although the response rates with the combination doxorubicin–cisplatin appear to be higher than those achieved with either agent alone, there is no evidence that survival is any longer with combination therapy. In the Gynecologic Oncology Group (GOG) trial comparing doxorubicin alone with doxorubicin–cisplatin, the response rates and progression-free survival were better with the combination regimen (42 *vs* 25%, 5.7 *vs* 3.8 months, respectively), but overall survival (OS) had not significantly improved (Thigpen *et al*, 2004).

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The taxanes, paclitaxel and docetaxel, are potent chemotherapeutic agents that block tubulin depolymerisation, leading to the inhibition of microtubule dynamics, and have significant clinical efficacy for various solid tumours. Paclitaxel has been evaluated as an active agent for endometrial cancer (Ball *et al*, 1996; Lissoni *et al*, 1996; Lincoln *et al*, 2003). However, preclinical data show that docetaxel has increased potency and an improved therapeutic index compared with paclitaxel (Bissery *et al*, 1995), and its short 1-h infusion time offers a substantial clinical advantage over the prolonged infusion durations required with paclitaxel. Docetaxel and paclitaxel also have substantially different toxicity profiles. In particular, docetaxel has a significant lower incidence of neurotoxicity in comparison to paclitaxel (Hsu *et al*, 2004).

The present phase II trial was designed to evaluate the clinical efficacy and tolerability of docetaxel 70 mg m^{-2} in patients with advanced or recurrent endometrial cancer.

PATIENTS AND METHODS

Eligibility criteria

Eligible patients aged between 20 and 74 years, with a life expectancy in excess of 3 months, and Eastern Cooperative

Oncology Group (ECOG) Performance Status (PS) of 0–2 had histologically documented primary stage III, IV or recurrent endometrial carcinoma. Tumours were staged according to the International Federation of Gynecology and Obstetrics criteria. All patients had measurable disease according to the response evaluation criteria in solid tumours (RECIST) (Therasse *et al*, 2000). Measurable lesions defined unidimensionally were $\geqslant 20$ mm using conventional imaging, or $\geqslant 10$ mm with spiral computed tomographic scan. Patients were either chemotherapy-naive or had received one prior chemotherapy regimen for endometrial cancer, with 4 weeks between prior therapy and study treatment. Prior treatment with a taxane was not allowed. Adequate organ function was required for study entry: neutrophil count $\geqslant 2000\,\mu l^{-1}$, platelet count $\geqslant 100\,000\,\mu l^{-1}$, haemoglobin $\geqslant 9.0\,g\,dl^{-1}$, serum bilirubin level $\leqslant 1.5\,mg\,dl^{-1}$, normal hepatic function (asparate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels $\leqslant 2.5$ times upper limit of the institutional normal (ULN)), serum creatinine level $\leqslant 1.5\,mg\,dl^{-1}$, PaO_2 $\geqslant 60$ mmHg and normal electrocardiogram. Patients with any of the following conditions were excluded from the study: sarcoma component, active infection, severe heart disease, interstitial pneumonitis, past history of hypersensitivity, peripheral neuropathy, malignant or benign effusions requiring drainage, active brain metastasis, or active concomitant malignancy. All patients gave informed consent before entering this study, which was approved by the institutional review boards at all participating institutions.

Treatment schedule

Docetaxel $70\,mg\,m^{-2}$ was infused over a 1-2-h period. The treatment was repeated every 3 weeks unless there was documented disease progression or unacceptable toxicity. Prophylactic medications for nausea, vomiting or hypersensitivity reactions were given if these symptoms occurred. No routine premedication was given for hypersensitivity reactions and fluid retention during the first cycle of treatment. The patient's physician identified all hypersensitivity reactions and, if deemed necessary, the investigator administered premedication drugs.

Treatment was delayed for up to 3 weeks in the event of toxicity, but was restarted when the neutrophil count was $\geqslant 1500\,\mu l^{-1}$, platelet count $\geqslant 100\,000\,\mu l^{-1}$, AST/ALT/ALP levels $\leqslant 2.5$ times ULN, and neuropathy or oedema $\leqslant$ grade 1. Docetaxel dosage was reduced by $10\,mg\,m^{-2}$ if febrile neutropenia occurred, if there was bleeding with grade 3–4 thrombocytopenia requiring a platelet transfusion, or if a patient experienced any grade 3–4 nonhaematologic toxicities except nausea, vomiting, anorexia, fatigue, alopecia or hypersensitivity.

Response and toxicity evaluation

The tumour response was assessed according to the standard RECIST criteria (Therasse *et al*, 2000). Target lesions included all measurable lesions up to a maximum of five lesions per organ and 10 lesions in total. Complete response (CR) was defined as the complete disappearance of all target and nontarget lesions, with no development of new disease. Partial response (PR) was defined as a reduction by $\geqslant 30\%$ in the sum of the longest diameter of target lesions. Complete response or PRss were confirmed by repeat assessments performed no less than 4 weeks after the criteria for response were first met. Progressive disease (PD) was defined as an increase by $\geqslant 20\%$ in the sum of the longest diameter of all target lesions, or the appearance of one or more new lesions and/or unequivocal progression of existing, nontarget lesions. Stable disease (SD) was defined as neither sufficient lesion shrinkage to qualify for a PR, nor sufficient increase to qualify for PD. Best response was defined as the most CR achieved by a patient (thus, each patient had a single best response: CR, PR, SD or PD), and the date of best response was the date it was first detected. Time to progression (TTP) was defined as the time from the first medication to the date of a PD event or death (due to endometrial cancer or study drugs). All tumours were radiographically assessed for response every 6 weeks. An independent response review committee (IRRC) evaluated all tumour responses after the investigators had completed their judgement.

Toxicities were evaluated with respect to incidence and severity using National Cancer Institute common toxicity criteria (NCI-CTC, version 2.0) (Trotti *et al*, 2000).

Statistical consideration

Assuming a response rate of 20%, the study was designed with 80% power such that the lower limit of the 95% confidence interval (CI) for the estimate of the response rate was greater than 0.05. A sample size of 32 evaluable patients was required.

The primary end point was overall tumour response (determined by the IRRC) with the corresponding 95% CI using the exact binominal method for the evaluable population. The secondary end point of this study was safety. The Kaplan–Meier (KM) method was used to determine the TTP and median survival time (MST) in the evaluable population.

RESULTS

Patient characteristics

A total of 33 patients were enrolled on the study from April 2001 to October 2003 and one patient was unevaluable as a result of having received prior treatment with paclitaxel and doxorubicin–platinum regimens. The median age of the intent to treat (ITT) population ($n = 33$) was 59 years (range 39–74) and 70% patients had ECOG PS 0 (Table 1). Several patients had unfavourable histologic characteristics: adenosquamous features (three) and uterine papillary serous cancers (two). Most patients (88%) had undergone total abdominal hysterectomy and bilateral salpingo-oophorectomy, and one-third of patients had prior radiotherapy. Of those patients who had received prior chemotherapy ($n = 14$), 10 had received combination doxorubicin–platinum in combination, three had received platinum alone and one had received oral fluorouracil. All 33 patients were evaluated for toxicity and survival, while 32 patients were evaluated for response and TTP.

Treatment delivery

Overall, 32 patients received a total of 133 cycles of docetaxel and the median number of cycles of docetaxel was four (range, 1–13). Five patients (15%) experienced dose reductions for the following reasons: two patients experienced febrile neutropenia (in one patient this occurred twice) and three patients had grade 3 nonhaematologic toxicities: diarrhoea (occurred twice in one patient), hyperglycaemia, hyperkalaemia and supraventricular tachycardia.

Response

Table 2 presents the assessment of response to treatment. Two patients, one who was chemotherapy-naïve and the other who had received prior therapy, were not assessable for response because they had received only one cycle of treatment. Before evaluation by the IRRC, primary physicians had reported two CRs and nine PRs. The IRRC judged one CR as a PR, two PR as SD and one SD as a PR. Therefore, the overall response rate for 10 of 32 patients was 31% (95% CI, 16.1–50.0%). Of 13 patients who had prior chemotherapy, three (23%) achieved a PR: two had received doxorubicin–platinum and one platinum alone. The histologic analysis revealed responses among the following tumour types: