total of 248 chromosomes from 124 postmenopausal Japanese women. Figure 1 indicates the location of each SNP analyzed in this study. All SNPs exist within the exon, thus resulting in amino acid substitution.

Although the genotypic distribution of I462V in the *CYP1A1* gene was in Hardy-Weinberg equilibrium, those of R48G and L432V in the *CYP1B1* gene were observed to deviate from Hardy-Weinberg equilibrium. The frequencies of the variant SNP alleles ranged from 19% to 23%. There were no variant alleles in four SNPs (G45D, A463G, A119S, and N453S [*CYP1B1*]) in the population analyzed in this study (Table 1). In addition, no significant differences were observed in the baseline characteristics with any genotypes tested in this study (Table 2). No significant differences were observed in either the baseline characteristics or the response to HT (data not shown).

To test whether these three exon SNPs might be involved in the response to HT, the percentage of changes in the lumbar BMD and the serum lipid profiles after HT were compared according to each genotype of the CYP genes (Table 3). The genotype L432V in the *CYP1B1* gene demonstrated significant associations with lumbar BMD and low-density lipoprotein cholesterol (LDL-C) responses after 12 months of HT. Neither the genotype I462V (*CYP1A1*) nor R48G (*CYP1B1*) demonstrated a significant association with the lumbar BMD or the serum lipid responses. The mean change in the BMD of all women after 12 months of treatment was 2.3 ± 0.5%. Although the absolute value of the BMD did not show any significantly difference among the different genotype groups, the participants with the homozygous (variant) genotype (GG) of L432V showed significantly less BMD change (−3.7 ± 2.4%) than those with the heterozygous (CG; 1.8 ± 1.0%) and homozygous (wild type) (CC; 3.4 ± 0.6%) genotypes. The serum LDL-C level of all women decreased (−13.5 ± 2.7%) after 12 months of treatment. In the women with the heterozygous (CG) and homozygous (CC; wild type) genotypes of L432V, the LDL-C level decreased, whereas that in women with the homozygous (variant) genotype (GG) of L432V inversely increased (11.1 ± 3.5%) after 12 months of treatment.

In the univariate analysis, some factors, other than the L432V polymorphism, significantly influenced the lumbar BMD and LDL-C responses. For example, with older age and a higher baseline BMD, there was less increase in BMD response to HT, and with a higher baseline LDL-C, there was less decrease in LDL-C. Body weight and BMI did not influence those responses to HT.

Finally, the effect of the L432V genotype on the responses of lumbar BMD and LDL-C were maintained after adjustment for the significant variables in the univariate analysis (Table 4). This confirms the independent effect of the L432V polymorphism in the *CYP1B1* gene on the response to HT.

To evaluate the relationship between the L432V SNP and the circulating hormone levels, serum estradiol, LH, and FSH levels after 12 months of HT were compared among the genotypes of L432V. Although the serum levels of estradiol and LH did not show any significant differences, the serum

TABLE 3. *Changes in the lumbar BMD and serum lipids after HT according to the CYP genotypes*

	% change (absolute value)											
	Genotype of I462V (*CYP1A1*)				Genotype of R48G (*CYP1B1*)				Genotype of L432V (*CYP1B1*)			
Variables	AA (n = 78)	AG (n = 42)	GG (n = 4)	P	CC (n = 90)	CG (n = 16)	GG (n = 18)	P	CC (n = 78)	CG (n = 36)	GG (n = 10)	P
L2-4 BMD, g/cm^3	2.4 ± 0.6 (0.78 ± 0.02)	2.1 ± 1.2 (0.77 ± 0.02)	3.9 ± 1.5 (0.81 ± 0.09)	0.833	2.4 ± 0.6 (0.78 ± 0.01)	2.6 ± 1.4 (0.79 ± 0.04)	1.7 ± 1.2 (0.76 ± 0.04)	0.872	3.4 ± 0.6 (0.78 ± 0.02)	1.8 ± 1.0 (0.77 ± 0.02)	−3.7 ± 2.4 (0.74 ± 0.06)	0.002
TC, mg/dL	−3.8 ± 2.3 (212.0 ± 4.6)	−4.8 ± 1.9 (211.3 ± 5.5)	−6.3 ± 6.6 (213.5 ± 5.3)	0.9330	−4.5 ± 2.0 (212.0 ± 3.5)	−4.0 ± 3.3 (213.4 ± 6.2)	−3.1 ± 4.2 (221.6 ± 8.7)	0.953	−4.2 ± 1.7 (210.3 ± 4.5)	−9.4 ± 3.5 (206.1 ± 4.7)	5.5 ± 4.4 (226.1 ± 11.4)	0.058
LDL-C, mg/dL	−11.0 ± 4.0 (116.8 ± 5.0)	−17.4 ± 3.2 (118.3 ± 3.2)	−16.6 ± 6.1 (114.0 ± 4.4)	0.455	−13.5 ± 3.2 (114.6 ± 4.3)	−6.3 ± 6.5 (125.0 ± 5.4)	−20.5 ± 7.4 (124.6 ± 6.9)	0.302	−15.6 ± 3.8 (115.6 ± 4.9)	−18.0 ± 4.2 (114.4 ± 4.3)	11.1 ± 3.5 (140.0 ± 10.9)	0.002
HDL-C, mg/dL	3.0 ± 2.9 (71.3 ± 2.0)	8.7 ± 3.5 (71.8 ± 2.8)	3.0 ± 3.1 (78.8 ± 4.0)	0.408	4.5 ± 2.8 (70.8 ± 1.7)	5.3 ± 2.5 (71.9 ± 3.3)	7.2 ± 4.1 (75.8 ± 5.2)	0.894	4.5 ± 1.9 (71.5 ± 2.0)	3.0 ± 6.0 (70.9 ± 2.6)	−2.5 ± 4.1 (68.4 ± 7.4)	0.827
TGs, mg/dL	15.7 ± 6.7 (129.6 ± 7.0)	14.5 ± 8.6 (115.7 ± 10.3)	38.3 ± 25.8 (111.5 ± 38.3)	0.698	19.8 ± 6.5 (127.9 ± 6.7)	1.9 ± 14.4 (113.1 ± 11.3)	10.7 ± 6.4 (115.0 ± 16.3)	0.252	21.2 ± 6.5 (122.1 ± 6.8)	5.9 ± 10.3 (124.5 ± 11.5)	16.8 ± 13.5 (137.0 ± 19.9)	0.357

BMD, bone mineral density; HT, hormone therapy, CYP, cytochrome P-450; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol. HDL-C, high-density lipoprotein cholesterol; TGs, triglycerides. Data are presented as mean ± SE.

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level of FSH showed significant differences among the L432V genotypes (Table 5). Compared with the women with the CC genotype (wild type, homozygous), women with the GG genotype (mutant, homozygous) had a significantly higher level of FSH ($P = 0.006$) after 12 months of HT.

DISCUSSION

Variations in the estrogen-metabolizing genes, such as *CYP1A1*, *CYP1B1*, *CYP17*, and *CYP19*, and catechol-*O*-methyltransferase genes have been reported regarding the susceptibility of women to breast cancer, and such variations were also found to influence the clinical course.[13,14] Furthermore, the SNPs of these genes have been evaluated in women using a variety of factors, such as the age at menarche and natural menopause,[15] breast density,[16] and plasma estrogen levels.[17,18]

Both the *CYP1A1* and *CYP1B1* loci appear to play a prominent role within the genes involved in estrogen metabolism. CYP1A1 catalyzes the C2-, C6-, and C15-α hydroxylation, whereas *CYP1B1* catalyzes the C4-hydroxylation of estradiol. Various polymorphic sites of the *CYP1A1* and *CYP1B1* genes have been described on either introns or exons.

In this study, women with a homozygous variant allele of L432V showed significantly poor responses to HT. The genotype frequency distributions of L432V in the *CYP1B1* gene were found to deviate from the Hardy-Weinberg equilibrium because of a variant homozygote excess. This variant in the *CYP1B1* gene is thus possibly an important candidate for an SNP predisposing to the development of either postmenopausal osteopenia or osteoporosis, although the baseline BMD did not significantly differ between the different genotypes in this study.

The catalytic activities of variant enzymes, especially the nucleotide changes in exon 2 (A119S polymorphism) and exon 3 (L432V polymorphism) of the *CYP1B1* gene, have been reported to be two- to fourfold higher than those of wild-type enzymes.[19-22] A significant decrease in the estradiol levels in postmenopausal women with the L432V variant homozygous genotype has been also reported.[18] In this study, significantly higher serum FSH levels during HT in women with an L432V variant genotype were observed, even though there was no significant difference in the serum estradiol level. Although several investigators have shown estradiol to be a predictor of bone loss,[23,24] there is a conflicting report in which there was no significant correlation of estradiol levels with BMD.[25] The peripheral levels of estradiol may not necessarily represent the estradiol levels in target tissues.[26] Thomsen et al[27] reported a strong correlation between the decrease in FSH and the change in BMD, whereas the association between BMD and the estradiol level was less clear. They also reported that women who have a favorable response in BMD during HT also tend to show a favorable change in the lipid profile, and this association is most likely driven by a common response of FSH to exogenous estrogen therapy. Therefore, the L432V variant that corresponds to the hyperactivity of *CYP1B1* accelerates estradiol metabolism, thus leading to higher serum FSH levels and thus may possibly affect the response to HT regarding the lumbar BMD and serum lipid profiles.

There are some limitations to our study. Gonadotropins are known to be secreted in an episodic fashion. The pulse amplitude of FSH in postmenopausal women with HT has been reported to be 5.7 ± 1.0 mIU/mL. Therefore, the validity of the gonadotropin determinations based on a single blood measurement may be questioned. In addition, the number of the L432V variants in this study was limited. Additional studies are therefore necessary to clarify the precise mechanisms by which the *CYP1B1* polymorphisms modulate the responsiveness of BMD and LDL-C to HT.

TABLE 4. *Baseline variables as predictors of the percent change in the lumbar BMD and serum LDL-C after HT: multivariate regression analysis*

Variables	Correlation coefficient *r*	*P*
BMD		
Age	0.130	0.107
Baseline BMD	−0.416	<0.001
L432V (CYP1B1) genotype	0.273	<0.001
LDL-C		
Baseline LDL-C	−0.501	<0.001
L432V (CYP1B1) genotype	0.182	0.039

BMD, bone mineral density; LDL-C, low-density lipoprotein cholesterol; HT, hormone therapy.

TABLE 5. *Serum hormone levels at 12 months after HT according to the genotype of L432V in the CYP1B1 gene*

	Genotype			
	CC (n = 20)	CG (n = 20)	GG (n = 10)	*P*
Estradiol, pg/mL	71.3 ± 7.3	74.3 ± 14.3	69.9 ± 16.8	0.971
LH, mIU/mL	11.2 ± 2.6	15.5 ± 3.2	16.2 ± 6.2	0.560
FSH, mIU/mL	9.4 ± 1.1	15.7 ± 3.3	24.1 ± 6.4	0.021

HT, hormone therapy; LH, luteinizing hormone; FSH, follicle-stimulating hormone.
Data are presented as mean ± SE. Controlling for age, date of blood draw, time of blood draw, fasting status, body mass index, and laboratory batch.

CONCLUSIONS

In summary, our genetic analyses of the genes *CYP1A1* and *CYP1B1* suggest that the L432V SNP in the *CYP1B1* gene might act as a marker of the drug response. An analysis of the *CYP1B1* gene SNPs might therefore prove to be useful in appropriately selecting HT for the management of either osteopenia or hyperlipidemia in Japanese postmenopausal women.

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GAMETE BIOLOGY

Increased apoptosis of germ cells in patients with AZFc deletions

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Abstract

Purpose AZFc deletions are associated with variable testicular histology ranging from the Sertoli cell only to spermatogenic arrest and hypospermatogenesis. Such variable phenotypes may be explained by progressive germ cell regression over time. Increased apoptosis is likely responsible for progressive regression of spermatogenic potential. This study evaluated germ cell apoptosis as a cause of the progressive decrease in the number of germ cells in patients with AZFc deletions.

Methods This study evaluated germ cell apoptosis in patients with AZFc deletions. A total of 151 patients who were diagnosed with either severe oligozoospermia or non-obstructive azoospermia were screened for Y chromosome microdeletions. Germ cell apoptosis was examined using terminal deoxy-nucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) on formalin-fixed 5-μm sections of testicular specimens.

Results Seven out of 117 (6.0%) patients with azoospermia and 4 of 34 (11.8%) patients with severe oligozoospermia had Y chromosome microdeletions. The percentage of apoptotic germ cells in the testes of patients with AZFc deletions were significantly increased compared to those of patients without AZFc deletions.

Conclusions These results suggest that increased apoptosis of germ cells is responsible for the progressive decline of spermatogenic potential in patients with AZFc deletions.

Capsule Males carrying AZFc deletions exhibit diminished sperm cell numbers due to an enhanced incidence of apoptosis.

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Introduction

AZFc deletions are the most frequent genetic cause of male infertility, observed with a prevalence of 10–15% in patients with severe oligozoospermia and azoospermia [1]. The DAZ gene family is thought to be the major candidate responsible for the AZFc phenotype. The DAZ gene encodes a protein with an RNA-binding domain that is expressed exclusively in germ cells [2]. The natural RNA substrates of DAZ proteins remain undefined, and the biological function of DAZ has not yet been elucidated.

AZFc deletions are associated with variable testicular histology, ranging from the Sertoli cell only to spermatogenic arrest and hypospermatogenesis. A possible explanation for such variable phenotypes is the progressive germ cell regression over time, which has been reported in patients with AZFc deletions [3–8].

The control of germ cell apoptosis plays an important role during normal spermatogenesis [9–12]. Increased apoptosis can induce a progressive decrease in the number of germ cells. No studies have thus far assessed the apoptosis of germ cells in patients with AZFc deletions. Therefore, the current study evaluated germ cell apoptosis as one of the causes of the progressive decrease in the number of germ cells in patients with AZFc deletions.

Materials and methods

Patients

A total of 151 patients who were diagnosed with severe oligozoospermia (sperm concentration of less than 1×10^6 per ml) or non-obstructive azoospermia were screened for Y chromosome microdeletions. Among these, 117 were azoospermics and 34 were oligzoospermics. Patients with iatrogenic azoospermia, varicocele or cryptochidism were excluded from this study. As controls, testicular samples were obtained from five patients with obstructive azoospermia who had normal spermatogenesis.

Specimens of bilateral testicular tissue were obtained by open biopsy. The biopsies were classified according to McLachlan *et al.* [13] as follows: hypospermatogenesis, all stages of spermatogenesis are present but reduced to a varying degree; germ cell arrest, the total arrest at a particular stage; Sertoli cell-only, no tubules containing germ cells. This study was approved by the hospital's Institutional Review Board and informed consent was obtained from all patients.

Y chromosome microdeletion assay

Genomic DNA was isolated from peripheral blood lymphocytes using standard procedures. Y chromosome microdeletions were evaluated using polymerase chain reaction of Y chromosome-specific STS markers. The STS markers used were as follows: AZFa: sY83, sY95, sY105; AZFb: sY118, sY126, sY136; AZFc: sY152, sY254, sY255, sY283.

In situ end labeling of testicular tissue sections

In order to detect apoptosis, terminal deoxy-nucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) was performed on formalin-fixed 5-μm tissue sections of specimens using an In Situ Apoptosis Detection Kit (Takara Bio Inc., Shiga, Japan). In brief, each section was deparaffinized and rehydrated. After incubation with 20 μg/ml Proteinase K (Boeheringer Mannheim, Mannheim, Germany), endogenous peroxidase were blocked with 2% H_2O_2 in methanol for 30 min. TdT enzyme was dropped on the sections and incubated at 37°C for 60 min. Then antifluorescein isothiocyanate horseradish peroxidase conjugate was placed on the sections and incubated at 37°C for 30 min. Slides were washed three times in PBS, developed with 0.05% diaminobenzidine (DAB), and stained for 10–15 min at room temperature. The specimens were then washed three times in distilled water, dehydrated and mounted. For quantitative evaluation, the percentage of labeled cells per total 200 cells of germ cells was evaluated for each patient.

Hormone assays

Semen samples were centrifuged (3000× g; 5 min) and the seminal plasma was stored at –20°C within one hr after ejaculation. Inhibin B was measured by two-site enzyme-linked immunoassay (Serotec Ltd., Oxford, UK).

Statistical analysis

The Mann-Whitney U test was used for statistical analyses using the StatView 5.0 statistical analysis program (Abacus Concepts, Berkeley, CA, USA). Statistically significant differences were confirmed for p values less than 0.05.

Results

Seven out of 117 (6.0%) patients with azoospermia and 4 of 34 (11.8%) patients with severe oligozoospermia had Y chromosome microdeletions (Table 1). AZFa, AZFb and AZFc were deleted in two azoospermic patients. AZFb and AZFc were deleted in one azoospermic patient. AZFc was deleted in four azoospermic patients and in four severe oligozoospermic patients. All patients with AZFa+b+c and AZFb+c deletions had a complete absence of spermatozoa upon testicular biopsy. Of the 8 patients with AZFc deletions, 6 had spermatozoa within the testis or ejaculate.

Serum and seminal plasma Inhibin B were undetectable in patients who lacked testicular spermatozoa. The seminal plasma Inhibin B level was greater than 15 pg/ml in all patients who had spermatozoa in testes or ejaculate (Table 2). Sequential seminological data was available in two patients with AZFc deletions. Patient 4 showed a

Table 1 Summary of DNA analysis of the twelve patients with Yq microdeletions

Markers	Patients										
	1	2	3	4	5	6	7	8	9	10	11
sY83	+	+	+	+	+	+	+	+	+	+	+
sY95	–	+	+	+	+	+	+	+	+	+	+
sY105	–	+	+	+	+	+	+	+	+	+	+
sY118	–	–	+	+	+	+	+	+	+	+	+
sY126	–	–	–	+	+	+	+	+	+	+	+
sY136	–	–	–	+	+	+	+	+	+	+	+
sY152	–	–	–	–	–	–	–	–	–	–	–
sY254	–	–	–	–	–	–	–	–	–	–	–
sY255	–	–	–	–	–	–	–	–	–	–	–
sY283	–	–	–	–	–	–	–	–	–	–	–
sY166	+	+	+	+	+	+	+	+	+	+	+

Table 2 Hormone values and clinical details of the ten patients with Yq microdeletions

Patients											
	1	2	3	4	5	6	7	8	9	10	11
Age (years)	45	44	43	42	35	36	36	55	46	34	48
Testicular volume (ml) right/left	5/8	8/10	4/3	17/15	7/7	17/16	14/13	18/11	10/9	8/7	5/5
Sperm count (X10^6/ml)	0	0	0	0.7	0.06	0	0.2	1.9	0	0	0
Deleted AZF regions	a,b,c	b,c	b,c	c	c	c	c	c	c	c	c
Inhibin B (pg/ml)											
Serum	<15	<15	<15	195	42	300	100	90	<15	<15	<15
Seminal plasma	<15	<15	<15	107	30	108	28	660	110	<15	<15
FSH (mIU/ml)	40.3	12.6	60.1	4.2	28.8	5.7	16.3	8.7	21.5	10.3	31.9
Histology	SCO	SCO	GA	GA	GA	HYPO			GA	GA	GA
Sperm recovery	–	–	–	+	+	+	+	+	+	–	–
Percentage of apoptotic cells (%)				2.5	5.0	4.0			7.5		7.0

SCO Sertoli cell-only, *GA* germ cell arrest, *HYPO* hypospermatogenesis

decline in the total sperm concentration from an average of 0.7 x 10^6 per ml to 0.02 x 10^6 per ml over 25 months. The serum and seminal plasma Inhibin B levels decreased from 195 pg/ml and 107 pg/ml to 35 pg/ml and 32 pg/ml, respectively. Patient 5 showed a decline in total sperm concentration from 0.06×10^6 per ml to azoospermia over 34 months. Serum and seminal plasma Inhibin B levels decreased from 42 pg/ml and 30 pg/ml to 18 pg/ml and 15 pg/ml, respectively.

Apoptosis was evaluated in the testes of 5 patients with AZFc deletions (patient 4, 5, 6, 9 and 11). Fifteen patients without AZFc deletions whose testicular histology were hypospermatogensis (3patients) or germ cell maturation arrest (12 patients) were also evaluated for apoptosis in testes. There was no significant difference in the testicular histology between these two groups.

The percentage of apoptotic germ cells in the testes of patients with AZFc deletions were significantly increased compared to those of patients without AZFc deletions and patients with obstructive azoospermia (5.2% vs. 2.1%, $p<$ 0.01; 5.2% vs. 1.0%, p=0.01; Table 3).

Table 3 Analysis of apoptosis in germ cells of testes

	Percentages of apoptotic cells (mean±SD)
Patients with AZFc deletions (n=5)	5.2±2.0^{a,b}
Patients without AZFc deletions (n=15)	2.1±0.9
Obstructive asoospermic patients (n=5)	1.0±0.7

[a] Significantly different from patients without AZFc deletions (P<0.01)

[b] Significantly different from obstructive asoospermic patients (P=0.01)

Discussion

In this study, seven out of 117 (6.0%) patients with azoospermia and 4 out of 34 (11.8%) patients with severe oligozoospermia had Y chromosome microdeletions. These findings were consistent with previous reports of microdeletion frequencies between 6.2 and 25.9% in Japanese males [14, 15]. In the present study population, the frequency of Y chromosome microdeletions was lower in azoospermic patients than in oligozoospermic patients. Other Japanese studies [14] also reported a low frequency of Y chromosome microdeletions in azoospermic patients (4.2%) in comparison to oligozoospermic patients (15.9%). Nagata *et al.* [16] reported that the sperm retrieval rate by testicular sperm extraction in Japanese azoospermic patients was low in comparison to other studies. Other common genetic causes may exist in Japanese azoospermic patients. Eight out of 11 patients with Y chromosome microdeletions had complete AZFc deletions (b2/b4 deletion). The seminal phenotype of patients with complete AZFc deletions varied from azoospermia to severe oligozoospermia. Progressive regression of the germinal epithelium over a period of time has been reported which may be an explanation for such variable phenotypes [5]. However, Oates *et al.* [17] reported that 4 patients with AZFc deletions had stable sperm production over time. The discrepancies between the studies may have been due to the small number of patients.

In this study, 2 patients with AZFc deletions were followed over 2 years. Both patients exhibited a decline in total sperm concentration over 2 to 3 years, associated with a decrease in serum and seminal plasma Inhibin B levels. This finding supports a hypothesis of progressive depletion of the seminiferous epithelium. There is an association between serum Inhibin B levels and testicular pathology in

patients with AZFc deletions [18]. The current study also suggested that Inhibin B is a good marker for spermatogenic potential in patients with AZFc deletions. However, further studies with a greater number of study patients will be required to confirm the progressive decline of spermatogenic potential in patients with AZFc deletions and the utility of Inhibin B as a marker of spermatogenesis.

Mammalian spermatogenesis is a highly regulated process, and apoptosis appears to play an essential role in maintaining an appropriate number of germ cells that can be adequately supported and matured by the Sertoli cells [19]. Several authors have reported accelerated apoptosis of germ cells in infertile men with impaired spermatogenesis [9–12]. In the present study, the percentages of apoptotic germ cells were comparable to those reported in other studies. Only Tesarik et al. [9] reported much higher percentages of apoptotic germ cells in patients with incomplete spermatogenesis. The discrepancy between the studies might have been due to the method of apoptosis detection. Tesarik et al. examined the germ cell apoptosis by analyzing cell smears from mechanically disintegrated testicular tissues and used a FITC-labeled nucleotide to detect DNA fragmentation.

The mechanisms of the germ cell apoptotic process underling spermatogenesis impairment are poorly understood. In the current study, increased germ cell apoptosis was observed in patients with AZFc deletions in comparison to patients without AZFc deletions and patients with obstructive azoospermia. This increase in apoptosis may be responsible for the progressive loss in spermatogenic potential. Rajpurkar *et al.* [20] demonstrated that chronic cigarette smoke induced apoptosis in rat testis. They concluded that increased apoptosis might be one of the pathogenic mechanisms responsible for defective spermatogenesis in the rat following chronic cigarette smoking. A varicocele has a progressively toxic effect on the testes that may ultimately result in irreversible infertitity [21]. Hassan et al. [22] reported that the percentage of apoptotic cells in seminiferous tubules of infertile patients with varicocele was significantly higher than in patients with obstructive azoospermia (6.29% vs. 2.71%). These percentages of apoptotic germ cells were comparable to those reported herein.

AZFc contains five protein-coding gene families (BPY2, CDY, DAZ, CSPG4LY and GOLGA2LY), which are all transcribed in testicular tissue [23]. These genes are thought to be associated with spermatogenesis, but their function is unknown. The best-characterized gene family in the AZFc region is the DAZ gene. The DAZ gene family encodes a protein with an RNA-binding motif, suggesting a functional role in mRNA stability or in the translational regulation of its target RNA. The CDC25 family has been recognized as the downstream target of DAZL, which is the autosomal DAZ family gene [24, 25]. CDC25 phosphatases play a key role in cell cycle progression by controlling the activation of cyclin-dependent kinases [26]. Of the CDC25 family, CDC25A is expressed at a high level in the testis, suggesting that CDC25A plays a crucial role in the mitotic or meiotic regulation of spermatogenesis [27, 28]. Inactivation of CDC25 induces cell cycle arrest and apoptosis of hepatocellular carcinoma cells [29]. The inhibition of the CDC25 function, owing to a loss of DAZ genes, may contribute to the accelerated germ cell apoptosis observed in patients with AZFc deletions.

This is the first paper reporting increased apoptosis of germ cells in patients with AZFc deletions. Further studies with a larger population are needed to confirm these results.

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Recurrent borderline ovarian tumor presenting as a pedunculated polyp at colonoscopy

Fig. 1 Colonoscopic view of the polypoid mass.

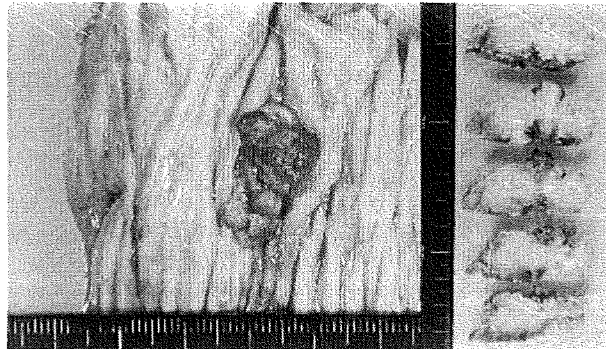
Fig. 2 Gross findings of the resected specimen. The polypoid mass is penetrating the anterior rectal wall.

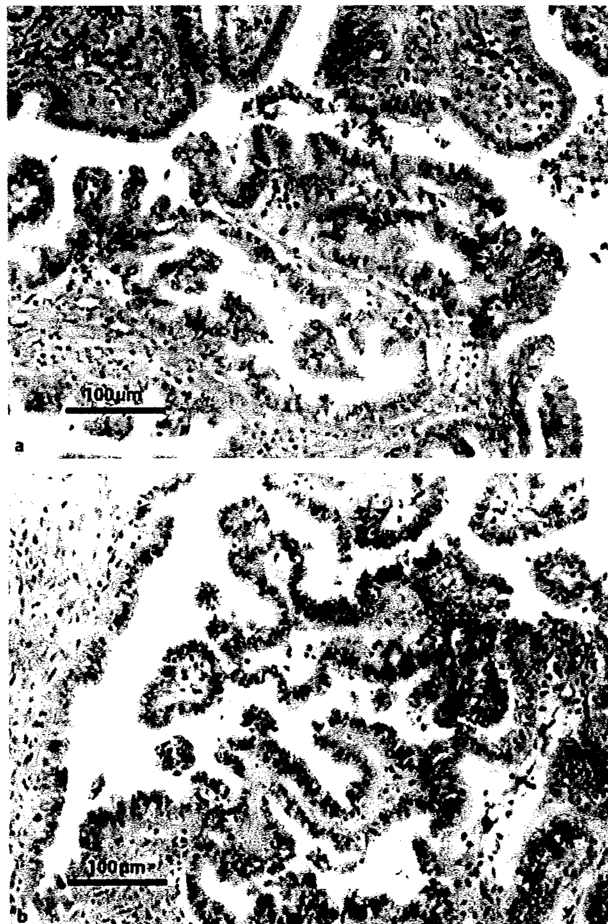

Fig. 3 Microscopic findings: (a) the rectal tumor and (b) the primary ovarian tumor (hematoxylin and eosin; magnification × 100). Both tumors show marked epithelial proliferation with a micropapillary and cribriform pattern.

A 69-year-old woman with a positive fecal occult blood test was referred for further investigations. She had been diagnosed as having a borderline serous ovarian tumor 8 years earlier, for which she had undergone complete debulking surgery. The tumor had originated in the left ovary and a pathological examination had revealed that it was confined to the left ovary, without capsule invasion. The patient was followed up for 7 years after the surgery without any evidence of recurrence.
Colonoscopy showed a hyperemic, polypoid lesion, 10 cm from the anal verge (**○ Fig. 1**) but the biopsy findings were nonspecific.
A computed tomography scan confirmed the presence of an intraluminal lesion in the rectum, and submucosal invasion was suspected. To rule out the possibility of recurrence of the borderline tumor or a primary rectal tumor, the patient underwent an exploratory laparotomy. There was no evidence of either carcinomatosis in the abdomen or involvement of adjacent organs. A low anterior resection was carried out with an end-to-end colorectostomy. The resected specimen included the pedunculated rectal polyp, which had invaded the entire rectal wall but was limited to the rectal serosa (**○ Fig. 2**).
On pathological review, the tumor was determined to be a borderline serous malignant tumor (**○ Fig. 3a**) and the findings were identical to those of tissue specimens taken from the original borderline ovarian tumor (**○ Fig. 3b**).
Since surgery, the patient has been doing well with no evidence of recurrence for 18 months.

Although epithelial proliferation in borderline ovarian tumors exceeds that found in benign tumors, they lack stromal invasion and generally behave in a benign fashion, different from invasive ovarian carcinoma. In patients undergoing primary pelvic clearance, the rate of recurrence is 2% – 13%; the major site of recurrence is the abdominal cavity owing to the exfoliation of tumor cells [1 – 3]. Recurrence with colorectal involvement is exceedingly rare, with only one case report of metastasis to the sigmoid colon 7 years after primary debulking surgery similar to the present case [4]. However, borderline ovarian tumors are slow growing, and 85% of recurrences

occur after the 5-year follow-up period [5]. A favorable prognosis can be expected with surgical resection in the case of both recurrence and distant metastasis.

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The prevalence of hereditary breast/ovarian cancer risk in patients with a history of breast or ovarian cancer in Japanese subjects

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Abstract

Aim: Women at high risk for hereditary breast/ovarian cancer require specific management strategies for cancer prevention and early detection. The authors sought to determine the prevalence of family histories suggestive of a hereditary breast/ovarian cancer syndrome in patients with a personal history of breast or ovarian cancer in Japanese women.

Methods: Family history (first- and second-degree relatives) data were collected by a self-administered questionnaire for women with a history of breast or ovarian cancer in six major cancer treating hospitals in Niigata prefecture, Japan.

Results: Data were obtained from 1463 women: 626 women with a history of breast cancer, 289 women with a history of ovarian cancer and 548 women without a history of any cancer as controls. Women with a family history of breast and/or ovarian cancer had OR of breast cancer of 2.3 (95% confidential interval [CI] 1.5–3.7) and ovarian cancer of 2.2 (95% CI 1.3–3.8). The risk was higher when the proband was younger or when two or more relatives were affected. Among women with a history of breast or ovarian cancer, 7.5% met the criteria for a 10% risk of a BRCA1 or BRCA2 mutation according to the Myriad model.

Conclusion: Obtaining a detailed breast and ovarian cancer family history and the application of the Myriad model is useful for identifying women at an elevated genetic risk of breast and ovarian cancer. The estimation for the prevalence of hereditary breast/ovarian cancer syndrome has significant implications for a patient's management, as well as for the capacity for risk assessment and testing.

Key words: breast cancer, genetic counseling, hereditary neoplastic syndromes, ovarian caner, risk assessment.

Introduction

Hereditary breast/ovarian cancer syndrome refers to families in which individuals have suffered from breast cancer and ovarian cancer (either one individual suffered from both, or several individuals in the pedigree suffered from one or the other disease). A better understanding of the characteristics of hereditary breast/ovarian cancer has increased the ability to identify families with a predisposition to these diseases. Identifying women at high risk of developing breast and ovarian cancer is increasingly important because

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specific management strategies, such as a prophylactic mastectomy,[1] prophylactic oophorectomy,[2] breast cancer screening by a combination of annual mammography and breast magnetic resonance imaging (MRI)[3] and ovarian cancer screening by transvaginal ultrasound examination with color Doppler and serum CA-125 concentration,[4,5] have demonstrated promise for decreasing the incidence of breast and ovarian cancer or in finding these tumors at an earlier stage.

There have been several studies regarding the prevalence of family history of breast and/or ovarian cancer,[6–10] but only a few reports have described a detailed family history in Japanese women.[11,12] The current study estimated the risk of breast and ovarian cancer using data from a multicentric case–control study and examined the prevalence of family histories suggestive of a hereditary breast/ovarian cancer syndrome in patients with a personal history of breast or ovarian cancer, to better understand the magnitude of the problem in Japanese women.

Methods

A case–control study of breast or ovarian cancer was conducted from 2007 to 2008 in six major cancer treating hospitals in Niigata prefecture (in central Japan). The data were prospectively collected from patients with a personal history of breast or ovarian cancer using a self-administered questionnaire that included information regarding personal cancer history, family history (first- and second-degree relative) of breast and ovarian cancer, age at the time of the diagnosis of cancer and the number of sisters.

This study enrolled 915 patients (626 breast cancer patients and 289 ovarian cancer patients) with histologically confirmed breast or ovarian cancer diagnosed within 3 years before the interview. The controls consisted of 548 subjects selected among the patients admitted to the same hospitals for benign gynecological disease such as bacterial vaginitis, climacteric disorders, and uterine myoma, or nurses that volunteered to join this study (125 patients with benign gynecological disease and 423 healthy nurses). None of the controls had been previously diagnosed with any cancer and were matched to the case series by age.

The odds ratios (OR) and 95% confidence intervals (CI) according to the type of cancer in the proband and relatives were estimated. Using the Myriad mutation prevalence tables (http://www.myriadtests.com/provider/brca-mutation-prevalence.htm) to estimate mutation risk, the prevalence of family histories with a 10% risk of a BRCA1 or BRCA2 mutation were evaluated according to the recommendations of the American Society of Clinical Oncology.[13] The Myriad mutation prevalence table represents observations of deleterious mutations of BRCA genes by Myriad Genetic Laboratories through its clinical testing service. It should be noted that these tables are constantly being updated. The tables were used as they existed in the spring of 2006. For purposes of data analysis, the age of diagnosis provided for patients or relatives with both breast and ovarian cancer was assumed to be the age at the time of the breast cancer diagnosis. The prevalence of family histories suggestive of a hereditary breast/ovarian cancer syndrome was evaluated according to the type of cancer in the proband. The proband indicates individuals clinically affected by either breast or ovarian cancer and all subjects also completed a self-administered questionnaire.

This study was reviewed and approved by the institutional review board of each hospital.

The OR of breast or ovarian cancer was estimated according to the history of cancer at selected sites in first-degree relatives using unconditional multiple logistic regression models. The model included the terms for the age at diagnosis, the study center and the number of sisters. The logistic procedure provided by the SAS Institute was utilized to perform the calculations.

Results

Tables 1 and 2 show the characteristics of the patients and controls and the distribution of 626 patients with a

Table 1 Patient characteristics

Characteristics	No. patients	%
Patients with breast cancer	626	
Median age at diagnosis in years (range)	55 (21–85)	
Age ≥50 years	235	38
Age <50 years	391	62
Patients with ovarian cancer	289	
Median age at diagnosis in years (range)	53 (20–77)	
Age ≥50 years	99	34
Age <50 years	189	66
Control	548	
Median age in years (range)	51 (19–92)	
Age ≥50 years	208	38
Age <50 years	340	62
Total	1463	

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Table 2 Women with first- or second-degree relatives with breast and/or ovarian cancer

Family history of breast and/or ovarian cancer	First-degree relatives	Second-degree relatives	Percent of total
Breast cancer probands			
Women without a family history of breast or ovarian cancer	465		74
Women with a family history	78	95	
Breast cancer		134	21
Ovarian cancer		20	3
Breast and ovarian cancer		7	1
Total	626		100
Ovarian cancer probands			
Women without a family history of breast or ovarian cancer	231		80
Women with a family history	33	26	
Breast cancer		46	16
Ovarian cancer		9	3
Breast and ovarian cancer		2	1
Total	288		100

personal history of breast cancer and 289 patients with a personal history of ovarian cancer according to the family history in first- and second-degree relatives. Seventy-eight (12.5%) patients with a personal history of breast cancer had a family history of breast and/or ovarian cancer in first-degree relatives and 161 (25.7%) patients in second-degree relatives. Thirty-three (11.5%) patients with a personal history of ovarian cancer had a family history of breast and/or ovarian cancer in first-degree relatives and 57 (19.8%) patients in second-degree relatives.

Table 3 presents the relationship between breast and ovarian cancer risk and several aspects of family history. The OR of breast cancer were elevated in subjects with a positive family history of breast cancer (OR 2.3, 95%CI 1.4–3.8) and ovarian cancer (OR 2.4, 95%CI 1.0–6.9). The OR of breast cancer were further elevated in subjects with a positive family history of breast cancer (OR 2.8, 95%CI 1.3–7.1) and ovarian cancer (OR 7.2, 95%CI 1.2–136.6), when a proband's age was <50. Similarly, the OR of ovarian cancer were elevated in the subjects with a positive family history of breast cancer (OR 2.5) and ovarian cancer (OR 3.7), when the proband's age was <50, although it was not significant. When the family history of breast and ovarian cancer were combined, the OR of breast and ovarian cancer were significantly elevated to 3.2 (95%CI 1.5–7.6) and 2.7 (1.1–7.0) when the proband's age was <50, and to 6.5 (1.1–121.7) and 10.5 (1.7–203.6) when there were two or more patients in the first-degree family, respectively.

Of the 915 patients with a personal history of breast or ovarian cancer, 7.5% had a 10% risk of carrying a BRCA1 or BRCA2 mutation according to the Myriad tables. In comparing cancer types, 7.0% of women with breast cancer versus 8.7% of women with ovarian cancer were considered to have a 10% risk for carrying a mutation (Table 4).

Discussion

Women at high risk for hereditary breast/ovarian cancer syndrome will likely benefit from management strategies designed for their specific level of risk. A prophylactic mastectomy and prophylactic oophorectomy are examples of effective strategies. Screening by a combination of annual mammography and breast MRI for breast cancer and transvaginal ultrasound examination with color Doppler and serum CA-125 concentration for ovarian cancer are recommended cancer screening strategies for high-risk women. Although the efficacy of a comprehensive strategy has not been assessed by randomized trials or case–control studies, these strategies will have an impact on the incidence, morbidity and mortality of high-risk women. Therefore, the identification of these women and the implementation of specific management strategies is crucial.

The current study estimated the risk of breast and ovarian cancer by a case–control study and examined the prevalence of family histories suggestive of hereditary breast/ovarian cancer syndrome in patients with a

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Table 3 Odds ratio (OR) of breast/ovarian cancer according to various aspects of the family history

	n (%)				OR (95% CI)		
	Breast cancer	Ovarian cancer	Total	Controls	Breast cancer	Ovarian cancer	Total
Family history† of breast cancer							
No	562 (89.8)	260 (90.3)	822 (89.9)	523 (95.4)	1 (reference)	1 (reference)	1 (reference)
Yes	64 (10.2)	28 (9.7)	92 (10.1)	25 (4.6)	2.3 (1.4–3.8)	2.2 (1.2–3.9)	2.3 (1.5–3.7)
Proband's age							
<50‡	24 (10.2)	9 (9.1)	33 (9.9)	8 (3.8)	2.8 (1.3–7.1)	2.5 (0.9–7.0)	2.8 (1.3–6.6)
≥50§	40 (10.2)	19 (10.1)	59 (10.2)	17 (5.0)	2.1 (1.2–3.9)	2.2 (1.1–4.4)	2.1 (1.2–3.8)
Family history† of ovarian cancer							
No	609 (97.3)	283 (98.3)	892 (97.6)	542 (98.9)	1 (reference)	1 (reference)	1 (reference)
Yes	17 (2.7)	5 (1.7)	22 (2.4)	6 (1.1)	2.4 (1.0–6.9)	1.5 (0.4–4.9)	2.2 (0.9–6.0)
Proband's age							
<50‡	7 (3.0)	2 (2.0)	9 (2.7)	1 (0.5)	7.2 (1.2–136.6)	3.7 (0.3–80.0)	5.8 (1.1–108.4)
≥50§	10 (2.6)	3 (1.6)	13 (2.2)	5 (1.5)	1.7 (0.6–5.5)	1.0 (0.2–4.1)	1.5 (0.5–4.7)
Family history† of B/O cancer							
No	548 (87.5)	255 (88.5)	803 (87.9)	518 (94.5)	1 (reference)	1 (reference)	1 (reference)
Yes	78 (12.5)	33 (11.5)	111 (12.1)	30 (5.5)	2.3 (1.5–3.7)	2.2 (1.3–3.8)	2.4 (1.6–3.6)
Proband's age							
<50‡	29 (12.3)	11 (11.1)	40 (12.0)	9 (4.3)	3.2 (1.5–7.6)	2.7 (1.1–7.0)	3.0 (1.5–6.9)
≥50§	49 (12.5)	22 (11.6)	71 (12.2)	21 (6.2)	2.1 (1.2–3.7)	2.1 (1.1–3.9)	2.1 (1.3–3.6)
No. affected relatives							
1	71 (11.3)	28 (9.7)	99 (10.8)	29 (5.3)	2.8 (1.7–4.6)	2.3 (1.3–4.1)	2.6 (1.7–4.2)
≥2	7 (1.1)	5 (1.7)	12 (1.3)	1 (0.2)	6.5 (1.1–121.7)	10.5 (1.7–203.6)	8.3 (1.6–151.3)

†Family history of first-degree relative. ‡Out of 235 Breast cancers, 99 Ovarian cancers, 334 B/O, 208 controls. §Out of 391 Breast cancers, 189 Ovarian cancers, 580 B/Os, 340 controls. Gray boxes show statistically significant. B/O cancer, breast and ovarian cancer; CI, confidence interval; OR, adjusted for age and number of sisters.

Table 4 Prevalence of patients with a ≥10% risk of a BRCA1 and BRCA2 mutation

Personal history	Prevalence (%)
Breast cancer	44/626 (7.0)
Ovarian cancer	25/289 (8.7)
Total	69/915 (7.5)
No cancer	1/548 (0.2)

personal history of breast or ovarian cancer, to better understand the magnitude of the problem in Japanese women.

In this study 12% of Japanese women with a personal history of breast cancer had a first-degree relative with breast and/or ovarian cancer and 11% of women with a personal history of ovarian cancer had a first-degree relative with breast and/or ovarian cancer. Consistent with the findings of the current study, Hirose *et al.*[11] reported a 7% incidence of positive breast cancer family history in first-degree relatives in Japanese women with a personal history of breast cancer. These incidents are lower than those reported in other studies in Western countries. In previous studies, 18% of women with a personal history of breast cancer had a first-degree relative with breast cancer and similarly 18% of women with a personal history of ovarian cancer had a first-degree relative with breast cancer in a large population-based control study in the USA.[7,14]

The OR of breast and ovarian cancer with a positive family with breast and/or ovarian cancer were 2.3 and 2.2, respectively. These results are consistent with those in previously reported studies. The OR of breast cancer were reported to be 1.6–2.1 and those of ovarian cancer were 1.4–3.1.[15,16] Although the OR of breast and ovarian cancer with a positive family history with ovarian cancer were not significant, they became significant (2.3 and 2.2) with a narrower 95% CI when a family history of breast and ovarian cancer were combined. These risks were significantly higher when the proband's age was <50 and when two or more relatives were affected with a positive family history of breast and/or ovarian cancer. As the incidence of ovarian cancer is much lower than that of breast cancer, collecting family history information not only for ovarian cancer but also breast cancer is needed to accurately assess the risk of breast and ovarian cancer.

There may be several limitations associated with this study. The first criticism is the selection bias between cancer patients and controls. Several well-established factors have been associated with an increased risk of

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breast or ovarian cancer including nulliparity, early menarche, advanced age, obesity, the use of hormone replacement therapy, and lifestyle characteristics other than family history. These factors were not included in the self-administered questionnaire used in this study. Although family history is a major risk factor for both breast and ovarian cancer, some selection bias may thus have influenced the results. The second limitation is the choice of referents. We used hospital-based patients as controls. It is sometimes pointed out that there are discrepant characteristics between the general population and hospital-based references. Such potential limitation should be considered before drawing definitive conclusions from the current study. The third criticism is the accuracy of self-reported family history data. It is important to determine how reliable these data are to avoid any possible recall bias. A number of studies have found that women can provide accurate (>90% accuracy) and reliable information about their family history of cancer.[17,18] It is reported if there was any recall bias operating in the reporting of family histories, the effect of the risk estimates would thus have been negligible.[19]

The hereditary breast/ovarian cancer syndrome is thought to be largely attributable to mutations in the BRCA1 and BRCA2 genes. Molecular genetic testing for BRCA1 and BRCA2 cancer-predisposing mutations is available on a clinical basis for probands who are identified to be at high risk for having a mutation of those genes. Approximately 7% of the patients with breast cancer and 10% of the patients with ovarian cancer are estimated to have a mutation in those cancer susceptibility genes.[20] Women who carry a deleterious BRCA1 or BRCA2 mutation have a 50–80% lifetime risk of breast cancer and a 10–40% lifetime risk of ovarian cancer.[21] The recognition of a BRCA mutation is often valuable in the decision making of patients with newly diagnosed breast or ovarian cancer. Once a mutation has been identified in the proband, genetic counseling and testing can be offered to unaffected family members. A systematic approach to collecting family histories and the use of risk models for mutation of those genes are required to identify those patients who need genetic testing.

Using several currently available models, from 3.3 to 6.0% of female patients without a history of breast or ovarian cancer have been shown to have family histories suggestive of a mutation and are therefore eligible for additional evaluation.[6,22]

Among the patients with a personal history of breast and/or ovarian cancer, the proportion with significant family histories may be much higher. Shannon *et al.*[23] reported a rate of 22% of high-risk patients among a cohort of 50 women with a history of breast or ovarian cancer, as determined by a genetic counselor. This incidence is highest among the patients with an Ashkenazi ancestry (47.3%) and among those with a personal history of ovarian cancer (35.9%).

Probability models have been developed to estimate the likelihood that an individual family has a mutation in BRCA1 or BRCA2. Among those models the Myriad mutation prevalence tables and the BRCAPRO model are the most widely used.

Dominguez *et al.*[24] reported that 20.6% of patients with a personal history of breast or ovarian cancer had a family history suggestive of a 10% risk of a BRCA1 or BRCA2 genetic mutation according to Myriad tables. In this study only 7.5% had a 10% risk of carrying a BRCA1 or BRCA2 mutation using the same model. Consistent with this result, the frequency of the BRCA1 mutation has been reported to be much lower in Japanese breast/ovarian cancer families.[25] The incidence rate of breast and ovarian cancer in Japan (74.4 per 100 000 women) is much lower than that in USA (120.2). The difference in the incidence rate may be partially due to the lower incidence of the BRCA mutation in Japan. Both BRCA1 and BRCA2 mutations have been reported to actually be more prevalent among women with ovarian cancer. The prevalence of BRCA mutations in ovarian cancer patients is reported to be approximately 12%[26] whereas that in breast cancer patients is approximately 5%.[20] Dominiquez *et al.*[24] reported that 16.9% of women (non-Ashkenazi) with breast cancer versus 30.9% of those with ovarian cancer were considered to have a 10% risk for carrying a mutation. The risk for carrying a mutation according to cancer type did not show a difference in the current study: 7.0% for breast cancer and 8.7% for ovarian cancer. It has been suggested that genetic testing should be considered for women who have been diagnosed with invasive ovarian cancer regardless of the family history, due to the high incidence of BRCA mutations.[27] The current results indicate that the incidence of a high-risk family history in Japanese ovarian cancer patients may be much lower due to the difference in genetic background. Various models are currently applied to evaluate the patient's risk and to assist in the decision of whether to recommend testing, however, ample discrepancies exist between them and the risk probabilities they generate. The Myriad model is a reasonable model to use as a first screening of high-risk women, because it is simple and fast.

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However, Asian women may not be accurately represented by this method because they represent only 1.1% of the individuals previously analyzed by Myriad laboratories. Moreover, the number of patients was relatively small in the current study, especially because the number of patients with ovarian cancer was only 289. A larger scale case–control study or cohort studies are required to confirm these results.

In conclusion, the incidence of a family history of breast and ovarian cancer and the prevalence of hereditary breast/ovarian cancer risk was assessed in Japanese women. The self-administered questionnaire is a simple, fast and effective method for detecting high-risk patients based on their family history. Obtaining a detailed breast and ovarian cancer family history and application of the Myriad model are useful for identifying women at elevated genetic risk of breast/ovarian cancer. Therefore, estimating the prevalence of hereditary breast/ovarian cancer syndrome is considered to have significant implications for patient management, as well as for the capacity of risk assessment and testing.

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ORIGINAL ARTICLE

Pharmacokinetic analysis of a combined chemoendocrine treatment with paclitaxel and toremifene for metastatic breast cancer

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Abstract
Background Multidrug resistance protein could be a target for improving the efficacy of paclitaxel (PXL). Toremifene (TOR) may moderate P-gp-related drug resistance in vitro. Some P-gp moderators may change the pharmacokinetic parameters of PXL in vivo. A pharmacokinetic (PK) study in metastatic breast cancer patients (MBC) was conducted to determine the safety and efficacy of PXL and TOR.

Method and patients Fifteen patients received 80 mg/m^2 PXL (i.v.) weekly and 120 mg/body TOR (p.o.) daily. For the pharmacokinetic study, PXL was administered on days 1, 8, 15, 32, and 39; TOR was given from day 18 to the end of study. On days 1, 8, 15, 18, 32, and 39, blood samples were collected from the patients who received either PXL alone or PXL + TOR, and these were analyzed by high-performance liquid chromatography.
Results Among the 15 patients enrolled in the study, one showed a partial response, and eight had a stable disease. TOR caused no specific adverse events that were greater than grade 3, and its toxicity profile in combination with PXL was similar to that of PXL monotherapy. The PK profile of PXL was similar with or without TOR. The PK parameters of PXL indicated no inter- or intra-patient variability in previously treated patients with MBC. No increased PXL toxicity was observed.
Conclusion The PK profile of combined PXL and TOR was similar to that of PXL monotherapy. The addition of TOR to PXL in previously treated patients with MBC appears safe.

Keywords Breast cancer · Chemoendocrine therapy · Drug resistance · Antiestrogens · Toremifene · Paclitaxel

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Introduction

ATP-binding cassettes (ABC) may play an important role in chemotherapy, because some malignant tumors have a congenital resistance to anticancer agents, which can be substrates of either P-glycoprotein (P-gp) or multidrug resistant protein 2 (MRP2) [1–7]. Chemotherapy may improve the survival rate of breast cancer patients, and

endocrine treatment may also be clinically beneficial [8–13]. Antiestrogens, tamoxifen, and toremifene (TOR) were shown to be effective in hormone-receptor-positive breast cancer patients [14]. For hormone-receptor-negative breast cancer, chemotherapy significantly improved the overall survival rate in primary breast cancer patients and progression-free survival in metastatic breast cancer patients [15–17]. Recently, a new category for endocrine responsiveness, "endocrine response uncertain," was identified in primary systemic treatment [18]. Chemoendocrine therapy is recommended because sensitivity to chemotherapy alone is relatively poor in breast cancer patients with hormone-receptor-positive breast cancer [12, 19]. However, the timing of chemotherapy combined with tamoxifen has been discussed [20]. The role of P-gp has been investigated in relation to hormone receptor status and drug-resistance, and P-gp may be involved in either endocrine response or chemosensitivity [19]. P-gp is considered to be one of the factors that predicts the success of chemotherapy; therefore, this protein remains a target in efforts to improve treatment failure in patients with advanced and recurrent breast cancer. To overcome drug resistance, P-gp modifiers will be needed for optimal chemotherapeutic results.

Antiestrogens may moderate P-gp-related drug resistance in vitro [21–23]. A synergistic effect of PXL and antiestrogens was observed in a multidrug-resistant cell line [24]. This synergistic effect was more potent when PXL was combined with TOR than with tamoxifen. In addition, TOR had a more synergistic effect than tamoxifen when used in combination on the proliferation of doxorubicin-resistant MCF-7 cells [25]. PXL was not effective when a P-gp gene was transfected into breast cancer cells [26]. Efflux of PXL from cancer cells might be mediated by P-gp, and the sensitivity of PXL might be mainly dependent on the expression of P-gp in breast cancer [7, 27, 28]. P-gp inhibitors may improve the sensitivity of PXL [29]. In this regard, antiestrogens may modify P-gp function and are potential candidates for P-gp inhibitors. However, some P-gp inhibitors such as valspodar and elacridar alter the pharmacokinetic parameters of anticancer agents, because these inhibitors moderate the function of P-gp in normal epithelial cells of renal microtubules or canalicular membrane of hepatocytes [30]. In addition, drug–drug interactions between PXL and TOR may affect the pharmacokinetic parameters of those two drugs, since both agents can be metabolized with CYP3A4 [31, 32]. To determine the pharmacokinetic parameters resulting from treatment with a combination of PXL and TOR, we conducted a pharmacokinetic study in metastatic breast cancer patients.

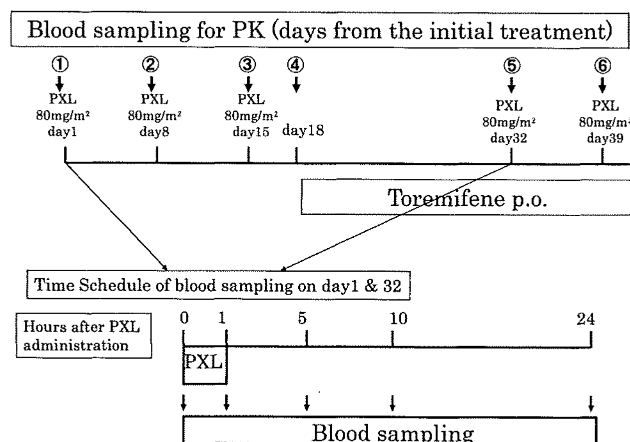

Fig. 1 Treatment and blood sampling schedule. Blood samples were collected on days 1, 8, 15, 18, 32, and 39. For the pharmacokinetic study, patients received paclitaxel monotherapy on days 1, 8, 15, 32, and 39. Patients were given toremifene (120 mg) orally from day 18 to the end of study. Blood collection was performed on days 1, 8, and 15 for the PK analysis of PXL. On day 18, blood sampling was performed before and after TOR administration for the PXL and TOR PK analyses. On days 32 and 39, we collected blood samples for the PK analyses of PXL and TOR

Material and methods

Patient eligibility criteria

Patients with histologically confirmed metastatic breast cancer, with a Eastern Clinical Oncology Group (ECOG) performance status of 0–2, 40–70 years of age, and with adequate liver and renal function, were eligible. In addition, patients who are planning to receive paclitaxel in practice and those who provided written informed consent were considered for the study.

Patient exclusion criteria

Patients were excluded if they had severe complications or were taking drugs known to be metabolized by CYP3A4, such as phenylalanine, phenobarbital, rifampicin, and carbamazepine.

Treatment

PXL formulated in Cremophor EL and dehydrated alcohol (1:1, v/v, 6 mg/mL, and taxol) was administered (i.v.). PXL (80 mg/m^2) was given for 1 h on days 1, 8, and 15 in a cycle. A cycle consisted of one week of treatment followed by one week off. Toremifene (TOR) (120 mg/(body day^{-1})) was administered orally from day 1 to day 21. Patients were repeatedly treated with a combination of PXL and TOR as long as disease

Table 1 Patient characteristics in 15 patients

Age (year)	53.0 ± 12.8 (range 33–77)
Performance status	
0–2	11
3	2
Menopausal state	
Premenopausal	6
Postmenopausal	9
Prior treatment	
Anthracycline	14
Taxane	11 (paclitaxel 9, docetaxel 9)
5FU	10
Endocrine	14
Metastatic site	
Bone	11
Lung	8
Liver	10
Others	13
ER	
Positive	11
Negative	4
Her2	
Positive	0
Negative	15

progression or unmanageable severe adverse events were defined.

For the pharmacokinetic study, PXL was initially administrated alone on days 1, 8, and 15. Beginning on day 18, TOR (120 mg) was given daily. On day 22, PXL administration in the first cycle was skipped. Blood samples were collected on day 32.

Blood sampling

Blood samples for pharmacokinetic (PK) analysis were collected on days 1, 8, 15, 18, 32, and 39 (Fig. 1). On day 1, blood samples were collected at six different time points for PK analysis of PXL alone: (1) before PXL administration, (2) 1 h after PXL administration, (3) at the end of PXL administration, (4) 3.5 h after the end of PXL administration, (5) 8.5 h after the end of PXL administration, and (6) 22.5 h after the end of PXL administration. On days 8 and 15, blood was collected 1 h after the end of PXL infusion. On day 18, blood samples were collected pre- and 1 h after TOR administration. Patients received 120 mg TOR at 8 a.m. after a meal. On day 32, blood samples from the patients who received 120 mg TOR p.o. at 8 a.m. were collected at six different time points for PK analysis of PXL, TOR and *N*-demethyltoremifene (TOR-1): (1) before PXL

Table 2 Pharmacokinetic parameters for paclitaxel (dose: paclitaxel 80 mg/m^2) without toremifene

Patient no.	$T_{1/2}$ Z (h)	AUC last (μg h/mL)	AUC inf. (μg h/mL)	CL tot [L/(h m^{-2})]	MRT (h)	V_{ss} (L/m^2)
1	9.4007	11.0846	11.4515	6.986	2.9095	20.3258
2	8.5093	6.0958	6.354	12.5906	3.6676	46.1774
3	6.9109	8.1346	8.3909	9.5341	3.384	32.2634
4	8.5592	8.1326	8.4103	9.5121	3.1104	29.5868
5	10.5148	5.9439	6.3298	12.6386	4.6365	58.5981
6	7.9377	6.594	6.8432	11.6905	3.4779	40.6582
7	10.0314	12.2528	12.9084	6.1975	4.0461	25.0759
8	8.9157	8.7407	9.1737	8.7206	4.0759	35.5442
9	8.6034	9.4411	9.8746	8.1016	3.8592	31.2651
10	10.0948	7.4109	7.983	10.0213	5.4547	54.663
11	8.4894	14.1294	14.7499	5.4238	3.7968	20.5932
12	8.3235	7.9264	8.1943	9.7629	3.1317	30.5742
13	7.9475	11.289	11.6655	6.8578	3.1531	21.6234
14	10.306	8.1792	8.562	9.3436	3.6288	33.9064
15	9.5512	5.7761	6.1572	12.9929	4.9199	63.9236
Mean	8.9	8.74	9.14	9.36	3.8	36.3
SD	1	2.47	2.56	2.39	0.7	13.8
Median	8.6	8.13	8.41	9.51	3.7	32.3
Maximum	10.5	14.13	14.75	12.99	5.5	63.9
Minimum	6.9	5.78	6.16	5.42	2.9	20.3

Paclitaxel 80 mg/m^2 was administrated intravenously for 1 h on day 1

The following pharmacokinetic parameters were evaluated: $T^{1/2}$ *Z* half-life in the terminal phase, *AUC last* area under the concentration–time curve up to the last measurement time point, *AUC inf.* area under the concentration–time curve up to infinite time, *MRT* mean residence time, *CL tot* total body clearance, V_{ss} volume of distribution at steady state

administration; (2) 1 h after PXL administration; (3) at the end of PXL administration; (4) 3.5 h after the end of PXL administration; (5) 8.5 h after the end of PXL administration; and (6) 22.5 h after the end of PXL administration. On day 39, a sample was collected 24 h after PXL infusion and 120 mg TOR p.o. administration for the PK analysis of PXL and TOR.

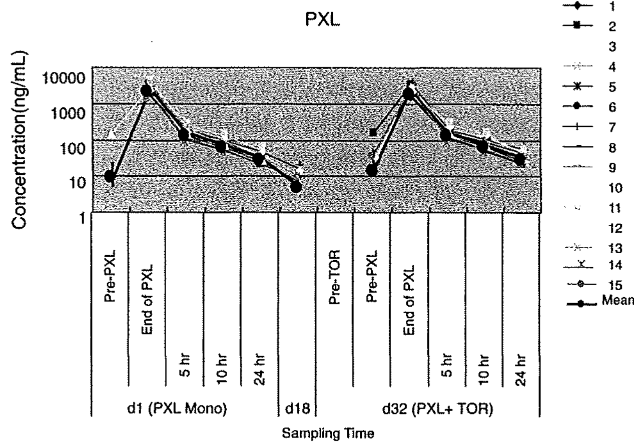

Fig. 2 Pharmacokinetic profile for paclitaxel in 15 patients. On day 1, the concentration of PXL without TOR at each sampling time is shown on the *left side* of this figure. The PXL pharmacokinetic profiles on day 32 with TOR are drawn on the *right side*

For the pharmacokinetic analysis of TOR and TOR-1, blood samples were collected in the morning after oral intake of 120 mg TOR. Samples were centrifuged at 2,100×*g* for 10 min at 4 °C, and the plasma fraction was collected and stored at −20 °C until analysis. PXL concentrations in plasma samples obtained from a peripheral vein were measured using a liquid chromatography–mass spectrometry or mass spectrometry assay.

Pharmacokinetic parameters

Concentrations of PXL, TOR, and TOR-1 were analyzed by high-performance liquid chromatography (HPLC). PK parameters were calculated using WinNonlin Professional software (v.5.0.1; Pharsight Corporation, Mountain View, CA, USA). Noncompartmental analysis was performed. Statistical analysis was performed using the *t* test.

Results

Patient background

Fifteen patients with metastatic breast cancer were enrolled. Fourteen patients received anthracycline, and 11 were treated with PXL and docetaxel (Table 1). In addition, 14 patients had previously received endocrine treatment.

Table 3 Pharmacokinetic parameters for paclitaxel (dose: paclitaxel 80 mg/m^2) with toremifene 120 mg/body

Patient no.	$T_{1/2}$ Z (h)	AUC last (μg h/mL)	AUC inf. (μg h/mL)	CL tot [L/(h m^{-2})]	MRT (h)	V_{ss} (L/m^2)
1	8.8038	5.3892	5.7266	13.9698	4.8959	68.395
2	8.6899	9.7528	10.0182	7.9854	2.6128	20.8646
3	8.0997	8.8134	9.1517	8.7416	3.5169	30.743
4	10.0986	7.7933	8.2006	9.7554	3.9509	38.5432
5	8.0958	8.2835	8.5647	9.3406	3.2274	30.1462
6	8.1442	5.8725	6.1207	13.0704	3.7166	48.578
7	11.1207	12.0039	12.8667	6.2176	4.911	30.5343
8	11.1569	6.45	7.1505	11.1881	6.8686	76.8468
9	8.0073	6.4636	6.7921	11.7783	4.3609	51.3635
10	9.7683	7.5252	8.2268	9.7243	6.4098	62.3314
11	7.3607	8.9564	9.4219	8.4909	4.861	41.2741
12	8.6712	7.8764	8.1776	9.7829	3.3407	32.6813
13	9.2115	8.2912	8.6769	9.2199	3.8067	35.0977
14	9.8566	7.8927	8.2434	9.7048	3.535	34.3065
15	8.6802	8.4938	8.7807	9.1109	3.0571	27.8527
Mean	9.1	7.99	8.41	9.87	4.2	42
SD	1.1	1.64	1.71	1.96	1.2	16.3
Median	8.7	7.89	8.24	9.7	3.8	35.1
Maximum	11.2	12	12.87	13.97	6.9	76.8
Minimum	7.4	5.39	5.73	6.22	2.6	20.9
Paired *t* test	NS	NS	NS	NS	NS	NS

On day 32, 15 patients received paclitaxel (80 mg/m^2) + toremifene (120 mg)

The following pharmacokinetic parameters were evaluated: $T_{1/2}$ Z half-life in the terminal phase, *AUC last* area under the concentration–time curve up to the last measurement time point, *AUC inf.* area under the concentration-time curve up to infinite time, *CL tot* total body clearance, V_{ss} volume of distribution at steady state. Each parameter was statistically analyzed between paclitaxel alone and paclitaxel + toremifene. *NS* $P > 0.05$ compared with paclitaxel alone