Table 3. Multivariable Cox's proportional hazards model analysis of prognostic factors for progression-free survival and overall survival in patients with advanced stage serous ovarian cancers ($n = 72$)

Variable	Hazard ratio (95% CI)	*P*-value
Progression-free survival		
ZEB2 expression	1.37 (1.07–1.78)	0.014*
Age	0.98 (0.96–1.00)	0.095
Optimal surgery (vs not optimal)	0.60 (0.44–0.82)	0.0011*
Grade 2 (vs Grade 1)	0.85 (0.58–1.24)	0.42
Grade 3 (vs Grade 1)	1.41 (0.98–2.06)	0.060
Overall survival		
ZEB2 expression	1.53 (1.05–2.22)	0.027*
Age	1.01 (0.96–1.04)	0.71
Optimal surgery (vs not optimal)	0.67 (0.41–1.05)	0.079
Grade 2 (vs Grade 1)	0.83 (0.47–1.50)	0.53
Grade 3 (vs Grade 1)	1.51 (0.93–2.62)	0.10

*Statistically significant ($P < 0.05$).

Table 4. Comparison of progression-free survival and overall survival in four groups with different expression profiles of *CDH1* and *ZEB2*

Serous ovarian cancer ($n = 72$)	Hazard ratio	95% CI	*P*-value
Progression-free survival			
CDH1 high/*ZEB2* low ($n = 23$)	1.00		
CDH1 high/*ZEB2* high ($n = 13$)	0.91	(0.53–1.43)	0.69
CDH1 low/*ZEB2* low ($n = 13$)	1.29	(0.83–1.94)	0.25
CDH1 low/*ZEB2* high ($n = 23$)	1.65	(1.18–2.35)	0.0035*
Overall survival			
CDH1 high/*ZEB2* low ($n = 23$)	1.00		
CDH1 high/*ZEB2* high ($n = 13$)	0.96	(0.37–1.96)	0.91
CDH1 low/*ZEB2* low ($n = 13$)	1.12	(0.63–1.95)	0.70
CDH1 low/*ZEB2* high ($n = 23$)	1.77	(1.12–2.92)	0.013*

subclass 2 ($n = 18$). This result was compatible with findings by Berchuck *et al.*[7] demonstrating similarities in gene expression between early stage serous ovarian cancers and a subset of advanced stage serous ovarian cancers that had favorable prognosis. Regarding the sample size in the current microarray analysis, one can realize that this may be first-stage evidence on ovarian expression profile associated with tumor progression. However, we successfully provided valuable insights that clarify the molecular mechanism of tumor progression using NMF algorithm.

Kurman *et al.* divide epithelial ovarian cancers into two groups designated type I and type II based on clinical, pathological, and molecular genetic studies.[21] Type I tumors are low grade and slow growing (including endometrioid, mucinous, and low-grade serous). Type II tumors (including high grade serous and undifferentiated) are rapidly growing, more aggressive, and are frequently associated with *TP53* mutation. In our experiments, the frequency of *TP53* mutation was higher in cases belonging to subclass 2 (9/18, 50%) compared to those belonging to subclass 1 + stage I (8/25, 32%). Although the frequency difference was not statistically significant, our novel subclassification based on gene expression profile might have a potential relationship with that of the two-type classification model of ovarian cancer proposed by Kurman *et al.*[21] Further study will be necessary to elucidate other biological and pathological implications except tumor progression in our subclassification.

After screening genes associated with tumor progression and subsequent validation of the association, we identified the expression of *ZEB2* and *CDH1* as prognostic factors for serous ovarian cancers. Although other genome-wide expression analyses[7–10] have identified gene expression profiles with prognosis values in patients with ovarian cancer, *ZEB2* and *CDH1* are not listed in their profiles. Previous studies using the expression microarrays investigate directly the association between gene expression level and survival time in patients with ovarian cancer, whereas we first extracted gene expression profiles reflecting tumor progression by a stepwise approach (Supplementary Fig. 1), and selected survival-associated genes with biological function from these genes. Furthermore, differences in microarray platforms, normalization methods, degrees of contamination by non-cancer cells in a given tumor specimen, and the patient populations under study[38] were observed between previous reports and ours. These points might contribute to the development of inconsistencies in lists of survival-associated genes from the microarray studies.

Our data also suggest that reduced *CDH1* expression is a key to subclassify advanced stage serous ovarian cancers. Recently Tothill *et al.* reported that six molecular subtypes of ovarian cancers, including serous and endometrioid histological types, were identified by a *k*-means clustering method according to genome-wide expression data from 285 ovarian cancer samples.[39] Of the six molecular subtypes, one subtype (C5 in the paper), comprising mainly high grade serous ovarian cancer samples, is characterized by reduced E-cadherin. Despite the difference in experimental design of the two studies, our data are compatible with their finding that a molecular subtype of ovarian cancers can be tagged by E-cadherin expression. E-cadherin is a hallmark of epithelial–mesenchymal transition, and a reduction of E-cadherin is thought to result in dysfunction of the cell–cell junction system, triggering cancer invasion in various human malignancies. In our experiment, E-cadherin expression was significantly associated with prognosis in patients with advanced stage serous ovarian cancer at both the mRNA and protein levels. Therefore, it is important to clarify the regulatory mechanisms of *CDH1* expression[40] in serous ovarian cancer in terms of tumor progression and prognosis, as well as subclassification.

Recent study shows that the interaction of Snail, ZEB, and bHLH factors regulates *CDH1* repression and epithelial–mesenchymal transition.[23] Besides *ZEB2*, other transcriptional repressors may reduce *CDH1* expression and lead to epithelial–mesenchymal transition.[41] Indeed, *SNAI2* was included in the 112 subclass-specific genes, and was found to directly interact with *CDH1* in the newly obtained IPA network (Fig. 2B). Previous reports show that other transcriptional repressors such as Snail 1 and Twist are related to prognosis in ovarian cancer, using immunohistochemical analysis.[35,42] Hosono *et al.*[42] have reported that expression of Twist is a significant prognostic factor in non-serous type but not in serous type tumors. Our results demonstrate that expression of *ZEB2* is negatively correlated with *CDH1* expression, and that the expression signature of increased *ZEB2* and reduced *CDH1* in ovarian tumor tissues is related to poor prognosis in serous ovarian cancer patients (Table 4). Furthermore, siRNA-mediated suppression of *ZEB2* in the serous type of ovarian cancer SKOV3 cells leads to an increase in *CDH1* expression (Supplementary Fig. 3), suggesting that *ZEB2* regulates *CDH1* expression in serous histological type tumors. To validate that *ZEB2* expression at the protein level is a significant prognostic factor, we would like to analyze *ZEB2* expression in a larger number of patients stratified according to individual histological types using immunohistochemical staining.

Park *et al.* have recently reported that microRNA-200 directly targets the mRNA of *ZEB2* as well as that of *ZEB1*, and indirectly controls the expression level of *CDH1* in cancer cell lines.[43] Further investigation is required to elucidate the more detailed mechanisms by which the *ZEB2–CDH1* axis in epithelial–mesenchymal transition is regulated in the process of ovarian cancer progression. Clarification of the mechanisms for the regulation of *ZEB2–CDH1* expression may provide plausible targets for the development of therapeutic strategies in the clinical management of serous ovarian cancers.

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Abbreviations

ACTA2	actin, alpha 2, smooth muscle, aorta
ACTB	actin, beta
bHLH	basic helix-loop-helix
BRCA1	breast cancer 1, early onset
CA125	carbohydrate antigen 125
CDH1	cadherin 1
COL16A1	collagen, type XVI, alpha 1
coph	cophenetic correlation coefficient
Cy3	cyamine 3-CTP
FN1	fibronectin 1
GO	gene ontology
IPA	Ingenuity Pathway Analysis
LTBP2	latent transforming growth factor beta binding protein 2
NMF	non-negative matrix factorization
SNAI1	snail homolog 1
TBP	TATA box binding protein
TP53	Tumor Protein p53
VIM	vimentin
ZEB2	zinc finger E-box binding homeobox 2

References

1 Disaia PJ, Creasman WT. Epithelial ovarian cancer. In: Disaia PJ, Creasman WT, eds. *Clinical Gynecologic Oncology*, 6th edn. St Louis: Mosby, 2002; 289–350.
2 Cannistra SA. Cancer of the ovary. *N Engl J Med* 2004; **351**: 2519–29.
3 Agarwal R, Kaye SB. Expression profiling and individualization of treatment for ovarian cancer. *Curr Opin Pharmacol* 2006; **6**: 345–9.
4 Olivier RI, van Beurden M, van't Veer LJ. The role of gene expression profiling in the clinical management of ovarian cancer. *Eur J Cancer* 2006; **42**: 2930–8.
5 Fehrmann RS, Li XY, van der Zee AG *et al.* Profiling studies in ovarian cancer: a review. *Oncologist* 2007; **12**: 960–6.
6 Spentzos D, Levine DA, Ramoni MF *et al.* Gene expression signature with independent prognostic significance in epithelial ovarian cancer. *J Clin Oncol* 2004; **22**: 4700–10.
7 Berchuck A, Iversen ES, Lancaster JM *et al.* Patterns of gene expression that characterize long-term survival in advanced stage serous ovarian cancers. *Clin Cancer Res* 2005; **11**: 3686–96.
8 Hartmann LC, Lu KH, Linette GP *et al.* Gene expression profiles predict early relapse in ovarian cancer after platinum-paclitaxel chemotherapy. *Clin Cancer Res* 2005; **11**: 2149–55.
9 Bonome T, Levine DA, Shih J *et al.* A gene signature predicting for survival in suboptimally debulked patients with ovarian cancer. *Cancer Res* 2008; **68**: 5478–86.
10 Le Page C, Ouellet V, Quinn MC, Tonin PN, Provencher DM, Mes-Masson AM. BTF4/BTNA3.2 and GCS as candidate mRNA prognostic markers in epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 913–20.
11 Dressman HK, Berchuck A, Chan G *et al.* An integrated genomic-based approach to individualized treatment of patients with advanced-stage ovarian cancer. *J Clin Oncol* 2007; **25**: 517–25.
12 Newton TR, Parsons PG, Lincoln DJ *et al.* Expression profiling correlates with treatment response in women with advanced serous epithelial ovarian cancer. *Int J Cancer* 2006; **119**: 875–83.
13 Partheen K, Levan K, Osterberg L, Horvath G. Expression analysis of stage III serous ovarian adenocarcinoma distinguishes a sub-group of survivors. *Eur J Cancer* 2006; **42**: 2846–54.
14 Okamoto A, Nikaido T, Ochiai K *et al.* Indoleamine 2,3-dioxygenase serves as a marker of poor prognosis in gene expression profiles of serous ovarian cancer cells. *Clin Cancer Res* 2005; **11**: 6030–9.
15 Donninger H, Bonome T, Radonovich M *et al.* Whole genome expression profiling of advance stage papillary serous ovarian cancer reveals activated pathways. *Oncogene* 2004; **23**: 8065–77.
16 Meinhold-Heerlein I, Bauerschlag D, Hilpert F *et al.* Molecular and prognostic distinction between serous ovarian carcinomas of varying grade and malignant potential. *Oncogene* 2005; **24**: 1053–65.
17 Bonome T, Lee JY, Park DC *et al.* Expression profiling of serous low malignant potential, low-grade, and high-grade tumors of the ovary. *Cancer Res* 2005; **65**: 10602–12.
18 Shridhar V, Lee J, Pandita A *et al.* Genetic analysis of early-versus late-stage ovarian tumors. *Cancer Res* 2001; **61**: 5895–904.
19 De Cecco L, Marchionni L, Gariboldi M *et al.* Gene expression profiling of advanced ovarian cancer: characterization of a molecular signature involving fibroblast growth factor 2. *Oncogene* 2004; **23**: 8171–83.
20 Lancaster JM, Dressman HK, Clarke JP *et al.* Identification of genes associated with ovarian cancer metastasis using microarray expression analysis. *Int J Gynecol Cancer* 2006; **16**: 1733–45.
21 Kurman RJ, Visvanathan K, Roden R, Wu TC, Shin IeM. Early detection and treatment of ovarian cancer: shifting from early stage to minimal volume disease based on a new model of carcinogenesis. *Am J Obstet Gynecol* 2008; **198**: 351–6.
22 Thiery JP. Epithelial mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002; **2**: 442–54.
23 Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* 2007; **7**: 415–28.
24 FIGO Cancer Committee. Staging Announcement: FIGO Cancer Committee. *Gynecol Oncol* 1986; **25**: 383–5.
25 Amikura T, Sekine M, Hirai Y *et al.* Mutational analysis of TP53 and p21 in familial and sporadic ovarian cancer in Japan. *Gynecol Oncol* 2006; **100**: 365–71.
26 Sekine M, Nagata H, Tsuji S *et al.* Mutational analysis of BRCA1 and BRCA2 and clinicopathologic analysis of ovarian cancer in 82 ovarian cancer families: two common founder mutations of BRCA1 in Japanese population. *Clin Cancer Res* 2001; **7**: 3144–50.
27 Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* 1995; **57**: 289–300.
28 Brunet JP, Tamayo P, Golub TR, Mesirov JP. Metagenes and molecular pattern discovery using matrix factorization. *Proc Natl Acad Sci USA* 2004; **101**: 4164–9.
29 Okada H, Tajima A, Shichiri K, Tanaka A, Tanaka K, Inoue I. Genome-wide expression of azoospermia testes demonstrates a specific profile and implicates ART3 in genetic susceptibility. *PLoS Genet* 2008; **4**: e26.
30 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* 2001; **25**: 402–8.
31 Inamura K, Fujiwara T, Hoshida Y *et al.* Two subclasses of lung squamous cell carcinoma with different gene expression profiles and prognosis identified by hierarchical clustering and non-negative matrix factorization. *Oncogene* 2005; **24**: 7105–13.
32 Daraï E, Scoazec JY, Walker-Combrouze F *et al.* Expression of cadherins in benign, borderline, and malignant ovarian epithelial tumors: a clinicopathologic study of 60 cases. *Hum Pathol* 1997; **28**: 922–8.
33 Faleiro-Rodrigues C, Macedo-Pinto I, Pereira D, Lopes CS. Prognostic value of E-cadherin immunoexpression in patients with primary ovarian carcinomas. *Ann Oncol* 2004; **15**: 1535–42.
34 Voutilainen KA, Anttila MA, Sillanpää SM *et al.* Prognostic significance of E-cadherin–catenin complex in epithelial ovarian cancer. *J Clin Pathol* 2006; **59**: 460–7.
35 Blechschmidt K, Sassen S, Schmalfeldt B, Schuster T, Höfler H, Becker KF. The E-cadherin repressor Snail is associated with lower overall survival of ovarian cancer patients. *Br J Cancer* 2007; **98**: 489–95.
36 Imamichi Y, König A, Gress T, Menke A. Collagen type I-induced Smad-interacting protein 1 expression downregulates E-cadherin in pancreatic cancer. *Oncogene* 2007; **26**: 2381–5.
37 Gao Y, Church G. Improving molecular cancer class discovery through sparse non-negative matrix factorization. *Bioinformatics* 2005; **21**: 3970–5.
38 Konstantinopoulos PA, Spentzos D, Cannistra SA. Gene-expression profiling in epithelial ovarian cancer. *Nat Clin Pract Oncol* 2008; **5**: 577–87.
39 Tothill RW, Tinker AV, George J *et al.* Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome. *Clin Cancer Res* 2008; **14**: 5198–208.
40 Liu YN, Lee WW, Wang CY, Chao TH, Chen Y, Chen JH. Regulatory mechanisms controlling human E-cadherin gene expression. *Oncogene* 2005; **24**: 8277–90.
41 Imai T, Horiuchi A, Wang C *et al.* Hypoxia attenuates the expression of E-cadherin via up-regulation of SNAIL in ovarian carcinoma cells. *Am J Pathol* 2003; **163**: 1437–47.

42 Hosono S, Kajiyama H, Terauchi M *et al.* Expression of Twist increases the risk for recurrence and for poor survival in epithelial ovarian carcinoma patients. *Br J Cancer* 2007; **96**: 314–20.

43 Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* 2008; **22**: 894–907.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Analytical process to extract 'subclass-specific genes'.

Fig. S2. Association between E-cadherin expression and prognosis of advanced stage serous ovarian cancers validated by immunohistochemical analyses.

Fig. S3. Interaction between *ZEB2* and *CDH1*.

Table S1. Comparison of clinicopathological characteristics between microarray set and validation set

Table S2. List of 23 transcripts analyzed by quantitative real-time RT-PCR in this study

Table S3. One hundred and twelve transcripts representing statistically significant expression differences between two subclasses of advanced stage serous ovarian cancers

Table S4. Expression levels of 23 genes by quantitative real-time RT-PCR were significantly different between subclass 1 (S1) and subclass 2 (S2)

Supplementary Methods Methods about GO analysis, Pathway analysis, siRNA experiments, and immunohistochemical analysis

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Relationship between single nucleotide polymorphisms in *CYP1A1* and *CYP1B1* genes and the bone mineral density and serum lipid profiles in postmenopausal Japanese women taking hormone therapy

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Abstract

Objective: The genetic variations of the genes encoding cytochrome P-450 enzymes are considered to play an important role in the metabolism of estradiol. The objective of this study was to evaluate the relationships among single nucleotide polymorphisms (SNPs) of cytochrome P-450 genes, lumbar bone mineral density (BMD), and serum lipids and to determine the effects of hormone therapy (HT).

Design: The participants were 124 Japanese women who had been diagnosed with osteopenia or osteoporosis and were taking HT for 12 months. Seven single nucleotide polymorphisms in the *CYP1A1* and *CYP1B1* genes were characterized. Lumbar BMD and the levels of serum lipids were measured before and after HT.

Results: A single nucleotide polymorphism in exon 3 of *CYP1B1* was found to be significantly associated with the effect of HT on BMD and low-density lipoprotein cholesterol both in univariate and multivariate analyses. In the women with the GG genotype of L432V, the responses to HT of BMD and low-density lipoprotein cholesterol markedly decreased. The serum follicle-stimulating hormone level after HT was significantly higher in the women with the GG genotype of L432V.

Conclusions: These results suggest that the L432V polymorphism in the *CYP1B1* gene could therefore be used to predict the effect of HT on lumbar BMD and low-density lipoprotein cholesterol in Japanese women.

Key Words: Single nucleotide polymorphism – *CYP1A1* – *CYP1B1* – Hormone therapy – Bone mineral density – Low-density lipoprotein cholesterol – Follicle-stimulating hormone.

Estrogen plays a significant role in bone and lipid metabolism, and its deficiency after menopause is the main reason for accelerated bone loss and deterioration of the serum lipid profiles, which are preventable by estrogen administration. A number of observational studies have suggested that hormone therapy (HT) reduces the risk of fractures and coronary events in postmenopausal women.[1-4] However, recently published results from randomized clinical trials of HT indicate that this therapy does not slow the progression of coronary atherosclerosis, whereas the reduction in the hip and clinical vertebral fracture rate is significant.[5,6] Our understanding is limited regarding why not all women benefit from such therapy. However, it is still possible that a genetically determined subgroup of the population could benefit from this therapy.

Postmenopausal HT is generally an effective treatment modality to prevent bone loss while also improving the serum lipid profiles; however, individual variations exist.[7-9] Some postmenopausal women respond strongly to HT, whereas approximately 8% who are compliant with this therapy are nonetheless nonresponders.[2] This raises the possibility that some genetic determinants as well as gene-environment interactions might modulate the responses to HT in individual participants.

Individual genetic variability of estradiol metabolism has been described as a significant contributor to the disease susceptibility with variations depending on ethnic background. Among others, the genetic variations of the genes encoding cytochrome P-450 (CYP) enzymes are considered to play an important role in this regard.[10] CYP enzymes play an important role in the production, bioavailability, and degradation of estradiol. A series of polymorphisms and mutations of the CYP enzyme complex have been identified. *CYP1A1* and *CYP1B1* catalyze the hydroxylation of estradiol and several single nucleotide polymorphic sites of those genes have been described.[11,12] Polymorphisms, especially single nucleotide polymorphisms (SNPs) exist in the exon with amino acid changes, thus leading to functionally relevant biochemical consequences that are therefore capable of influencing the responses to HT.

In this study, we attempted to clarify whether SNPs in the exons of the *CYP1A1* and *CYP1B1* genes affected the change in bone mineral density (BMD) and serum lipid profiles in postmenopausal Japanese women during HT.

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METHODS

Design

The participants were 124 Japanese women, ranging in age from 40 to 64 years (49.8 ± 1.0 y, mean ± SEM) who had been diagnosed with osteopenia or osteoporosis and were willing to take HT for 12 months. The diagnoses of osteopenia and osteoporosis were based on the criteria recommended by the Japanese Society of Bone and Mineral Research: a lumbar BMD (L2-4) of less than 80% and less than 70% in younger adults (20-44 y), respectively. In all cases, more than 6 months had elapsed since the last menstrual period, the serum estradiol level was lower than 20 pg/mL, and the serum follicle-stimulating hormone (FSH) level was more than 50 mIU/mL. The exclusion criteria were a history of metabolic disease (including hyperparathyroidism, previously diagnosed osteoporosis, or nontraumatic vertebral fracture on baseline radiograph), chronic disease (uncontrolled hypo- or hyperthyroidism, liver disease, or unstable cardiac disease), cancer or thromboembolic disease, a history of treatment with glucocorticoids for more than 6 months, current HT use or HT use within the past 3 months, or a metabolic or other endocrine disease that could influence lipid metabolism. None of the women smoked or drank alcohol to excess, and none engaged in regular strenuous exercise. Furthermore, none had a history of illness or medical therapy, apart from HT, that might affect bone turnover or lipid metabolism. The women were not genetically related. HT was administered either in a sequential regimen (50 women) consisting of 0.625 mg conjugated equine estrogens for 24 days (days 1-24) and 5 mg medroxyprogesterone acetate for 10 days (days 15-24) or a continuous regimen (74 women) consisting of 0.625 mg conjugated equine estrogens and 2.5 mg medroxyprogesterone acetate for 28 days, according to the woman's preference.

Measures

Bone densitometry

BMD, expressed as the mass per unit area (g/cm^2), was measured in the anteroposterior plane of the lumber spine (L2-4), using dual-energy x-ray absorptiometry with a QDR-2000 analyzer (Hologic Inc., Waltham, MA); absorptiometries were examined by the same observer. The average coefficients of variation of the phantom measurements of bone mineral content, bone area, and BMD during the study period were 1.1%, 0.7%, and 0.6%, respectively. In addition, in the control women, the coefficient of variation of the in vivo precision of BMD between two measurements (mean interval: 2.6 ± 1.2 y) was 0.9%. There was no scanner drift observed during the study period. BMD change (ΔBMD) was expressed as the percentage of BMD change compared with the pretreatment baseline.

Analysis of lipids

After an overnight fast (a minimum 12-h fast), blood was collected from each woman to estimate the lipids and lipoproteins. We measured the total cholesterol (Determiner L-TCN; Kyowa Medex, Tokyo, Japan) and triglyceride (L-type Wako TG-H; Wako Pure Chemical, Osaka, Japan) concentrations by enzymatic methods, and the high-density lipoprotein cholesterol concentration by a homogeneous method (Determiner L HDL-C, Kyowa Medex) using a Hitachi 7450 automated analyzer. Low-density lipoprotein cholesterol was calculated using Friedewald's equation.

Hormones and assays

The serum hormone levels were evaluated after 12 months of HT. Blood samples were drawn in the morning after an overnight fast. The serum was separated immediately and frozen at −80°C for future analysis. The hormone levels were measured using an electrochemiluminescent immunoassay for estradiol and a chemiluminescent immunoassay for luteinizing hormone (LH) and FSH. The hormone fractions were measured in three different batches, and a laboratory batch was also treated to determine the random effect in all hormone analyses. The sensitivity, expressed as the minimal detectable dose, was 11.0 pg/mL, 0.11 mIU/mL, and 0.06 mIU/mL for estradiol, LH, and FSH, respectively. The intra- and interassay coefficients of variation were 1.63% and

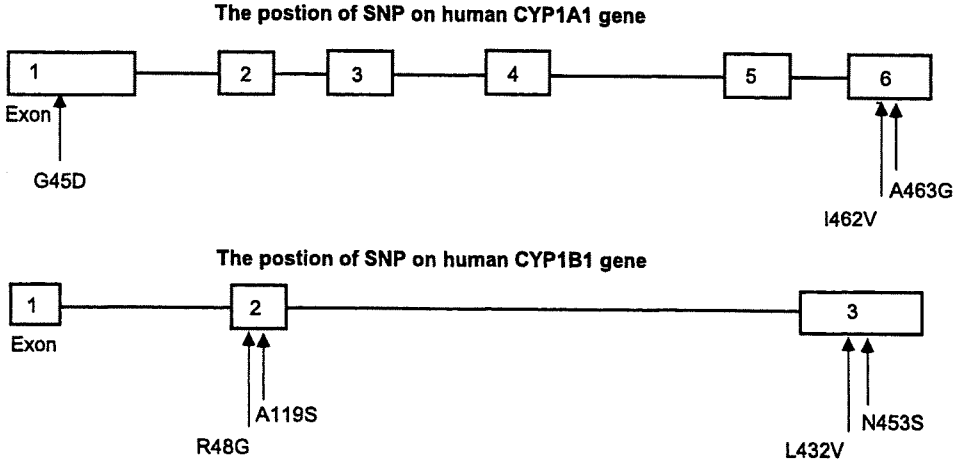

FIG. 1. The position of each single nucleotide polymorphism (SNP) in the *CYP1A1* and *CYP1B1* genes.

TABLE 1. *Genotype and allele frequencies of seven SNPs of CYP gene in Japanese participants*

Gene	Polymorphism	Genotype	Homo (major)	Hetero	Homo (minor)	Allele frequency (major allele) In this study	Sasaki et al	In dbSNP	JSNP ID	dbSNP
CYP1A1	G45D	GA	124 (100%)	0 (0%)	0 (0%)	1.00	—	—	ssj0003953	rs4646422
	I462V	AG	78 (62.9%)	42 (33.9%)	4 (3.2%)	0.80	—	0.902	ssj0007951	rs1048943
	A463G	CG	124 (100%)	0 (0%)	0 (0%)	1.00	—	—	IMS-JST026484	rs2278970
CYP1B1	R48G	CG	90 (72.6%)	16 (12.9%)	18 (1.5%)	0.79	0.68	0.653	ssj0007955	rs10012
	A119S	GT	124 (100%)	0 (0%)	0 (0%)	1.00	0.85	0.648	ssj0007956	rs1056827
	L432V	CG	78 (62.9%)	36 (29.0%)	10 (8.1%)	0.77	0.82	0.592	IMS-JST085313	rs1056836
	N453S	AG	124 (100%)	0 (0%)	0 (0%)	1.00	1.00	0.889	—	rs1800440

SNPs, single nucleotide polymorphisms; CYP, cytochrome P-450; Homo, homozygous; Hetero, heterozygous; JSNP, Japanese Single Nucleotide Polymorphism (database); dbSNP, Single Nucleotide Polymorphism database.

2.24% for estradiol, 3.37% and 3.62% for LH, and 3.50% and 5.28% for FSH.

DNA isolation and genotyping

The peripheral blood samples were collected after informed consent was obtained from each woman. Genomic DNA was extracted from the peripheral blood leukocytes using a DNA purification kit (QIAamp DNA Blood Mini kit; Qiagen, Valencia, CA) according to the manufacturer's instructions. All polymerase chain reactions were performed on a Perkin Elmer GeneAmp 9700 system, and the presence of amplicons was checked on agarose gel. A single nucleotide primer extension assay was performed to analyze SNPs using a SNaPshot Kit (Applied Biosystems, Foster City, CA). The extended primers were analyzed on an ABI 3100 device (Applied Biosystems). The primer sequences for the polymerase chain reactions and primer extension reactions are available in the Japanese Single Nucleotide Polymorphism database. Initial denaturation was performed at 95°C for 2 minutes, followed by 35 cycles each consisting of denaturation at 95°C for 30 seconds, annealing at 60°C, and extension at 72°C for 1 minute, followed by final extension at 72°C for 8 minutes. This study was approved by the Niigata University Human Investigation Committee.

Statistical analysis

Differences in the baseline characteristics, the absolute BMD value, and the serum lipid concentrations among genotypes were tested using an analysis of covariance with age and BMI as covariates. The values of triglycerides were not normally distributed and needed to be log-transformed for the statistical comparisons but, for clarity for presentation, the nontransformed values are presented in the text and tables. To evaluate the relationships between CYP polymorphisms and the change in BMD or serum lipid concentrations during HT, we used repeated-measures analysis of variance. A multiple linear regression model was used to evaluate the simultaneous contributions of different variables. Only those variables that had values of $P < 0.05$ in the univariate analysis were included in the multivariate analyses. All data are expressed as the mean ± SEM. Differences of $P < 0.05$ were considered to indicate statistical significance. All data management and statistical computations were performed with the StatView 4.0 (Abacus Concepts, Berkeley, CA) or the SPSS 10.0 software program (SPSS Inc., Chicago, IL).

RESULTS

In this study, we characterized seven SNPs, three SNPs in the *CYP1A1* gene and four SNPs in the *CYP1B1* gene, from a

TABLE 2. *Baseline characteristics according to the CYP genotypes*

Variables	Genotype of I462V (*CYP1A1*) AA (n = 78)	AG (n = 42)	GG (n = 4)	*P*	Genotype of R48G (*CYP1B1*) CC (n = 90)	CG (n = 16)	GG (n = 18)	*P*	Genotype of L432V (*CYP1B1*) CC (n = 78)	CG (n = 36)	GG (n = 10)	*P*
Age, y	50.1 ± 0.8	49.2 ± 0.8	51.3 ± 1.8	0.73	49.6 ± 0.7	51.5 ± 1.4	49.4 ± 1.2	0.53	50.6 ± 0.8	49.0 ± 0.9	47.8 ± 1.4	0.74
Age at menopause, y	47.4 ± 0.6	47.7 ± 0.6	49.0 ± 1.9	0.89	47.6 ± 0.5	46.3 ± 1.6	48.3 ± 0.5	0.46	47.8 ± 0.6	47.2 ± 0.6	46.6 ± 1.9	0.39
Height, cm	154.9 ± 0.6	151.6 ± 2.4	157.8 ± 7.6	0.19	154.9 ± 0.6	154.9 ± 1.5	153.3 ± 0.9	0.51	153.6 ± 0.9	156.1 ± 1.2	158.5 ± 1.1	0.66
Weight, kg	52.5 ± 0.7	51.7 ± 1.0	51.0 ± 6.0	0.76	51.9 ± 0.7	53.4 ± 1.6	52.5 ± 1.6	0.64	52.0 ± 0.7	52.2 ± 1.2	53.2 ± 2.5	0.40
BMI, kg/m^2	21.9 ± 0.26	25.3 ± 3.5	20.3 ± 0.9	0.38	21.6 ± 0.2	22.3 ± 0.6	22.4 ± 0.6	0.34	23.7 ± 1.9	21.7 ± 0.4	22.1 ± 0.7	0.74
L2-4 BMD, g/cm^3	0.76 ± 0.02	0.76 ± 0.02	0.79 ± 0.08	0.96	0.76 ± 0.02	0.79 ± 0.05	0.75 ± 0.05	0.78	0.77 ± 0.02	0.76 ± 0.02	0.78 ± 0.07	0.23
TC, mg/dL	224.4 ± 4.2	227.2 ± 6.5	231.0 ± 13.5	0.89	223.8 ± 3.9	224.9 ± 8.1	234.5 ± 11.8	0.54	226.2 ± 4.3	228.6 ± 6.8	216.1 ± 10.9	0.65
LDL-C, mg/dL	132.6 ± 4.7	137.3 ± 5.9	132.5 ± 7.2	0.82	130.8 ± 3.8	140.1 ± 0.8	147.3 ± 13.0	0.24	136.1 ± 4.8	135.2 ± 6.2	123.9 ± 10.6	0.60
HDL-C, mg/dL	67.6 ± 1.9	67.0 ± 2.9	77.0 ± 6.8	0.51	67.5 ± 1.8	67.0 ± 2.9	69.6 ± 6.1	0.88	67.4 ± 1.9	68.0 ± 3.1	67.0 ± 6.1	0.98
TGs, mg/dL	122.9 ± 8.8	115.8 ± 11.3	84.8 ± 12.5	0.55	121.2 ± 8.4	114.4 ± 14.3	102.1 ± 11.8	0.60	115.7 ± 9.0	125.3 ± 11.4	125.7 ± 24.5	0.76

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

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^2	2.4 ± 0.6	2.1 ± 1.2	3.9 ± 1.5	0.833	2.4 ± 0.6	2.6 ± 1.4	1.7 ± 1.2	0.872	3.4 ± 0.6	1.8 ± 1.0	−3.7 ± 2.4	0.002
	(0.78 ± 0.02)	(0.77 ± 0.02)	(0.81 ± 0.09)		(0.78 ± 0.01)	(0.79 ± 0.04)	(0.76 ± 0.04)		(0.78 ± 0.02)	(0.77 ± 0.02)	(0.74 ± 0.06)	
TC, mg/dL	−3.8 ± 2.3	−4.8 ± 1.9	−6.3 ± 6.6	0.9330	−4.5 ± 2.0	−4.0 ± 3.3	−3.1 ± 4.2	0.953	−4.2 ± 1.7	−9.4 ± 3.5	5.5 ± 4.4	0.058
	(212.0 ± 4.6)	(211.3 ± 5.5)	(213.5 ± 5.3)		(212.0 ± 3.5)	(213.4 ± 6.2)	(221.6 ± 8.7)		(210.3 ± 4.5)	(206.1 ± 4.7)	(226.1 ± 11.4)	
LDL-C, mg/dL	−11.0 ± 4.0	−17.4 ± 3.2	−16.6 ± 6.1	0.455	−13.5 ± 3.2	−6.3 ± 6.5	−20.5 ± 7.4	0.302	−15.6 ± 3.8	−18.0 ± 4.2	11.1 ± 3.5	0.002
	(116.8 ± 5.0)	(118.3 ± 3.2)	(114.0 ± 4.4)		(114.6 ± 4.3)	(125.0 ± 5.4)	(124.6 ± 6.9)		(115.6 ± 4.9)	(114.4 ± 4.3)	(140.0 ± 10.9)	
HDL-C, mg/dL	3.0 ± 2.9	8.7 ± 3.5	3.0 ± 3.1	0.408	4.5 ± 2.8	5.3 ± 2.5	7.2 ± 4.1	0.894	4.5 ± 1.9	3.0 ± 6.0	−2.5 ± 4.1	0.827
	(71.3 ± 2.0)	(71.8 ± 2.8)	(78.8 ± 4.0)		(70.8 ± 1.7)	(71.9 ± 3.3)	(75.8 ± 5.2)		(71.5 ± 2.0)	(70.9 ± 2.6)	(68.4 ± 7.4)	
TGs, mg/dL	15.7 ± 6.7	14.5 ± 8.6	38.3 ± 25.8	0.698	19.8 ± 6.5	1.9 ± 14.4	10.7 ± 6.4	0.252	21.2 ± 6.5	5.9 ± 10.3	16.8 ± 13.5	0.357
	(129.6 ± 7.0)	(115.7 ± 10.3)	(111.5 ± 38.3)		(127.9 ± 6.7)	(113.1 ± 11.3)	(115.0 ± 16.3)		(122.1 ± 6.8)	(124.5 ± 11.5)	(137.0 ± 19.9)	

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.

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.

REFERENCES

1. Kiel DP, Felson DT, Anderson JJ, et al. Hip fracture and the use of estrogens in postmenopausal women. The Framingham Study. *N Engl J Med* 1987;317:1169-1174.
2. Rosen CJ, Kessenich CR. The pathophysiology and treatment of postmenopausal osteoporosis. An evidence-based approach to estrogen replacement therapy. *Endocrinol Metab Clin North Am* 1997;26: 295-311.
3. Nabulsi AA, Folsom AR, White A, et al. Association of hormone-replacement therapy with various cardiovascular risk factors in post-

menopausal women. The Atherosclerosis Risk in Communities Study Investigators. *N Engl J Med* 1993;328:1069-1075.
4. Stampfer MJ, Colditz GA, Willett WC, et al. Postmenopausal estrogen therapy and cardiovascular disease. Ten-year follow-up from the Nurses' Health Study. *N Engl J Med* 1991;325:756-762.
5. Herrington DM, Reboussin DM, Brosnihan KB, et al. Effects of estrogen replacement on the progression of coronary-artery atherosclerosis. *N Engl J Med* 2000;343:522-529.
6. Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *JAMA* 2002;288:321-333.
7. Hassager C, Jensen S, Christiansen C. Non-responders to hormone replacement therapy for the prevention of postmenopausal bone loss: do they exist? *Osteoporos Int* 1994;4:36-41.
8. Herrington DM, Howard TD, Hawkins GA, et al. Estrogen-receptor polymorphisms and effects of estrogen replacement on high-density lipoprotein cholesterol in women with coronary disease. *N Engl J Med* 2002;346:967-974.
9. Komulainen M, Kroger H, Tuppurainen MT, et al. Identification of early postmenopausal women with no bone response to HRT: results of a five-year clinical trial. *Osteoporos Int* 2000;11:211-218.
10. Huber JC, Schneeberger C, Tempfer CB. Genetic modelling of the estrogen metabolism as a risk factor of hormone-dependent disorders. *Maturitas* 2002;42:1-12.
11. Sasaki M, Tanaka Y, Kaneuchi M, et al. Alleles of polymorphic sites that correspond to hyperactive variants of CYP1B1 protein are significantly less frequent in Japanese as compared to American and German populations. *Hum Mutat* 2003;21:652.
12. Hefler LA, Tempfer CB, Grimm C, et al. Estrogen-metabolizing gene polymorphisms in the assessment of breast carcinoma risk and fibroadenoma risk in Caucasian women. *Cancer* 2004;101:264-269.
13. Huang CS, Chern HD, Chang KJ, et al. Breast cancer risk associated with genotype polymorphism of the estrogen-metabolizing genes CYP17, CYP1A1, and COMT: a multigenic study on cancer susceptibility. *Cancer Res* 1999;59:4870-4875.
14. Goode EL, Dunning AM, Kuschel B, et al. Effect of germ-line genetic variation on breast cancer survival in a population-based study. *Cancer Res* 2002;62:3052-3057.
15. Gorai I, Tanaka K, Inada M, et al. Estrogen-metabolizing gene polymorphisms, but not estrogen receptor-α gene polymorphisms, are associated with the onset of menarche in healthy postmenopausal Japanese women. *J Clin Endocrinol Metab* 2003;88:799-803.
16. Haiman CA, Hankinson SE, De Vivo I, et al. Polymorphisms in steroid hormone pathway genes and mammographic density. *Breast Cancer Res Treat* 2003;77:27-36.
17. Haiman CA, Hankinson SE, Spiegelman D, et al. The relationship between a polymorphism in CYP17 with plasma hormone levels and breast cancer. *Cancer Res* 1999;59:1015-1020.
18. De Vivo I, Hankinson SE, Li L, et al. Association of CYP1B1 polymorphisms and breast cancer risk. *Cancer Epidemiol Biomark Prev* 2002;11:489-492.
19. Shimada T, Hayes CL, Yamazaki H, et al. Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1. *Cancer Res* 1996;56:2979-2984.
20. Hayes CL, Spink DC, Spink BC, et al. 17 β-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc Natl Acad Sci U S A* 1996;93:9776-9781.
21. Hanna IH, Dawling S, Roodi N, et al. Cytochrome P450 1B1 (CYP1B1) pharmacogenetics: association of polymorphisms with functional differences in estrogen hydroxylation activity. *Cancer Res* 2000;60:3440-3444.
22. Ingelman-Sundberg M, Oscarson M, McLellan RA. Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment. *Trends Pharmacol Sci* 1999;20:342-349.
23. Garcia-Perez MA, Moreno-Mercer J, Tarin JJ, et al. Relationship between PTH, sex steroid and bone turnover marker measurements and bone density in recently postmenopausal women. *Maturitas* 2003; 45:67-74.
24. Ahlborg HG, Johnell O, Turner CH, et al. Bone loss and bone size after menopause. *N Engl J Med* 2003;349:327-334.
25. Blain H, Vuillemin A, Guillemin F, et al. Serum leptin level is a predictor of bone mineral density in postmenopausal women. *J Clin Endocrinol Metab* 2002;87:1030-1035.
26. Kuhl H. Pharmacokinetics of oestrogens and progestogens. *Maturitas* 1990;12:171-197.
27. Thomsen AB, Silvestri S, Haarbo J, et al. Associated response in bone and lipids during hormone replacement therapy. *Maturitas* 2004;47: 39-45.

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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.

Keywords Apoptosis · AZF genes · Germ cells · Inhibin B · Microdeletions

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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References

1. Ferlin A, Arredi B, Foresta C. Genetic causes of male infertility. Reprod Toxicol. 2006;22:133–41.
2. Huang WJ, Lin Y, Hsiao KN, Eilber KS, Salido EC. Restricted expression of the human DAZ protein in premeiotic germ cells. Hum Reprod. 2008;23:1280–9.
3. Girardi SK, Mielnik A, Schlegel PN. Submicrocsopic deletions in the Y chromosome of infertile men. Hum Reprod. 1997;12:1635–41.
4. Simoni M, Carani C, Gromoll J, Meschede D, Dwomiczak B, et al. Screening for deletions of the Y chromosome involving the DAZ gene in azoospermia and severe oligozoospermia. Fertil Steril. 1997;67:542–7.
5. Calogero AE, Garofalo MR, Barone N, Palma AD, Vicari E, et al. Spontaneous regression over time of the germinal epithelium in a Y chromosome-microdeleted patient. Hum Reprod. 2001;16:1845–8.
6. Dada R, Gupa NP, Kucheria K. Molecular screening for Yq microdeletion in men with idiopathic oligozoospermia and azoospermia. J Biosci. 2003;28:163–8.
7. Dada R, Gupta NP, Kucheria K. Yq microdeletions—Azoospermia factor candidate genes and spermatogenic arrest. J Biomol Tech. 2004;15:176–83.
8. Ferilin A, Arredi B, Speltra E, Cazzadore C, Selice R, et al. Molecular and clinical characterization of Y chromosome microdeletions in infertile men. A 10-year experience in Italy. J Clin Endocrinol Metab. 2007;92:762–70.
9. Tesarik J, Greco E, Cohen-Bacrie P, Mendoza C. Germ cell apoptosis in men with complete and incomplete spermiogenesis failure. Mol, Hum Reprod. 1998;4:757–62.
10. Lin WW, Lipshultz LI, Lamb DJ, Kim ED, Wheeler TM. In situ end-labeling of human testicular tissue demonstrates increased apoptosis in conditions of abnormal spermatogenesis. Fertil Steril. 1997;68:1065–9.
11. Takagi S, Itoh N, Kimura M, Sasao T, Tsukamoto T. Spermatogonial proliferation and apoptosis in hypospermatogenesis associated with nonobstructive azoospermia. Fertil Steril. 2001;76:901–7.
12. Kim S, Yoon Y, Park Y, Seo JT, Kim JH. Involvement of the Fas-Fas ligand system and active caspase-3 in abnormal apoptosis in human testis with maturation arrest and Seltoli cell-only syndrome. Fertil Steril. 2007;87:547–53.
13. McLachlan RI, Meyts ER, Hoei-Hansen CE, Kretser DM, Skakkebaek NS. Histological evaluation of the human testis—approaches to optimizing the clinical value of the assessment. Hum Reprod. 2007;22:2–16.

14. Sawai H, Komori S, Koyama K. Molecular analysis of the Y chromosome AZFc region in Japanese infertile males with spermatogenic defects. J Reprod Immunol. 2002;53:37–44.
15. Carvalho CMB, Fujisawa M, Shirakawa T, Gotoh A, Kamidono S, et al. Lack of association between Y chromosome haplogroups and male infertility in Japanese men. Am J Med Genet A. 2003;116:152–8.
16. Nagata Y, Fujita K, Banzai J, Kojima Y, Kashima K, et al. Seminal plasma inhibin B level is a useful predictor of the success of conventional testicular sperm extraction in patients with non-obstructive azoospermia. J Obstet Gynaecol Res. 2005;31:384–8.
17. Oates RD, Silber S, Brown LG, Page DC. Clinical characterization of 42 oligospermic or azoospermic men with microdeletion of the AZFc region of the Y chromosome, and of 18 children conceived via ICSI. Hum Reprod. 2002;17:2813–24.
18. Frydelund-Larsen L, Krausz C, Leffers H, Andersson AM, Carlsen E, et al. Inhibin B: A marker for the functional state of the seminiferous epithelium in patients with asoospermia factor c microdeletions. J Clin Endocrinol Metab. 2002;87:5618–24.
19. Dunkel L, Hirvonen V, Erkkila K. Clinical aspects of male germ cell apoptosis during testis development and spermatogenesis. Cell Death Differ. 1997;4:171–9.
20. Rajpurkar A, Jiang Y, Dhabuwala CB, Dunbar JC, Li H. Cigarette smoking induces apoptosis in rat testis. J Environ Pathol Toxicol Oncol. 2002;21:243–8.
21. Cozzolino DJ, Lipshultz LI. Varicocele as a progressive lesion: positive effect of varicocele repair. Hum Reprod Update. 2001;7:55–8.
22. Hassan A, EL-Nashar EM, Mostafa T. Programmed cell death in varicocele-bearing testes. Androlagia. 2009;41:39–45.
23. Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, et al. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature. 2003;423:825–37.
24. Maines JZ, Wasserman SA. Post-transcriptional regulation of the meiotic Cdc25 protein Twine by the Dazl orthologue Boule. Nat Cell Biol. 1999;1:171–4.
25. Venables JP, Ruggiu M, Cooke HJ. The RNA-binding specificity of the mouse Dazl protein. Nucleic Acids Res. 2001;29:2479–83.
26. Morgan DO. Pmciples of CDK regulation. Nature. 1995;374:131–4.
27. Wickramasinghe D, Becker S, Ernst MK, Resnick JL, Centanni JM, et al. Two CDC25 homologues are differentially expressed during mouse development. Development. 1995;121:2047–56.
28. Mizoguchi S, Kim KH. Expression of cdc25 phosphatases in germ cells of the rat testis. Biol Reprod. 1997;56:1474–81.
29. Kar S, Wang M, Carr BI. 2-methoxyestradiol inhibits hepatocellular carcinoma cell growth by inhibiting Cdc25 and inducing cell cycle arrest and apoptosis. Cancer Chemother Pharmacol. 2008;62:831–40.

Recurrent borderline ovarian tumor presenting as a pedunculated polyp at colonoscopy

Fig. 1 Colonoscopic view of the polypoid mass.

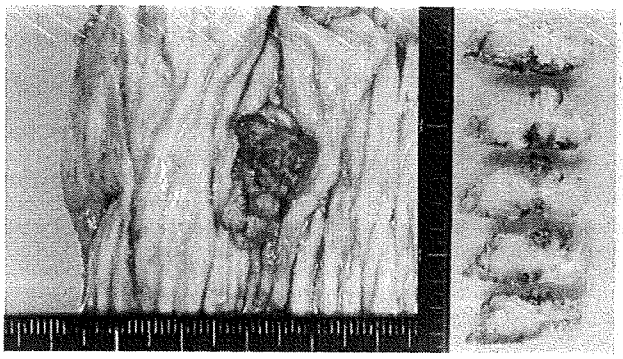

Fig. 2 Gross findings of the resected specimen. The polypoid mass is penetrating the anterior rectal wall.

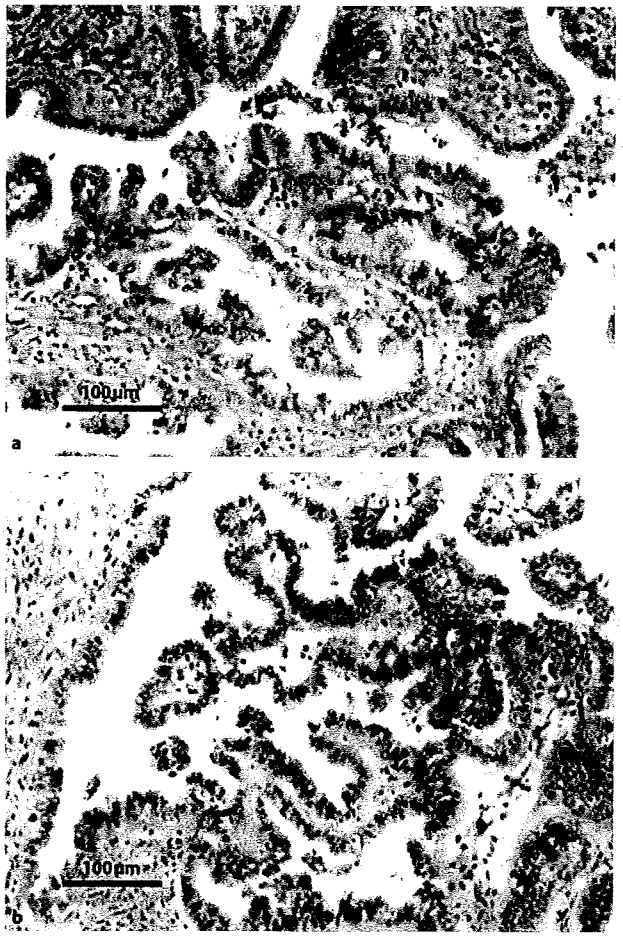

Fig. 3 Microscopic findings: (a) the rectal tumor and (b) the primary ovarian tumor (hematoxylin and eosin; magnification × 100). Both tumors show marked epithelial proliferation with a micropapillary and cribriform pattern.

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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References

1 *Wong HF, Low JJ, Chua Y et al.* Ovarian tumors of borderline malignancy: a review of 247 patients from 1991 to 2004. Int J Gynecol Cancer 2007; 17: 342–349
2 *Longacre TA, McKenney JK, Tazelaar HD et al.* Ovarian serous tumors of low malignant potential (borderline tumors): outcome-based study of 276 patients with long-term (> or = 5-year) follow-up. Am J Surg Pathol 2005; 29: 707–723
3 *Ayhan A, Guvendag Guven ES, Guven S et al.* Recurrence and prognostic factors in borderline ovarian tumors. Gynecol Oncol 2005; 98: 439–445
4 *Lee EJ, Deavers MT, Hughes JI et al.* Metastasis to sigmoid colon mucosa and submucosa from serous borderline ovarian tumor: response to hormone therapy. Int J Gynecol Cancer 2006; 16 (Suppl. 1): 295–299
5 *Silva EG, Gershenson DM, Malpica A et al.* The recurrence and the overall survival rates of ovarian serous borderline neoplasms with noninvasive implants is time dependent. Am J Surg Pathol 2006; 30: 1367–1371

Bibliography
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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	

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

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