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Polymorphisms of estrogen metabolism-related genes *ESR1*, *UGT2B17*, and *UGT1A1* are not associated with osteoporosis in surgically menopausal Japanese women

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Abstract

Introduction: Bilateral salpingo-oophorectomy (BSO) is a risk factor for osteoporosis. Previous studies have reported an association between genetic polymorphisms and the risk of developing osteoporosis. However, the relationship between osteoporosis and genetic polymorphisms in Japanese women treated with BSO is not well understood. To improve the quality of life for post-BSO patients, it is important to determine the genetic factors that influence their risk for osteoporosis. The aim of this study was to investigate the association between gene variations of estrogen metabolism-related genes and osteoporosis in surgically menopausal patients, which may improve their quality of life.

Material and methods: This study included 203 menopausal women treated with BSO because of gynecologic disorders. One hundred and twenty-six women with artificial (surgical) menopause, who had undergone BSO in the premenopausal period, were compared with 77 women with natural menopause, who had undergone BSO in the postmenopausal period. The women were tested for bone mineral density to diagnose osteoporosis. Polymorphisms of estrogen receptor 1 (*ESR1*) and UDP-glucuronosyl transferase (UGT) genes *UGT2B17* and *UGT1A1* were analyzed, and their association with bone mass and osteoporosis was statistically evaluated.

Results: No significant association was found between osteoporosis and polymorphisms in *ESR1*, *UGT2B17*, or *UGT1A1* in both groups, suggesting that BSO might be a more significant physiological factor in influencing bone mass density compared to genetic variations.

Conclusions: These results suggest that the *ESR1*, *UGT2B17*, and *UGT1A1* polymorphisms are not genetic factors affecting osteoporosis in postmenopausal Japanese women.

Key words: bilateral salpingo-oophorectomy, bone mineral density, gene polymorphism, menopause, osteoporosis.

Introduction

Since the aging population in developed countries is growing, strategies to improve the quality of life for the elderly are urgently needed. Bone fracture due to osteoporosis is one of the most important causes of disability in postmenopausal women, usually requiring long-term clinical support.

Artifical (surgical) menopause due to premenopausal bilateral salpingo-oophorectomy (BSO) is a risk factor for osteoporosis. Previous studies have reported an association between genetic polymorphisms of estrogen metabolism-related genes and the risk of developing osteoporosis. However, the relationship between osteoporosis and genetic polymorphisms in Japanese women with artifical menopause is not well understood. It is important to determine the genetic factors that influence their risk of osteoporosis.

The estrogen endocrine system has long been known to play an important role in the regulation of bone mass and the occurrence of osteoporosis, and it has been established that continuous estrogen deficiency causes osteoporosis in women. Previous studies have reported an association between polymorphisms of the genes related to estrogen metabolism and osteoporosis or osteoporotic fractures [1-5].

Bilateral salpingo-oophorectomy, together with total abdominal hysterectomy, is the predominant treatment for many estrogen-dependent gynecologic tumors, especially ovarian and endometrial cancers, and is performed in such cases whenever feasible [6].

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Artifical menopause due to BSO puts premenopausal women at a high risk for osteoporosis and subsequent bone fracture.

Recent genome-wide association studies have revealed a link between polymorphisms in several genes related to estrogen metabolism and the risk of osteoporosis and bone fracture. The estrogen receptor 1 (*ESR1*) gene is one of the most extensively studied candidate genes showing a convincing relationship with osteoporosis, as confirmed by meta-analyses of genome-wide association studies [1, 7]. The other potential biomarkers are UDP-glucuronosyltransferases (UGTs), a family of steroid hormone-metabolizing enzymes that catalyze glucuronidation, the final step in the inactivation of steroid hormones. The *UGT1A1* and *UGT2B7* genes are expressed in the uterus and are involved in the conjugation and elimination of estrogens [8]. *UGT2B17* has been shown to inactivate androgens and to stimulate bone formation [5]. In a genome-wide copy number variation study, a higher *UGT2B17* copy number was found to be associated with lower bone mineral density (BMD) [5].

The relationship between genomic polymorphisms and the risk of osteoporosis in Japanese women treated with BSO is not well understood. The FRAX® tool is a widely used prediction model developed by the World Health Organization to evaluate the risk of osteoporosis and hip fracture based on patient clinical parameters and anthropometrics (http://www.shef.ac.uk/FRAX/index.aspx); however, it does not consider genetic variations in estrogen metabolism-related genes. To improve the care for BSO-treated women, it is essential to elucidate the link between genetic variability and risk of osteoporosis in these patients. Here, we aimed to reveal the relationship between the genetic variations of estrogen metabolism-related genes and bone mass to suggest an effective osteoporosis risk surveillance system for women with artifical menopause.

Material and methods

This study was conducted with bioresources from Keio Women's Health Biobank (KWB) in the Keio University School of Medicine (Tokyo, Japan). The clinical data were collected from 203 menopausal women who visited the menopausal clinic of the Department of Obstetrics and Gynecology, Keio University Hospital from 2006 to 2011 (Table I). One hundred and twenty-six women with artifical menopause, i.e., who had undergone BSO in the premenopausal period (premenopausal BSO group), were compared with 77 women with natural menopause, who had undergone BSO in the postmenopausal period (postmenopausal BSO group).

The eligibility criterion for inclusion in the study was treatment with BSO for any gynecologic disorder. Exclu-

Tab. I. Characteristics of the study patients according to the time of bilateral oophorectomy

Characteristics	Total	Premenopausal bilateral oophorectomy	Postmenopausal bilateral oophorectomy
Total	203	126	77
Age: mean (SD%)	56.0 (12.0)	49.7 (9.5)	66.4 (7.9)
		$p < 0.0001$	
Time (y) after menopause: mean (SD%)		5.9 (5.5)	14.8 (8.4)
		$p < 0.0001$	
Disease			
Cervical cancer	21	18	3
Ovarian cancer	37	23	14
Endometrial cancer	124	66	58
Ovarian and endometrial double cancer	7	5	2
Myoma or endometriotic cyst	14	14	0
Body mass index (kg/m^2): mean (SD%)		22.3 (4.3)	22.4 (3.1)
		$p = 0.21$	
Normal bone mass	114	75	39
Osteopenia	71	46	25
Osteoporosis	18	5	13

sion criteria were the presence of diagnosed secondary osteoporosis, treatment with hormone replacement therapy, steroid use, ongoing treatment for osteoporosis, and chemotherapy before BSO.

Age, time period after menopause, disease, and body mass index (BMI) were analyzed (Table I).

Bone mineral density of the lumbar spine or proximal femur was measured using dual energy X-ray absorptiometry (PRODIGY; GE Healthcare Japan, Tokyo, Japan). Young adult mean values (%) were calculated, and osteoporosis was diagnosed according to diagnostic criteria for primary osteoporosis (2012 revision) proposed by the Japanese Society for Bone and Mineral Research and Japan Osteoporosis Society Joint Review Committee for the Revision of the Diagnostic Criteria for Primary Osteoporosis to diagnose primary osteoporosis [9].

Genomic DNA was extracted using standard procedures. We analyzed copy number variations (CNVs) of the *ESR1* and *UGT2B17* genes, and polymorphisms of the *UGT1A1* gene, including single nucleotide polymorphisms (SNPs) *UGT1A1*6* (211G>A), *UGT1A1*27* (686C>A), *UGT1A1*60* (-3263T>A), and TA repeat variation of *UGT1A1*28* ($A(TA)_7TAA$) (Table II).

To estimate copy numbers of the *ESR1* exon 5 and *UGT2B17* intron 2, the TaqMan® Copy Number Assay was used. In order to include a two-copy endogenous control within each real-time PCR reaction, the RNAse P Copy Number Reference Assay (Applied Biosystems, Carlsbad, CA, USA) was used. For each assay, 10 ng of genomic DNA was assayed in duplicate in 10-µl reactions containing 1× TaqMan® Genotyping Master Mix (Applied Biosystems). Cycling was performed under default conditions in 384-well optical plates using the ABI 7900HT system. To calculate copy numbers, we used the CopyCaller v.1.0 software (Applied Biosystems); copy numbers for each sample and for each locus were determined using the ΔΔCt method.

*UGT1A1*6*, *UGT1A1**27, and *UGT1A1**28 SNPs were genotyped using the Invader® UGT1A1 Molecular Assay kit (Sekisui Medical Co. Ltd, Tokyo, Japan) as previously described [10]. *UGT1A1*60* was detected by PCR and direct sequencing (Supplementary Tab. I).

Statistical analysis was performed using Excel for Mac 2011 (Microsoft, USA) with the add-in software Statcel 3 (OMS, Japan) and Prism 6 (GraphPad Software, La Jolla, CA, USA). The *F* test was used to verify the homogeneity of variance. Normally distributed vari-

Tab. II. Genetic variations detected in women treated with bilateral salpingo-oophorectomy

Gene polymorphism			Total	Premenopausal BSO	Postmenopausal BSO
ESR1	CNV exon 5	0	0	0	0
		1	0	0	0
		2	200	124	76
UGT2B17	CNV intron 2	0	158	101	57
		1	1	0	1
		2	37	22	16
		4	4	2	2
*UGT1A1*6*	SNP 211G>A	–/–	117	70	47
		*6/–	45	27	18
		*6/*6	7	3	4
*UGT1A1*27*	SNP 686C>A	–/–	167	98	69
		*27/–	0	0	0
		*27/*27	0	0	0
*UGT1A1*28*	TA repeat $A(TA)_7TAA$	–/–	131	73	58
		*28/–	36	26	10
		*28/*28	2	1	1
*UGT1A1*60*	SNP -3263T>A	–/–	92	52	40
		*60/–	57	40	17
		*60/*60	10	6	4

BSO – bilateral salpingo-oophorectomy, CNV – copy number variation, SNP – single nucleotide polymorphism

Supplementary Tab. I. Primers and method of direct sequencing for *UGT1A1*60*

Primers	
	Sequences (5'→3')
*UGT1A1*60* Forward	5'-ttaaccaaagaacattctaamgg-3'
*UGT1A1*60* Reverse	5'-caaaatcccagaaaaacagc-3'
Reaction mixture for PCR	(µl)
10X Ex Taq Buffer (Takara code RR001)	1
dNTP Mixture (2.5 mM each) (Takara)	0.8
TaKaRa Ex Taq (5 units/µl) (Takara)	0.1
Primer 10 µM fwd/rev	1
Template DNA	100 ng ≧
Sterilized distilled water	up to 10 µl
Total vol:	10
PCR program	
94°C 2 min	
94°C 30 sec	×40 cycles
60°C 30 sec	
72°C 2 min	
72°C 5 min	
4°C	
Purification of PCR product	
PCR product 1 µl	
Exo-SAP IT (usb cat# 78200/01/02/05/50) 1 µl	
Sterilized distilled water	
Total 5 µl	
37°C 30 min	
85°C 20 min	
Sequence PCR	
Purification of PCR product	5 µl
Big Dye Terminater V3.1 5X sequence buffer (Applied Biosystems)	2 µl
Big Dye terminaterv 3.1 (Applied Biosystems cat#4376496)	0.5 µl
*UGT1A1*60* PCR Primer (Rivers 10 µM)	0.32 µl
Sterilized distilled water	2.2 µl
Total	10 µl
Sequence PCR temp	
96°C 1 min	
96°C 10 sec	×35 cycles
50°C 5 sec	
60°C 4 min	
4°C	
Purification of Big Dye	
SAM solution (Applied Biosystems cat#4376496)	32 µl
X-terminator solutiom (Applied Biosystems cat#4379323)	6 µl
Vortex	
RT 30 min	
Sequensing with ABI310 or ABI3130	

ables were compared using Student's *t* test, and non-parametric distributed variables were compared by the Mann-Whitney *U* test. The χ^2 test or Fisher's exact test was performed for contingency tables. Results with *p* values < 0.05 were considered statistically significant.

Patients who provided blood had received a verbal explanation of the study objectives and had read corresponding documents before giving written informed consent. Access to personal data that could permit identification of the participants was under strict control of the manager of personal information in the department. We obtained permission to use the specimens, and the study protocol was approved by the ethics committee of the Keio University School of Medicine (approval number: 20050135 and 20070081).

Results

The average age and time interval after menopause in the postmenopausal BSO group were significantly higher than those in the premenopausal BSO group ($p < 0.0001$). In contrast, BMI was not significantly different between the patients with premenopausal and postmenopausal BSO ($p = 0.21$).

One hundred and fourteen women had normal bone mass, 71 – osteopenia, and 18 – osteoporosis.

Table II shows the result of genetic variation analysis in women treated with BSO. Because none of the patients had variations in the *ESR1* gene or the *UGT1A1*27* SNP, the relationships between bone mass and CNVs of the *UGT2B17* gene or the *UGT1A1*6*, *UGT1A1*28*, and *UGT1A1*60* SNPs were analyzed (Table III). No association between *UGT2B17* and *UGT1A1* polymorphisms and bone mass was detected in either the total analyzed population or premenopausal and postmenopausal BSO groups.

Discussion

Several meta-analysis studies have demonstrated an association between genetic polymorphisms and osteoporosis or osteoporotic fractures, although many of these studies analyzed the general population and were not specific to women with artifical menopause.

Estrogens bind to and activate ESR1, resulting in the upregulation of the expression of many cancer-related genes. Meta-analyses of genome-wide association studies have shown that the *ESR1* genotype is associated with BMD [1, 7]; however, the contribution of the *ESR1* CNV was inconclusive because all the analyzed patients had two *ESR1* copies. In our study, we could not detect CNV of the *ESR1* gene because of the low frequency of the genetic variation in this region. Among 164 Japanese patients, only three CNVs of *ESR1* (chr6: 151959784-152182026) have been found within the

Tab. III. Relationship between gene variation and bone mass in BSO patients

Gene	Polymorphism		Total								Premenopausal BSO								Postmenopausal BSO							
			Normal	Osteo-penia + Osteo-porosis	*p* value	Odds ratio (95% CI)	Normal	Osteo-penia + Osteo-porosis	*p* value	Odds ratio (95% CI)	Normal	Osteo-penia + Osteo-porosis	*p* value	Odds ratio (95% CI)	Normal	Osteo-penia + Osteo-porosis	*p* value	Odds ratio (95% CI)	Normal	Osteo-penia + Osteo-porosis	*p* value	Odds ratio (95% CI)	Normal	Osteo-penia + Osteo-porosis	*p* value	Odds ratio (95% CI)
UGT2B17	CNV	0	84	74	0.12	0.57	144	14	1.00	1.08	58	43	0.40	0.67	97	4	0,33	2,21	26	31	0.19	0.49	47	10	1.00	0.88
		≥ 1	28	14		(0.28–1.16)	38	4		(0.34–3.48)	16	8		(0.26–1.72)	22	2		(0.38–12.81)	12	7		(0.17–1.42)	16	3		(0.22–3.61)
UGT2B17	CNV	0,1	85	74	0.15	0.60	145	14	0,77	1.12	58	43	0.41	0.67	97	4	0,32	2.20	27	31	0.28	0.55	48	10	0.95	0.96
		≥ 2	27	14		(0.29–1.22)	37	4		(0.35–3.60)	16	8		(0.26–1.72)	22	2		(0.38–12.81)	11	7		(0.19–1.63)	15	3		(0.23–3.95)
*UGT1A1*6*	SNP 211G>A	–/–, *6/–	92	70	0.24	3.29	147	15	1.00	0.63	58	39	0.57	2.97	93	4	1.00	2.67	34	31	0.36	3.29	54	11	1.00	0.53
		*6/*6	2	5		(0.62–17.44)	7	0		(0.03–11.66)	1	2		(0.26–33.96)	3	0		(0.13–66.64)	1	3		(0.32–33.33)	4	0		(0.03–10.48)
*UGT1A1*6*	SNP 211G>A	–/–	63	54	0.49	0.79	107	10	0.78	1.14	39	31	0.31	0.63	67	3	1.00	0.77	24	23	0.93	1.04	40	7	0.73	1.27
		*6/–, *6/*6	31	21		(0.41–1.53)	47	5		(0.37–3.51)	20	10		(0.26–1.54)	29	1		(0.08–7.72)	11	11		(0.38–2.87)	18	4		(0.33–4.89)
*UGT1A1*28*	TA repeat $A(TA)_7TAA$	–/–, *28/–	92	75	0.50	0.25	152	15	1.00	1.97	58	41	1.00	0.47	95	4	1.00	7.04	34	34	1.00	0.33	57	11	1.00	1.67
		*28/*28	2	0		(0.01–5.19)	2	0		(0.09–42.89)	1	0		(0.02–11.83)	1	0		(0.25–119.3)	1	0		(0.01–8.48)	1	0		(0.06–43.57)
*UGT1A1*28*	TA repeat $A(TA)_7TAA$	–/–	71	60	0.49	0.77	118	13	0.53	0.50	43	30	0.97	0.99	70	3	1.00	0.90	28	30	0.51	0.53	48	10	0.68	0.48
		*28/–, *28/*28	23	15		(0.37–1.61)	36	2		(0.11–2.34)	16	11		(0.04–2.42)	26	1		(0.09–9.02)	7	4		(0.14–2.02)	10	1		(0.06–4.19)
*UGT1A1*60*	SNP -3263T>A	–/–, *60/–	81	68	1.00	0.79	135	14	100	1.07	53	39	1.00	0.68	89	3	0.23	5.93	28	29	1.00	0.97	46	11	1.00	0.45
		*60/*60	6	4		(0.22–2.93)	9	1		(0.13–9.09)	4	2		(0.12–3.90)	5	1		(0.52–67.83)	2	2		(0.13–7.34)	4	0		(0.02–8.96)
*UGT1A1*60*	SNP -3263T>A	–/–	52	40	0.59	1.19	82	10	0.59	0.66	34	18	0.12	1.89	51	1	0.34	3.56	18	22	0.37	0.61	31	9	0.31	0.36
		*60/–, *60/*60	35	32		(0.63–2.23)	62	5		(0.22–2.03)	23	23		(0.84–4.26)	43	3		(0.36–35.48)	12	9		(0.21–1.78)	19	2		(0.07–1.86)

BSO – bilateral salpingo-oophorectomy, CNV – copy number variation, SNP – single nucleotide polymorphism

CNV regions listed in the Human Genome Validation Database (https://gwas.biosciencedbc.jp/index.html). Further large-scale studies may be required to clarify the relationship of *ESR1* CNVs with osteoporosis in the Japanese population.

UGT2B17 encodes the enzyme that metabolizes steroid sex hormones and is closely related to androsterone, testosterone, and dihydrotestosterone turnover [11]. In a genome-wide association study in an elderly Chinese population, Yang *et al*. found that *UGT2B17* CNV was strongly associated with osteoporotic fractures [5]. They also analyzed the correlation of serum testosterone and estradiol concentrations with *UGT2B17* copy numbers in 236 young Chinese men, confirming that those without *UGT2B17* deficiency had a significant association with higher testosterone and estradiol levels. However, Chew *et al*. observed no such association of the *UGT2B17* gene copy number with estrogen and free estradiol index; they also reported that homozygous or heterozygous deletion of the *UGT2B17* gene did not correlate with the increased risk of incident fragility fracture in elderly Caucasian women [12]. The difference in these results suggests that racial and sexual diversity may affect the relationship between the *UGT2B17* genotype and osteoporosis.

Polymorphisms in the *UGT1A1* coding region are known to reduce UGT enzymatic activity, resulting in a significantly slower elimination of β-estradiol [8]. The *UGT1A1*28* SNP in the promoter region has been shown to negatively affect the transcriptional activity of the *UGTlA1* gene. The frequency of the *UGT1A1*28* variant is considerably lower in the Japanese compared to the Caucasian population. In contrast, the *UGT1A1*6* and *UGT1A1*27* polymorphisms are fairly common in the Japanese population but absent in Caucasians [13]. Still, the relationship between the *UGT1A1* genotype and osteoporosis has not been well understood. Recently, the *UGT1A1*28* polymorphism has been reported to significantly influence the pharmacokinetics of raloxifene, a selective estrogen modulator used for the treatment of postmenopausal women with osteoporosis, and an increase in BMD after raloxifene treatment has also been observed [14]. Further pharmacogenomics studies are required to establish an individualized approach to the treatment of osteoporosis.

In conclusion, our analysis could not find a relationship between the *ESR1*, *UGT2B17*, and *UGT1A1* genotypes and the decrease in bone mass among Japanese women treated with BSO. However, our study had certain limitations. First, we did not evaluate lifestyle factors such as smoking, alcohol consumption, diet, and exercise habits, which might have an impact as environmental factors; large-scale analyses are needed to adjust for these cofounding effects by multiple regression analysis and to validate our result. Second, some patients might have had a history of ovarian dysfunction or early menopause, as well as adjuvant chemotherapy after BSO, which could have influenced the bone mass index. Finally, polymorphisms of the estrogen metabolism-related genes analyzed in our study serve as risk factors for gynecologic disorders. The *UGT1A1* polymorphism is hypothesized to influence the development of endometrial and ovarian cancers because of the ability to induce estrogen glucuronidation. Some controversial data on the relationship between polymorphisms in the UGT genes and endometrial and ovarian cancers have been reported [15-22]. These findings suggest that common abnormalities in estrogen metabolism may increase the risk of osteoporosis, but detailed metabolomics data are limited. Further studies are required to confirm our results.

Incidence rates of ovarian and endometrial cancers in Japan have been steadily increasing since the 1970s [Cancer Statistics in Japan'13, Center for Cancer Control and Information Services, National Cancer Center, Japan (http://ganjoho.jp/public/statistics/backnumber/2011_en.html). In addition, because of the increase in the onset of endometrial cancer at a young age in many countries [23], the number of premenopausal endometrial cancer survivors who will undergo BSO is expected to grow in the near future. Furthermore, BSO is also indicated for women with hereditary breast and ovarian cancer (HBOC) syndrome as risk-reductive salpingo-oophorectomy (RRSO). The genetic basis of HBOC is an inherited mutation in the *BRCA1* or *BRCA2* (*BRCA1/2*) tumor suppressor genes, and RRSO may be performed in the framework of standard medical treatment for *BRCA1/2* mutation carriers in the near future [24]. Therefore, premenopausal women with *BRCA1/2* mutations treated with BSO also contribute to the increase in the population of young women with surgically-induced menopause.

Bone fractures due to osteoporosis can be a serious cause of disability or even death in survivors of endometrial cancer. Therefore, prevention of osteoporotic fractures is essential for improving the quality of life of a growing number of cancer survivors, especially women with artifical menopause. These measures can also positively affect healthy postmenopausal women. Future metabolome and pathway analyses may clarify the mechanisms linking estrogen, bone mineral metabolism, and carcinogenesis.

Bilateral salpingo-oophorectomy represents a significant factor that influences bone mineral metabolism in women. A recent report has revealed that postmenopausal ovaries are hormonally active in women for as long as 10 years after menopause [25]. Chaki *et al*. have found that bone turnover is more aggressive in postmenopausal BSO-treated women than in natural menopausal women [26]. This may be one of the reasons why we did not find the association between the polymorphism of the estrogen metabolism-related

genes and osteoporosis in BSO-treated women. Bilateral salpingo-oophorectomy performed before or after menopause is likely to have a more significant impact on the hormonal condition in women than genetic variations in steroid metabolism-related factors.

Our report highlights the importance of bone mass surveillance in women after BSO and suggests that the clinicians who participate in the care of patients with artifical menopause should check bone mass indexes rather than analyze genetic biomarkers validated for the general population. Further large-scale studies may result in the development of predictive models to identify post-BSO women at risk of osteoporosis for early intervention and management.

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Disclosure

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