Rs1008805 polymorphism of *CYP19A1* gene is associated with the efficacy of hormone therapy in stage I-II and operable stage III breast cancer

XIYING SHAO[1*], LEI LUO[2*], YONG GUO[3*], XIAOHONG XU[4], DEHOU DENG[1], JIANGUO FENG[5], YUHENG DING[3], HANZHOU MOU[5], PING HUANG[1], LEI SHI[1], YUAN HUANG[1], WEIWU YE[1], CAIJIN LOU[1], ZHANHONG CHEN[1], YABING ZHENG[1] and XIAOJIA WANG[1]

[1]Department of Medical Oncology, Zhejiang Cancer Hospital, Hangzhou, Zhejiang 310022; [2]Zhejiang Institute for Food and Drug Control, Hangzhou, Zhejiang 310052; [3]Department of Medical Oncology, The First Affiliated Hospital of Zhejiang Traditional Chinese Medical University, Hangzhou, Zhejiang 310006; [4]Clinical Laboratory; [5]Cancer Research Institute, Zhejiang Cancer Hospital, Hangzhou, Zhejiang 310022, P.R. China

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Abstract. It has been hypothesized that single nucleotide polymorphisms in *CYP19A1* gene may alter aromatase activity and circulating steroid hormone levels in females. Therefore, it is biologically reasonable that *CYP19A1* rs1008805 (A/G) polymorphism may be associated with the clinical outcome of hormone therapy. Genotyping for the *CYP19A1* rs1008805 polymorphism was performed for 287 females with hormone receptor (HR)-positive early breast cancer, and potential associations were evaluated between *CYP19A1* rs1008805 genotypes and disease-free survival (DFS). Based on the analysis of the whole cohort, no significant differences were observed between rs1008805 genotypes and DFS. However, in postmenopausal females, rs1008805 variants were significantly associated with DFS (AA vs. AG vs. GG, 89.2 vs. 58.2 vs. 32.7 months; P=0.019). In addition, when the population was divided into two cohorts, females with the GG variant exhibited a significantly poorer DFS [GG vs. AA or AG, 32.7 vs. 70.6 months; hazard ratio (HR), 3.613; 95% confidence interval (CI), 1.380-9.457; P=0.005]. Furthermore, when adjusted for other patient features in multivariate analyses, GG genotype remained an independent prognostic marker for DFS (HR, 3.439; 95% CI, 1.251-9.456; P=0.017). However, there were no significant differences in DFS between patients harboring the minor allele and those with the homozygous common allele (AG or GG vs. AA, 52.4 vs. 89.2 months; HR, 1.288; 95% CI, 0.705-2.353; P=0.408). There were also no associations between rs1008805 polymorphism and DFS for premenopausal females. In conclusion, the homozygous minor allele (GG) of *CYP19A1* rs1008805 was identified to be significantly associated with an inferior clinical outcome of hormone therapy in postmenopausal hormone receptor-positive patients with early breast cancer. If confirmed by further study, genotyping for *CYP19A1* rs1008805 polymorphism may provide predictive information to improve the selection of endocrine treatment.

Correspondence to: Professor Xiaojia Wang or Dr Yabing Zheng, Department of Medical Oncology, Zhejiang Cancer Hospital, 38 Guangji Road, Hangzhou, Zhejiang 310022, P.R. China
E-mail: wxiaojia0803@163.com
E-mail: zhengyabing@sina.com

*Contributed equally

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Introduction

Over the last three decades, the number of breast cancer cases has increased worldwide (1) to become the most likely cause of cancer mortality and morbidity in females (2). Increased levels of aromatase expression have been observed in breast lesions compared with normal breast tissue (3,4) and alterations in aromatase expression are associated with the pathogenesis of breast cancer (5,6).

Approximately two thirds of breast cancer cases overexpress estrogen receptors (ER) and/or progesterone receptors (PgR) (7,8). Consequently, endocrine therapy, including tamoxifen or aromatase inhibitors (AIs), has become an effective treatment for these patients. For decades, 5-year tamoxifen administration was the gold standard for the adjuvant endocrine treatment of breast cancer (9). More recently, postmenopausal patients have also had the option of receiving AIs as an alternative to tamoxifen, or following tamoxifen treatment (10). The presence and intensity of ER and/or PgR are useful predictive markers for the response to hormone therapy in clinical practice. Identification of more accurate biomarkers for the most efficient patient selection to exclude non-responsive patients remains crucial.

Aromatase, encoded by *CYP19A1* gene, catalyzes the final step of the conversion from androgens into estrogens (3,11,12). In premenopausal females, estrogen is predominantly produced

by the ovary, with a small amount generated by the aromatization of adrenal and ovarian androgens in extragonadal tissue. Conversely, in postmenopausal females, the aromatization of androgens from extragonadal tissue becomes the prime origin of estrogen as the ovary ceases to function (7,12,13).

It has previously been suggested that genetic polymorphisms in *CYP19A1* gene were associated with aromatase activity as well as circulating steroid hormone levels in females (3,11,12,14-16). Therefore, it is biologically reasonable that *CYP19A1* gene polymorphism may be associated with the clinical outcome of hormone therapy. Population-based studies of *CYP19A1* gene polymorphisms have revealed controversial results regarding their potential association with the therapeutic efficacy of endocrine treatment. Kuo *et al* (17) revealed that the A allele of *CYP19A1* rs4646 was significantly in association with poorer distant disease-free survival rate (P<0.05) and marginally associated with shorter overall survival (OS; P=0.06) or disease-free survival (DFS; P=0.07) in lymph node-negative, hormone receptor-positive patients with endocrine therapy. In addition, a study conducted by Garcia-Casado *et al* (18) estimated that the same variant was significantly associated with poorer progression-free survival (PFS) in patients with letrozole neoadjuvant therapy, and patients with genotypic variants of rs4646 were more frequently represented in the non-responder cohort (48 vs. 26%). However, Liu *et al* (19) suggested that A allele of rs4646 was significantly associated with longer time to progression (TTP) and OS when assessed in patients with metastatic breast cancer (MBC) receiving anastrozole treatment, consistent with the study of Colomer *et al* (20), which indicated that TTP was significantly prolonged in patients with the minor T allele of *CYP19A1* rs4646 compared with those with homozygous common allele (GG) from a cohort of postmenopausal MBC with letrozole administration.

CYP19A1 gene is located at chromosomal locus 15q21.1 and has a complex structure with a regulatory region that includes 10 tissue-specific non-coding upstream exons, with separate promoters, which regulate transcription in different tissues (21). Haplotype blocks 1 to 4 are located in this regulatory region. The rs1008805 polymorphism (A/G) is located in block 3. The frequency of the minor allele (G) is approximately 29.5% in Chinese females (22). A study conducted in Chinese population demonstrated that single nucleotide polymorphisms (SNPs) in block 1 and 2 of *CYP19A1* gene were associated with the plasma levels of estrogen in postmenopausal females (23). Furthermore, it has been identified that the G allele of the rs1008805 SNP was significantly associated with the risk of breast cancer (24). Consequently, hypotheses were formulated that rs1008805 polymorphism may be associated with response of hormone therapy, which is still lacking supporting data.

Accordingly, a genetic analysis of *CYP19A1* rs1008805 polymorphism was performed with a cohort of patients with hormone receptor-positive early breast cancer in order to elucidate whether rs1008805 variants were associated with the clinical outcome of hormone therapy.

Patients and methods

Study cohort and data. A total of 287 Chinese females with hormone receptor-positive stage I-II and operable stage III breast cancer, according to the tumor-node-metastasis stage classification (25), were enrolled in the present study between 1 April 2004 and 31 July 2010 at Zhejiang Cancer Hospital (Hangzhou, Zhejiang). All of the patients received hormone therapy. In brief, 250 patients received tamoxifen therapy and 37 received third-generation aromatase inhibitors. A total of 274 (95.5%) of the patients received adjuvant chemotherapy, whereas 130 (45.3%) received radiotherapy. A total of 274 patients (95.5%) received chemotherapy including cyclophosphamide, doxorubicin and fluoracil or cyclophosphamide, epirubicin and fluoracil or doxorubicin, cyclophosphamide (AC) or fluoracil, epirubicin and cyclophosphamide followed by docetaxel or weekly paclitaxel treatment or docetaxel, doxorubicin and cyclophosphamide, cyclophosphamide and epirubicin or AC followed by docetaxel or weekly paclitaxel, 8 (2.5%) with no chemotherapy and 6 (2.0%) remained unknown. Human epidermal growth factor receptor-2 positive females received Trastuzumab treatment. A 3-mlperipheral blood sample was obtained and processed for DNA extraction in the Department of Oncology. The pathologic review, blood samples and genetic studies were approved by the institutional review board of Zhejiang Cancer Hospital. All patients provided written informed consent according to the guidelines of the Ethics Committee of Zhejiang Cancer Hospital.

DNA extraction and genotyping. Genomic DNA was extracted from peripheral blood with the AxyPrep Blood Genomic DNA Miniprep kit (Axygen; Corning Life Sciences, Union City, CA, USA). Genotyping was conducted through the Sequenom MassARRAY matrix-assisted laser desorption/ionization-time of flight mass spectrometry platform (Sequenom, San Diego, CA, USA) as previously described (26). Primers (5'-TCCTTA CCGAATCACTACCC-3' and 5'-CCTGCTATTACTTCC AACCC-3') and single base extensions were designed with Assay Designer software (version 3.0; Sequenom) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Multiplex PCR was performed in 5 μl volumes containing 10 ng whole-genome-amplified genomic DNA, 2.5 pmol of each PCR primer, 0.1 unit of HotStar Taq polymerase (Qiagen GmbH, Hilden, Germany) and 2.5 μmol deoxynucleotides (dNTP; Qiagen GmbH). Thermo cycling was performed at 94˚C for 15 min followed by 45 cycles at 94˚C for 20 sec, 56˚C for 30 sec and 72˚C for 1 min and a final incubation at 72˚C for 3 min. Unincorporated dNTPs were deactivated with 0.3 units of shrimp alkaline phosphatase (Sequenom) followed by primer extension using 5.4 pmol of each primer extension probe, 50 μmol of the appropriate ddNTP combination, and 0.5 units of iPLEX enzyme (Sequenom). The extension reactions were performed at 94˚C for 30 sec and then 94˚C for 5 sec, followed by 5 cycles at 52˚C for 5 sec and 80˚C for 5 sec for a total of 40 cycles, and then 72˚C for 3 min. A cation exchange resin was used to remove residual salt from the reactions. Purified primer extension reaction products were spotted onto a 384-well spectro CHIP using the Mass ARRAY Nano dispenser and determined by the mass spectrometer. Genotype analysis was performed in real time using MassARRAY RT software (version 3.0.0.4) and analyzed using MassARRAY Typer software (version 3.4; Sequenom).

Statistical analysis. The deviation from Hardy-Weinberg equilibrium (HWE) was assessed with Pearson's χ^2 test using the HWE calculator described in Rodriguez *et al* (27).

Follow-up data as available on 31 December 2014, were analyzed. DFS was defined as the date of the original surgery for breast cancer to the date of recurrence or mortality from any causes (28). DFS plots were produced using the Kaplan-Meier estimator method. Differences in median DFS were compared using the log-rank test.

Cox's regression analyses were conducted to estimate the hazard ratio (HR) and corresponding 95% confidence interval (CI) for each variable. The multivariate-adjusted HR for relapse associated with the individual genotypes was examined for the groups subsequent to adjusting for other variables (lymph node positivity, tumor size >2 cm, negative hormone receptor status, human epidermal growth factor receptor-2 positive status, chemotherapy, hormone therapy, radiotherapy and body mass index ≥24). These analyses were performed using the SPSS statistical software package (version 17.0; SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Patient features. A total of 287 females with hormone receptor-positive early breast cancer were enrolled in the present study, and the median age was 46 years (range, 20-73 years). As presented in Table I, 217 patients were premenopausal and 70 were postmenopausal. The clinicopathological features details are also listed in Table I.

In total, there were 145 patients with AA genotype, 124 with AG variant, and 18 with GG genotype. Genotype frequencies observed in our patient cohort were consistent with Hardy-Weinberg equilibrium (P>0.05, data not shown).

CYP19A1 rs1008805 polymorphism and DFS in the whole cohort. Based on the analysis of all patients, no significant differences were observed between rs1008805 genotypes and DFS (AA vs. AG vs. GG, 58.3 vs. 57.7 vs. 42.7 months; P=0.638; Table II). In addition, there was no difference for DFS between patients with the minor allele, i.e., AG or GG, and those carrying the homozygous common allele AA (AG or GG vs. AA, 56.4 vs. 58.3 months; HR, 0.891; 95% CI, 0.675-1.177; P=0.417; Table II). Furthermore, when the population was split into two groups, one with GG variant and the other with AG or AA genotypes, there was no association between genotypes and DFS (GG vs. AG or AA, 42.7 vs. 58.0 months; HR, 1.092; 95% CI, 0.593-2.010; P=0.777; Table II).

CYP19A1 rs1008805 polymorphism and DFS in premenopausal patients. In premenopausal females, there was no significant association between rs1008805 genotypes and DFS (AA vs. AG vs. GG, 56.2 vs. 57.7 vs. 98.2 months; P=0.252; Table II). When the patients were clustered into two groups, one with AA variant, and the other with AG or GG genotypes, no significant differences in DFS were evident between these subgroups (AG or GG vs. AA, 58.6 vs. 56.2 months; HR, 0.786; 95%CI, 0.574-1.076; P=0.132; Table II). Besides these, there were also no differences for DFS between patients with the homozygous minor allele (GG) and those carrying the common allele (GG vs. AA or AG, 98.2 vs. 56.4 months; HR, 0.648; 95%CI, 0.286-1.468; P=0.294; Table II).

CYP19A1 rs1008805 polymorphism and DFS in postmenopausal patients. In postmenopausal patients, rs1008805 genotypes were significantly associated with DFS (AA vs. AG vs. GG, 89.2 vs. 58.2 vs. 32.7 months; P=0.019; Table II; Fig. 1). When the population was subdivided to two cohorts, the patients carrying GG variant had a significantly poorer DFS than those harboring AA or AG genotypes (GG vs. AA or AG, 32.7 vs. 70.6 months; HR, 3.613; 95% CI, 1.380-9.457; P=0.005; Table II; Fig. 2). Furthermore, being adjusted by patient features in multivariate analyses, GG genotype remained an independent prognostic marker for DFS (HR, 3.439; 95% CI, 1.251-9.456; P=0.017; Table II). However, there was no significant difference in DFS between females harboring the minor allele and those with the homozygous common allele in a univariate analysis (AG or GG vs. AA, 52.4 vs. 89.2 months; HR, 1.288; 95% CI, 0.705-2.353; P=0.408; Table II).

Discussion

The results of the present study describe an association between rs1008805 variants of *CYP19A1* gene and the efficacy of hormone therapy in postmenopausal females with early breast cancer. Previous studies attempting to identify the association between the expression of aromatase mRNA or protein and aromatase enzyme activity levels with therapeutic response have been indefinite and contradictory (17-20). In the present study, postmenopausal females with the homozygous GG variant of *CYP19A1* rs1008805 exhibit a poorer DFS when compared with those carrying AG or AA genotypes. This difference was further confirmed by multivariate analysis. These findings are biologically reasonable when considering the essential role of *CYP19A1* gene in estrogen metabolism, its potential activity in tumor growth and progression, as well as the potential functional significance of *CYP19A1* genetic polymorphisms.

Several genotypic polymorphisms of *CYP19A1* gene have generated inconsistent results with regard to their potential association with clinical outcome. Liu *et al* (19) demonstrated that TTP and OS were significantly improved in patients with the variant alleles of rs4646 (TT or TG) when compared with patients carrying the wild-type allele (GG) in 272 females with MBC treated with anastrozole. Besides, the data from Colomer *et al* (20) revealed that patients with the rare T allele of rs4646 had a TTP that was three times that of those harboring the homozygous common genotype (GG). However, the data from 95 consecutive postmenopausal females with stage II-III hormone receptor-positive breast cancer revealed that the T allele of rs4646 was associated with poorer response to letrozole neoadjuvant therapy, and the rare allele also appeared to be associated with shorter PFS when compared with those carrying the homozygous common allele, and this effect was particularly significant among elderly patients with no operation following letrozole induction (18). In contrast, Ghimenti *et al* (29) identified that rs6493497 and rs7176005 polymorphisms were not associated with the efficacy of anastrozole neoadjuvant therapy, aromatase mRNA basal expression level or expression alteration caused by therapy.

Estrogen serves a key role in the growth and progression of breast cancer through disrupting the processes of

Table I. Association of *CYP19A1* polymorphisms with clinical characteristics.

Characteristic	n	Polymorphism type, n (%)			P-value
		AA	AG	GG	
Total	287	145 (50.5)	124 (43.2)	18 (6.3)	
Menopausal status					0.902
Premenopausal	217	111 (76.6)	93 (75.0)	13 (72.2)	
Postmenopausal	70	34 (23.4)	31 (25.0)	5 (27.8)	
Tumor size, cm					0.672
≤2	102	52 (35.9)	44 (35.5)	6 (33.3)	
>2	168	82 (56.6)	74 (59.7)	12 (66.7)	
Unknown	17	11 (7.6)	6 (4.8)	0	
Lymph node invasion status					0.853
Negative	82	41 (28.3)	37 (29.8)	4 (22.2)	
Positive	199	100 (69.0)	85 (68.5)	14 (77.8)	
Unknown	6	4 (2.8)	2 (1.6)	0	
TNM stage					0.266
I-II	156	83 (57.2)	65 (52.4)	8 (44.4)	
III	108	48 (33.1)	50 (40.3)	10 (55.6)	
Unknown	23	14 (9.7)	9 (7.3)	0	
Estrogen receptor status					0.266
Negative	36	16 (11.0)	20 (16.1)	0	
Positive	245	125 (86.2)	102 (82.3)	18 (100.0)	
Unknown	6	4 (2.8)	2 (1.6)	0	
Progesterone receptor status					0.861
Negative	65	33 (22.8)	29 (23.4)	3 (16.7)	
Positive	216	108 (74.5)	93 (75.0)	15 (83.3)	
Unknown	6	4 (2.8)	2 (1.6)	0	
Erb-B2 receptor tyrosine kinase-2 status					0.095
Negative	174	79 (54.5)	83 (66.9)	12 (66.7)	
Positive	70	37 (25.5)	30 (24.2)	3 (16.7)	
Unknown	43	29 (20.0)	11 (8.9)	3 (16.7)	
Body mass index, kg/m^2					0.671
<24	159	84 (57.9)	64 (52.0)	11 (61.1)	
≥24	126	61 (42.1)	58 (47.2)	7 (38.9)	
Unknown	2	0	2 (0.8)	0	

cell differentiation and proliferation (30). Lønning *et al* (31) demonstrated that circulating estrogen levels were significantly associated with poorer DFS in postmenopausal patients. In a case-control cohort study, Rock *et al* (32) indicated that total estradiol, bioavailable estradiol and free estradiol circulating concentrations were associated with the risk of recurrence. Aromatase catalyzes the biosynthesis of estrogen in the adipose tissues through the conversion of androgens (3,11,12). In addition, elevated levels of aromatase expression have been detected in malignant breast lesions compared with in normal breast tissue (4,33). Besides, a number of previous studies have demonstrated that *CYP19A1* gene polymorphisms may be associated with increased aromatase activity. Wang *et al* (34) and Gennari *et al* (35) indicated that rs6493497 and rs7176005 were associated with marked decrease in aromatase activity, and Kristensen *et al* (13) observed that longer TTTA repeats were associated with increased aromatase activity. A population-based and *in vitro* study revealed that a Thr364 mutation caused a sharp decrease in aromatase protein levels and activity, whereas a Cys264 mutation was associated with a slight decrease in allozyme activity. Furthermore, the mechanism by which non-synonymous SNPs interfere with the aromatase enzymatic activity was a consequence of an alteration in the aromatase protein level (36). Of note, it has been suggested that *CYP19A1* polymorphisms were significantly associated with hormone levels (14,15,37). Previous studies have indicated that the rs4646 may be associated with circulating hormone levels in postmenopausal breast cancer (18,20). An analysis of five

Table II. Association of *CYP19A1* rs1008805 polymorphism with disease-free survival.

		Univariate		Multivariate[a]	
CYP19A1 polymorphism	Median DFS, months	HR (95% CI)	P-value	HR (95% CI)	P-value
All patients					
GG vs. AG vs. AA	42.7 vs. 57.7 vs. 58.3	AG:AA,0.875 (0.657-1.167) GG:AA,1.030 (0.553-1.919)	0.638	AG:AA, 0.794 (0.586-1.075) GG:AA, 1.014 (0.540-1.902)	0.309
GG vs. AA/AG	42.7 vs. 58.0	1.092 (0.593-2.010)	0.777	1.121 (0.605-2.077)	0.716
AG/GG vs. AA	56.4 vs. 58.3	0.891 (0.675-1.177)	0.417	0.817 (0.610-1.094)	0.175
Premenopausal patients					
GG vs. AG vs. AA	98.2 vs. 57.7 vs. 56.2	AG:AA, 0.811 (0.587-1.120) GG:AA, 0.590 (0.258-1.353)	0.252	AG:AA, 0.093 (0.7430-0.526) GG:AA, 0.646 (0.279-1.495)	0.183
GG vs. AA/AG	98.2 vs. 56.4	0.648 (0.286-1.468)	0.294	0.737 (0.323-1.684)	0.469
AG/GG vs. AA	58.6 vs. 56.2	0.786 (0.574-1.076)	0.132	0.783 (0.557-1.102)	0.161
Postmenopausal patients					
GG vs. AG vs. AA	58.2 vs. 89.2 32.7 vs. 89.2	AG:AA, 3.750 (1.371-10.256) GG:AA, 1.086 (0.572-2.062)	0.019	AG:AA, 1.015 (0.476-2.165) GG:AA, 3.468 (1.160-10.369)	0.116
GG vs. AA/AG	32.7 vs. 70.6	3.613 (1.380-9.457)	0.005	3.439 (1.251-9.456)	0.017
AG/GG vs. AA	52.4 vs. 89.2	1.288 (0.705-2.353)	0.408	1.843 (0.875-3.883)	0.108

[a]Adjusted for lymph node invasion positivity, tumor size >2 cm, negative hormone receptor status, Erb-B2 receptor tyrosine kinase-2-positive status, chemotherapy, hormone therapy, radiotherapy and body mass index ≥24. DFS, disease-free survival; HR, hazard ratio; CI, confidence interval.

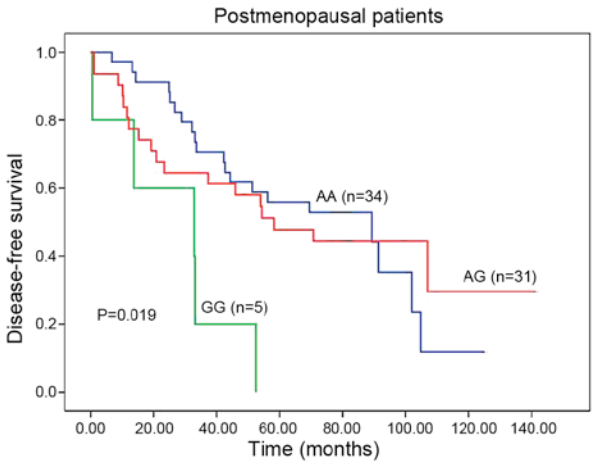

Figure 1. Kaplan-Meier estimator survival curves for postmenopausal patients. Disease-free survival of the patients grouped according to cytochrome P450 19A1 rs1008805 genotypes (AA vs. AG vs. GG).

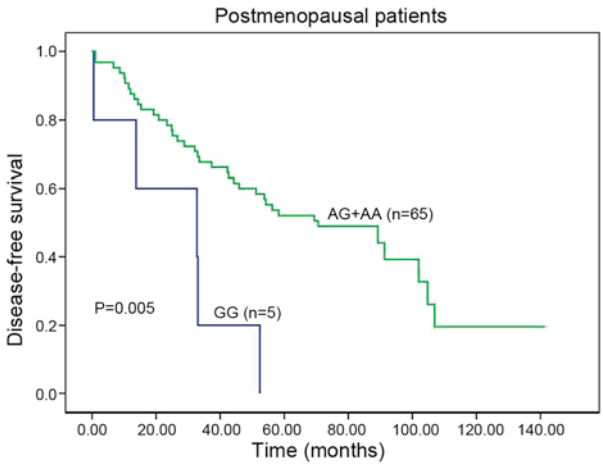

Figure 2. Kaplan-Meier estimator survival curves for postmenopausal patients. Disease-free survival of the postmenopausal females stratified by cytochrome P450 19A1 rs1008805 genotypes (AA and AG vs. GG).

large prospective cohort studies demonstrated that the A allele of rs727479 and rs749292 were significantly associated with elevated levels of estradiol and estrone (11). Haiman *et al* (38) revealed that females carrying 8-repeat allele of the TTTA polymorphism exhibited increased estrogen levels compared with those harboring the 7-repeat allele. Cai *et al* (23) demonstrated that SNPs in block 1 and 2 of *CYP19A1* gene were associated with plasma estrogen levels in postmenopausal Chinese females. Analysis of a comprehensive evaluation of majority variants in whole *CYP19A1* gene revealed that a two

SNP haplotype (rs749292-rs727479 A-A) was associated with a 15% high erestrogen levels in postmenopausal females (39).

The presence of the G allele at rs1008805 has been identified to be significantly associated with an increase in the risk of breast cancer (40). Additionally, Haiman *et al* (24) identified that the same genotype was marginally significant associated with breast cancer risk. The present study demonstrated that postmenopausal females with GG variant of *CYP19A1* rs1008805 have a poorer DFS for hormone therapy compared with those carrying AG or AA genotypes. The majority of SNPs are silent, and thus may not cause alterations to the function or the expression of mRNA (41). However, *CYP19A1* rs1008805 SNP investigated in the present study appears to have an active effect. This maybe linked with an advantage structural alteration to the aromatase protein structure that causes it to be more active (42). A number of other mechanisms are also possible, including an alteration to a DNA-binding site (43,44), mRNA stabilization, splicing or folding alterations, and the modification of transcriptional and post-translational regulation (45-47).

On the basis of these previous studies and the results of the present study, it is hypothesized that *CYP19A1* rs1008805 G SNP may result in elevated aromatase activity, higher protein levels, and thus an increase in circulating estrogen concentrations in postmenopausal females. It is a plausible hypothesis that the GG variant of rs1008805 may cause diminished functional efficacy of hormone therapy when used at the recommended dose, allowing estrogen synthesis to be maintained in the subgroup of postmenopausal patients carrying the GG genotype of rs1008805 during endocrine treatment. However, the presumed difference in estrogen level would be decreased between the premenopausal patients with the GG variant and those with AA or AG, as estrogen is predominantly generated by the ovary in premenopausal females, perhaps causing the effect of *CYP19A1* variants on estrogen levels to be negligible for this group. Therefore, the GG genotype was associated with decreased DFS in postmenopausal patients with hormone therapy, whereas there was no significant association between rs1008805 genotypes and DFS for premenopausal females receiving endocrine treatment.

To conclude, it was demonstrated that the GG genotype of rs1008805 SNP in the first exon of *CYP19A1* gene was significantly associated with inferior DFS in postmenopausal females with hormone therapy. Testing for *CYP19A1* gene rs1008805 SNP as a predictive marker for the response to endocrine therapy in hormone receptor-positive early breast cancer warrants a larger independent prospective clinical evaluation.

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