Association of Novel Gene Polymorphisms RRM1 –756T>C and –269 C>A With Breast Cancer

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Background: Genetic variants are reported to play an important role in the susceptibility of breast cancer. Ribonucleotide reductase 1 (RRM1) is suggested to play an essential role in the regulation of cancer development. The purpose of this study was to identify novel gene polymorphisms of RRM1 –756T>C and RRM1 –269 C>A specific to patients with breast cancer and healthy controls. **Methods:** A total of 833 subjects, including 321 healthy controls and 512 patients with breast cancer, were recruited in this study. Allelic discrimination of RRM1 –756T>C (rs11030919) and RRM1 –269C>A (rs12806698) polymorphisms of the RRM1 gene was assessed with the real-time polymerase chain reaction. **Results:** The adjusted odds ratios and 95%

confidence intervals were 1.20 (0.71–2.04) and 1.10 (0.65–1.86) to have breast cancer among individuals with CC alleles of RRM1 –756T>C and individuals with AA alleles of RRM1 –269C>A gene polymorphism, respectively, compared to individuals having wild type of RRM1 gene polymorphisms. Also, there was no significant genetic interaction effect on the susceptibility of breast cancer and nonassociation between genetic polymorphisms and clinical statuses of breast cancer. **Conclusion:** Gene polymorphisms of RRM1 –756T>C and RRM1 –269C>A may be not an important factor for the susceptibility of breast cancer. J. Clin. Lab. Anal. 28:287–293, 2014. © 2014 Wiley Periodicals, Inc.

Key words: ribonucleotide reductase 1; single nucleotide polymorphism; breast cancer

INTRODUCTION

Ribonucleotide reductase (RNR), nucleotide metabolism enzyme for DNA synthesis and DNA repair, consists of a regulatory subunit ribonucleotide reductase 1 (RRM1) and two catalytic subunits ribonucleotide reductase 2 (RRM2) or p53R2 (1, 2). RRM1 is suggested to be contributed to the regulation of cancer cell proliferation, migration, and metastasis (1–7). Gautam et al. reported that RRM1 induced cell cycle arrest in G₂ phase for triggering a G₂ cell cycle checkpoint response with increased DNA repair

and cell apoptosis and suppression of carcinogenesis (1). Among mice with carcinogen-induced lung tumor, RRM1 transgenic-positive mice had a significantly longer survival compared to RRM1 transgenic-negative mice

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(1). A complete loss of RRM1 is deadly for cells because of total inhibition of RNR activity, but a partial loss of RRM1 could induce tumor development (2, 4). However, Reid et al. found a significantly growth inhibition effect in cancer cell line after transfection with RRM1-specific siRNA and cells transfected with RRM1-targeting siRNA failed to form tumors among nude mice with xenograft induced tumor (3). Also, Bennett et al. found a 30–50% inhibition of cell proliferation in RRM1 siRNA transfected MDA-MB-231 cells, human breast cancer cell line (5). They suggested that RRM1 promotes cancer cell proliferation and is a potential target for cancer therapies (3, 5). We hypothesized that the expression level of RRM1 could be a key regulator for breast cancer development.

Breast cancer with highly aggressive and metastatic potential is one of the most frequently occurring malignant neoplasms and is the essential leading cause of cancer death worldwide (8, 9). Some of epidemiologic studies have reported that genetic variants of genes involved in DNA replication or DNA damage repair play an important role in the susceptibility of breast cancer (10–13). RRM1 gene is located on chromosome 11p15.5 and spans approximately 44.1 kb (4, 14). To the best of our knowledge, only two studies have estimated the risk of RRM1 gene polymorphism, including RRM1 1082C>A, 2455A>G, 2464G>A, rs1980412-C>T, rs2304891-T>C, and rs1474500-T>C, on the susceptibility of breast cancer (15, 16). Rha et al. found a poor overall survival among patients having the double single nucleotide polymorphisms (SNPs) of 2455A>G and 2464G>A compared to patients with wild type of 1082C>A, 2455A>G, and 2464G>A, respectively, or single SNP patients (15). Also, Feng et al. found that subjects with rs1474500(RRM1)-TT had a 3.19-fold risk of breast cancer (16), which indicates that the screening of RRM1 gene polymorphism could be one of the strategies for detecting the candidates who are likely susceptible to breast cancer.

The novel SNPs RRM1 –756T>C (rs11030918) and RRM1 –269C>A (rs12806698) are, respectively, on the promoter region of RRM1 gene, which shows minor allele frequencies to be close to 10% in Asian ethnic population (14). We suggested that –756T>C and –269C>A gene polymorphisms in the promoter region of RRM1 could modulate transcription process and alter RRM1 protein production (17, 18) and significantly affect the individual sensitivity to breast cancer (1–5). However, these two polymorphisms of RRM1 –756T>C and –269 C>A have never been examined in breast cancer patients. Therefore, in this present study, we determine whether genetic variations in RRM1 –756T>C and –269 C>A are associated with the susceptibility and clinicopathological development of breast cancer in Taiwanese.

MATERIALS AND METHODS

Subjects and Specimen Collection

This was a hospital-based case-control study. A total of 512 patients with breast cancer diagnosed at Chung Shan Medical University Hospital, Taichung, Taiwan or Changhua Christian Hospital, Changhua, Taiwan, were recruited as a case group between June 2008 and February 2012. A total of 321 healthy individuals, whose demographic data including race, ethnic, and resident area were the same as those of the study cases, visited the Department of Family Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan or Changhua Christian Hospital, Changhua, Taiwan for health examination, were randomly collected. These control groups had neither self-reported history of cancer of any sites. Personal information and characteristics collected from the study subjects using interviewer-administered questionnaires contained questions involving demographic characteristics. The whole blood specimens, collected from healthy controls and breast cancer patients, were placed in tubes containing EDTA and immediately centrifuged and stored at –80°C. The histological types of the primary tissues and the clinicopathologic stage of the breast cancer were both determined by pathology, according to a system based on a modification of the WHO classification and the TNM system, respectively. Associated clinicopathological characteristics, such as the clinical stage of breast cancer, cell-differentiation status, lymph node metastasis, and distant metastasis, were verified by chart review. The study was conducted with the approval of the Changhua Christian Hospital Institutional Review Board and the Chung Shan Medical University Hospital Institutional Review Board and informed written consent was obtained from each individual.

Selection of RRM1 Polymorphisms

The novel SNPs RRM1 –756T>C (rs11030918) and RRM1 –269C>A (rs12806698) are, respectively, on the promoter region of RRM1 gene, which shows minor allele frequencies to be close to 10% in Asian ethnic population (14). Moreover, these SNPs of RRM1 gene were selected in this study since these SNPs were found in the cancer patients (17, 18).

Genomic DNA Extraction

Genomic DNA was extracted from whole blood samples collected from study subjects by QIAamp DNA blood mini kits (Qiagen, Valencia) according to the manufacture's instructions (19). DNA was dissolved in TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA] and then quantitated by a measurement of OD₂₆₀. Final preparation was stored

at -20°C and used as templates in polymerase chain reaction (PCR).

Real-Time PCR

Allelic discrimination of the RRM1 $-756\text{T}>\text{C}$ (rs11030918) and RRM1 $-269\text{C}>\text{A}$ (rs12806698) gene polymorphisms was assessed with the ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) and analyzed using SDS vers. 3.0 software (Applied Biosystems) with the TaqMan assay. The primers of RRM1 $-756\text{T}>\text{C}$ (rs11030918) and RRM1 $-269\text{C}>\text{A}$ (rs12806698) were 5'-FAM-CCCTGCTTAAAATCCTCTCA and 5'-FAM-CAGTCTGTGAAGACTACCCCG, respectively. The final volume for each reaction was 5 μL , containing 2.5 μL TaqMan Genotyping Master Mix, 0.125 μL TaqMan probe mix, and 10 ng genomic DNA. The real-time PCR included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Moreover, for each assay, appropriate controls (non-template and known genotype) were included in each typing run to monitor reagent contamination and as a quality control. To validate results from real-time PCR, around 5% of assays were repeated and several cases of each genotype were confirmed by the DNA sequence analysis (20).

Immunohistochemistry Analysis

Gene expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal receptor 2 (HER2) in cancerous tissue was analyzed based on standard immunohistochemistry (21). Immunostaining was performed with anti-ER (Dako, ID5), anti-PR (Dako, PGR 636), and anti-HER2 (Dako, Carpinteria, CA), respectively, using an autostaining and semiquantitative scoring system (Ventana Medical System, Inc., Arizona). All hormone-related receptors statuses were evaluated according to breast pathology guidelines. For estimation of ER and PR gene expression, analysis in which a value was less than 10% of expression was considered as a negative staining for hormone receptor and a percentage of expression equal to or more than 10% of expression was considered as a positive staining. In the assessment of the staining of HER2, a strong complete membrane staining in more than 30% of invasive tumor cells was considered a positive staining. In score 2+ cases, fluorescent in situ hybridization (FISH) was performed and the HER2 status was judged on that basis.

Statistical Analysis

Hardy-Weinberg equilibrium was assessed using a goodness-of-fit χ^2 test for biallelic markers. The average age is presented as the mean $\pm$ SE. The odds ratios (ORs)

with their 95% confidence intervals (CIs) of the association between genotype frequencies and breast cancer risk as well as clinical characteristics were estimated by multiple logistic regression models after controlling for other covariates. A P value < 0.05 was considered significant. The data were analyzed on SAS statistical software (Version 9.1, 2005; SAS Institute Inc., Cary, NC).

RESULTS

In our recruited control group, the frequencies of genetic polymorphisms such as RRM1 $-756\text{T}>\text{C}$ ($P > 0.05$, χ^2 value: 0.22) and RRM1 $-269\text{C}>\text{A}$ ($P > 0.05$, χ^2 value: 0.93) were in Hardy-Weinberg equilibrium, respectively.

The distributions of demographical characteristics and gene polymorphisms in breast cancer patients and healthy controls are shown in Table 1. The adjusted ORs and 95% CIs were 1.20 (0.71–2.04) and 1.10 (0.65–1.86) to have breast cancer among individuals with CC alleles of RRM1 $-756\text{T}>\text{C}$ and individuals with AA alleles of RRM1 $-269\text{C}>\text{A}$ gene polymorphism, respectively, compared to individuals having wild types of RRM1 gene polymorphisms. Except for age ($P < 0.001$), there was no significant difference distribution of gender, race, and genetic polymorphisms between breast cancer patients and healthy controls, even we furthermore combined different genetic distributions of these two genes for estimating the genetic interaction effect on the susceptibility of breast cancer (Table 1).

Furthermore, we calculated the relations between genotypic frequencies and clinical statuses, including clinical stage, lymph node metastasis, cell-differentiated grade, recurrence status, and the hormone statuses (negative vs. positive), including ER, PR, and HER2. Also, there was no significant association between these two genotypic frequencies and clinical pathological markers (Tables 2 and 3).

DISCUSSION

To the best of our knowledge, this is the first study to provide novel information of RRM1 $-756\text{T}>\text{C}$ and RRM1 $-269\text{C}>\text{A}$ genetic polymorphism impacts on susceptibility and clinicopathological development of breast cancer. Assuming 95% CI, P value = 0.05, our sample size has at least 80% power to detect a 2.0-fold risk in gene polymorphisms of RRM1 $-756\text{T}>\text{C}$ and RRM1 $-269\text{C}>\text{A}$ on the susceptibility of breast cancer.

RRM1 has been demonstrated to play an essential role in determining breast carcinogenesis (5). To date, few studies have investigated the impact of RRM1 gene polymorphism on breast cancer risk. Rha et al. recruited 74 patients with advanced breast cancer and 56 healthy subjects to estimate genetic effects of RRM1 1082C>A,

TABLE 1. Adjusted Odds Ratio (AOR) and 95% CIs of Breast Cancer Associated With Genotypic Frequencies of RRM1 –756T>C and RRM1 –269C>A

Variable	Controls (<i>n</i> = 321) (%)	Patients (<i>n</i> = 512) (%)	OR (95% CI)	AOR (95% CI)	<i>P</i> value
RRM1 –756					
TT	173 (53.9)	272 (53.1)	1.00	1.00	
TC	123 (38.3)	193 (37.7)	0.99 (0.74–1.34)	1.01 (0.75–1.37)	0.91
CC	25 (7.8)	47 (9.2)	1.19 (0.71–2.01)	1.20 (0.71–2.04)	0.49
TT	173 (53.9)	272 (53.1)	1.00	1.00	
TC or CC	148 (46.1)	240 (46.9)	1.03 (0.78–1.36)	1.04 (0.78–1.39)	0.74
RRM1 –269					
CC	174 (54.2)	275 (53.7)	1.00	1.00	
CA	120 (37.4)	190 (37.1)	1.00 (0.74–1.35)	1.03 (0.76–1.39)	0.84
AA	27 (8.4)	47 (9.2)	1.10 (0.66–1.83)	1.10 (0.65–1.86)	0.69
CC	174 (54.2)	275 (53.7)	1.00	1.00	
CA or AA	147 (45.8)	237 (46.3)	1.02 (0.77–1.35)	1.04 (0.78–1.39)	0.76
RRM1 genes combination					
Group 1	171 (53.3)	266 (52.0)	1.00	1.00	
Group 2	5 (1.6)	15 (2.9)	1.92 (0.68–5.39)	1.81 (0.63–5.16)	0.26
Group 3	145 (45.1)	231 (45.1)	1.02 (0.77–1.35)	1.04 (0.78–1.39)	0.76
Age (years)					
Mean ± SE	44.15 ± 0.55	51.05 ± 0.48			<0.001
Gender					
Women		Women			
Race					
Asian		Asian			

The ORs with their 95% CIs were estimated by logistic regression models.

The AORs with their 95% CIs were estimated by multiple logistic regression models, after controlling for age.

Group 1: individuals with TT of RRM1 –756T>C and CC of RRM1 –269C>A; Group 2: individuals with at least one of the following, including T/C or C/C of RRM1 –756T>C, or C/A or A/A of RRM1 –269C>A; Group 3: individuals with T/C or C/C of RRM1 –756T>C, and C/A or A/A of RRM1 –269C>A.

TABLE 2. AOR and 95% CI of Clinical Statuses and RRM1 –756T>C Genotype Frequencies in Breast Cancer Patients (*n* = 512)

Variable	<i>n</i> (%) TT (<i>n</i> = 272)	<i>n</i> (%) TC or CC (<i>n</i> = 240)	OR (95% CI)	AOR (95% CI)	<i>P</i> value
Clinical Stage					
Stage < II	111 (40.8)	109 (45.4)	1.00	1.00	
Stage ≥ II	161 (59.2)	131 (54.6)	0.89 (0.61–1.29)	0.87 (0.60–1.27)	0.49
Lymph node metastasis					
No	233 (85.7)	215 (89.6)	1.00	1.00	
Yes	39 (14.3)	25 (10.4)	0.71 (0.41–1.26)	0.72 (0.41–1.27)	0.26
Cell-differentiated grade					
Grade I/II	91 (33.5)	65 (27.1)	1.00	1.00	
Grade III	181 (66.5)	175 (72.9)	1.41 (0.92–2.14)	1.42 (0.93–2.15)	0.10
ER					
Negative	76 (27.9)	64 (26.7)	1.00	1.00	
Positive	196 (72.1)	176 (73.3)	0.91 (0.51–1.64)	0.91 (0.51–1.62)	0.74
PR					
Negative	78 (28.7)	65 (27.1)	1.00	1.00	
Positive	194 (71.3)	175 (72.9)	0.98 (0.56–1.72)	1.00 (0.57–1.77)	0.98
HER2					
Negative	196 (72.1)	179 (74.6)	1.00	1.00	
Positive	76 (27.9)	61 (25.4)	0.89 (0.59–1.35)	0.88 (0.58–1.34)	0.56
Recurrence status					
No	260 (95.6)	229 (95.4)	1.00	1.00	
Yes	12 (4.4)	11 (4.6)	1.09 (0.45–2.62)	1.09 (0.45–2.62)	0.83

The ORs with their 95% CIs were estimated by logistic regression models.

The AORs with their 95% CIs were estimated by multiple logistic regression models, after controlling for age.

Cell-differentiated grade: Grade I: well differentiated; Grade II: moderately differentiated; Grade III: poorly differentiated.

HER2, human epidermal growth factor receptor 2.

TABLE 3. AOR and 95% CI of Clinical Statuses and RRM1 –269C>A Genotype Frequencies in Breast Cancer Patients (*n* = 512)

Variable	<i>n</i> (%) CC (<i>n</i> = 275)	<i>n</i> (%) CA or AA (<i>n</i> = 237)	OR (95% CI)	AOR (95% CI)	<i>P</i> value
Clinical stage					
Stage < II	110 (40.0)	110 (46.4)	1.00	1.00	0.29
Stage ≥ II	165 (60.0)	127 (53.6)	0.82 (0.57–1.20)	0.81 (0.56–1.18)	
Lymph node metastasis					
No	235 (85.5)	213 (89.9)	1.00	1.00	0.25
Yes					
Cell-differentiated grade					
Grade I/II	93 (33.8)	63 (26.6)	1.00	1.00	0.11
Grade III	182 (66.2)	174 (73.4)	1.39 (0.91–2.12)	1.40 (0.92–2.13)	
ER					
Negative	80 (29.1)	60 (25.3)	1.00	1.00	0.98
Positive	195 (70.9)	177 (74.7)	1.00 (0.55–1.80)	0.99 (0.55–1.78)	
PR					
Negative	82 (29.8)	61 (25.7)	1.00	1.00	0.81
Positive	193 (70.2)	176 (74.3)	1.04 (0.59–1.84)	1.07 (0.60–1.88)	
HER2					
Negative	197 (71.6)	178 (75.1)	1.00	1.00	0.55
Positive	78 (28.4)	59 (24.9)	0.89 (0.58–1.35)	0.88 (0.58–1.34)	
Recurrence status					
No	263 (95.6)	226 (95.4)	1.00	1.00	0.73
Yes	12 (4.4)	11 (4.6)	1.15 (0.48–2.78)	1.16 (0.48–2.78)	

The ORs with their 95% CIs were estimated by logistic regression models.

The AORs with their 95% CIs were estimated by multiple logistic regression models, after controlling for age.

Cell-differentiated grade: Grade I: well differentiated; Grade II: moderately differentiated; Grade III: poorly differentiated.

HER2, human epidermal growth factor receptor 2.

2455A>G, and 2464G>A gene polymorphisms on breast cancer susceptibility, they found no significantly different distribution of SNPs between breast cancer patients and healthy controls. Also, there was no significant association between SNP and tumor response after gemcitabine monotherapy. However, a strong association was found between the lower frequency of neutropenia and a haplotype containing double SNPs of 2455A>G and 2464G>A, and a poor overall survival was found in patients having the double SNPs compared to wild-type or single SNP patients. They suggested that breast cancer patients with these two SNPs were genetically less susceptible to gemcitabine treatment, which resulted in a tendency toward a poor survival in patients having these two SNPs (15). Feng et al. conducted a case-control study based on a dataset of longitudinal study called Nurses' Health Study to estimate RRM1 genetic impact, including rs1980412-C>T, rs2304891-T>C, and rs1474500-T>C, on the susceptibility of breast cancer. They obtained 1,231 patients with invasive breast cancer and 1,203 healthy controls derived from 32,826 subjects who provided blood samples between 1989 and 1990 and who were followed until May 2004 in Nurses' Healthy Study. They found that individuals with rs1474500(RRM1)-TT had a 3.19-fold risk to have breast cancer. On the other hand, a signifi-

cant SNP–SNP interaction, which related to the susceptibility of breast cancer, was found. Subjects with double SNPs of rs1980412(RRM1)-CC and rs7372736(MLH1)-GG, rs1980412(RRM1)-CC and rs9852810(MLH1)-TC, rs1980412(RRM1)-CC and rs7611106(MLH1)-AA, rs1980412(RRM1)-CC and rs7632760(MLH1)-GG, rs1980412(RRM1)-CC and rs6789043(MLH1)-CC, rs2304891(RRM1)-TT and rs7372736(MLH1)-GG had a protect effect for subjects from breast cancer, but individuals with double SNPs of rs2304891(RRM1)-CC and rs1981929(MSH2)-AA had a 1.4-fold risk to have breast cancer (16).

In this present study, we found that the allelic frequencies of RRM1 –756T>C and RRM1 –269C>A gene polymorphisms were not significantly associated with breast cancer, even we furthermore combined different genetic distributions of these two genes for estimating the genetic interaction effect on the susceptibility of breast cancer. Few of studies investigate RRM1 –756T>C and RRM1 –269C>A gene polymorphism impacts on disease. Ryu et al. and Soo et al. have recruited 298 and 53 patients with nonsmall cell lung cancer (NSCLC), respectively, to estimate the association of RRM1 –756T>C and RRM1 –269C>A gene polymorphisms with overall survival among patients treated with a gemcitabine

combination (14,22). Both of these studies showed no significant association between these two SNPs and overall survival among NSCLC patients treated with gemcitabine or gemcitabine combination (14, 22). We suggested that RRM1 -756T>C and RRM1 -269C>A genetic variants could not be considered as a vital factor related to an increased susceptibility to breast cancer, and there are other possible explanations for why an individual is not susceptible to breast cancer, aside from these two SNPs, such as linked genes.

Recently, hormone-related factors, including ER, PR, and HER2 have been considered to be associated with the development and prognosis of breast cancer (23–26). Triple-negative breast cancer according to ER, PR, and HER-2 expression is an aggressive subtype of breast cancer and it is related to abundant DNA aberration or lower protein expression of DNA repair system genes (27, 28). We hypothesized that RRM1 -756T>C and RRM1 -269C>A genetic variants could influence breast cancer progression and gene expression of ER, PR, and HER2 (15). Therefore, we further estimated the relationship between these two gene polymorphisms and clinical statuses, including clinical stage, lymph node metastasis, cell-differentiated grade, recurrence status, and negative or positive gene expression of ER, PR, and HER2. Also, there was no significant association between RRM1 -756T>C and RRM1 -269C>A genetic polymorphism and clinical statuses of breast cancer. Kim et al. recruited 230 tissue samples of patients with invasive breast cancer to estimate the protein expression of RRM1 in breast carcinoma tissues. They found that RRM1 expression was not significantly different between the subtypes of breast cancer and there was no association between RRM1 expression and disease-free survival and overall survival (28). Also, Metro et al. have examined protein expressions in breast tissues among 55 metastatic breast cancer patients treated with gemcitabine-based chemotherapy and evaluated its relationship with clinical outcome. They found that there was no significant association between RRM1 protein expression and tumor stage, histology, hormone receptor status (positive vs. negative), and HER2 status (positive vs. negative) (29). We suggested that it is probably RRM1 -756T>C and RRM1 -269C>A genetic variants without an observable influence on breast cancer progression and gene expressions of ER, PR, and HER2.

One of the limitations of our study is the low sample size. Furthermore, the functional role of RRM1 in growth or metastasis of breast cancer is worth for further investigation, which will be included in our future work. Clones containing various genotypes of RRM1 SNPs will be constructed to elucidate the possible functions of RRM1 in breast cancer cell lines, as well as the underlying mechanisms. In conclusion, RRM1 -756T>C and RRM1

–269C>A gene polymorphisms could not be considered as a factor related to an increased susceptibility to breast cancer.

CONFLICT OF INTEREST

None declared.

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