A genetic polymorphism (rs17251221) in the calcium-sensing receptor is associated with ovarian cancer susceptibility

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Abstract. Calcium-sensing receptor (CaSR) is a G-protein-coupled receptor that senses blood calcium. *In vivo*, CaSR is required for normal epidermal differentiation by mediating calcium signaling. CaSR was confirmed to be a tumor suppressor in colon and breast cancer. The single-nucleotide polymorphism (SNP) rs17251221, located on the intron, is a genetic variation of the *CaSR* gene. We analyzed rs17251221 in ovarian cancer using an allelic discrimination assay. Cycling probes were used for genotyping 290 ovarian cancer patients and 312 age-matched cancer-free females. rs17251221 and clinicopathological characteristics of ovarian cancer were analyzed statistically. The AG and GG genotypes were confirmed to appear in fewer cancer cases than in controls and the genotype distribution between cases and controls was statistically significant. The AG+GG genotype was correlated with low ovarian cancer risk, while rs17251221 was not associated with clinicopathological variables including age at diagnosis, tumor size, histologic type, pathological subtype, lymph node metastasis, CA-125 expression, clinical stage, or degree of differentiation. The rs17251221 polymorphism genotype was not correlated with survival in ovarian cancer. These results suggest that the G allele of the CaSR rs17251221 polymorphism is protective against ovarian cancer and the homozygous GG genotype may be a protective genotype as well. The rs17251221 may play an important role in the development of ovarian cancer and could be used as a biomarker for predicting ovarian cancer.

Introduction

Ovarian cancer is the most lethal cancer of gynecologic cancers even though it causes fewer deaths than breast cancer

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and precursor lesions of the uterine cervix (1). New cases of ovarian cancer are estimated to be 21,980 in the United States in 2014 and deaths due to ovarian cancer totaled 14,270, which represent 5% of deaths due to female malignancies (2). Most patients with ovarian cancer are not diagnosed at early stages due to lack of obvious symptoms, few effective diagnostic approaches and no tumor markers (3). Ovarian cancer is difficult to treat and patient prognosis is poor unless diagnosis occurs early, conferring a survival rate of 90-95% (4). Thus, earlier detection is crucial. Ovarian cancer is influenced by many factors such as physical and chemical exposures, biotic factors, and heredity (5). Genetic variants in *AURKA*, *BRCA1*, and *CCNE1* genes are associated with ovarian cancer risk (6). Women with hereditary ovarian cancer syndrome, such as mutations in BRCA1 and BRCA2, are estimated to confer a 40% greater risk of ovarian cancer, but these mutations are found in only 0.05% of the female population (1). Increased frequency of single-nucleotide polymorphisms (SNP) rs11954856 and rs351771 of adenomatous polyposis coli (APC) can increase the risk of ovarian cancer in Polish women (7). Therefore, more studies are needed to identify potential markers to predict ovarian cancer susceptibility and this may improve treatment strategies and increase survival.

Calcium-sensing receptor (CaSR) is a G-protein-coupled receptor initially cloned from the bovine parathyroid gland in 1993 (8). Its structure has three domains: extracellular (coded for by the first 6 exons of the *CaSR* gene), a membrane-spanning motif, and an intracellular tail (coded for by the 7th exon) (9). CaSR has been characterized as a sensor for calcium and parathyroid hormone regulation via the parathyroid gland and kidney in response to blood calcium (8,10,11). CaSR function is required for normal epidermal differentiation via mediation of calcium signaling *in vivo* (12). Mutations in the *CaSR* gene lead to loss or gain of function, but most cause alterations in extracellular calcium (13). Disruption of CaSR function contributes to alterations in the physiology of neoplastic cells (14), which modifies tumor development and progression. SNP rs17251221 is located in an intron of the *CaSR* gene on chromosome 3, and it is significantly associated with serum calcium regulation (15,16). Evidence suggests a significant association between CaSR rs17251221 and stone multiplicity in nephrolithiasis patients (17). CaSR rs17251221

Table I. rs17251221 genotype and allele distribution in ovarian cancer and control.

Genotype	Ovarian (%)[a]	Control (%)[a]	P-value	OR 95% CI
AA	277 (95.52)	275 (88.14)		1 (reference)
AG	13 (4.48)	36 (11.54)	0.001	0.359 (0.186-0.691)
GG	0 (0)	1 (0.32)	0.001	
AA	277 (95.52)	275 (88.14)		1 (reference)
AG+GG	13 (4.48)	37 (11.86)	0.001	0.349 (0.181-0.671)
AA	277 (100)	275 (99.64)		
GG	0	1 (0.36)	0.499	
A	567 (97.76)	586 (93.91)		1 (reference)
G	13 (2.24)	38 (6.09)	0.001	0.354 (0.186-0.671)

[a]The values of χ^2 in healthy control and ovarian cancer cases were 0.02 and 0.15, respectively, both with a P-value of >0.05; OR, odds ratio; CI, confidence interval.

has also been verified to be associated with hepatocellular carcinoma (HCC) risk, and the rs17251221 G allele genotype offers a better prognosis for HCC treated with transcatheter hepatic arterial chemoembolization (18). Data also show that rs17251221 was strongly associated with prostate cancer (15). Previously, we reported that genetic variations in rs17251221 for the *CaSR* gene are associated with breast cancer risk and may be prognostic indicators for patient outcomes (19). In the present study, we analyzed rs17251221 of the *CaSR* gene to clarify any association between CaSR rs17251221 and ovarian cancer susceptibility.

Materials and methods

Patients and samples. Study participants (n=290) (mean age, 52.58±13.08 years) were diagnosed with ovarian cancer at the Qilu Hospital of Shandong University between September 2008 and December 2014. Patient data are presented in Table I. Subjects were age-matched with 312 cancer-free females (mean age 51.7±12.58 years) who were recruited from women who had annual physical examinations. Participants involved were Han Chinese residents. The study was approved by the Ethics Committee of Shandong University, and written informed consent was obtained from all the participants in the present study.

DNA extraction. Blood was obtained from each subject and DNA was extracted from the samples according to the protocol of the TIANamp Genomic DNA kit (Tiangen, Beijing, China). DNA concentration and purity were measured by using an ultraviolet spectrophotometer (GE Healthcare, Pittsburgh, PA, USA). DNA samples were stored at -80°C as previously described (20,21).

SNP genotyping analysis of CaSR. Cycling probes were synthesized by Takara Biotechnology (Dalian, China), and an allelic discrimination assay (Cycleave PCR® Core kit, CY505S, Takara) was performed using an ABI 7900HT thermal cycler for SNP genotyping. Each 20-μl reaction contained: 1X Cycleave PCR reaction mixture, 0.2 μm PCR forward primer, 0.2 μm PCR reverse primer, 0.4 μm cycling probe, and 50 ng

DNA template. PCR amplification conditions were as follows: 95°C for 30 sec, 45 cycles of 95°C for 5 sec, 55°C for 10 sec, and 72°C for 25 sec. Sequence Detection systems software version 2.4.1 was used for data analysis. To confirm genotyping data, several DNA samples were randomly selected for sequencing analysis.

Statistical analysis. Data analysis was performed as previously described (21). The genotype and allele frequency of CaSR were examined using a χ^2 test for Hardy-Weinberg equilibrium (HWE) (22). P>0.05 was set as a non-deviation from HWE. The genotype and allele distribution were analyzed using the χ^2 test between ovarian cancer and control groups. When 25% of analyzed cells had counts <5, the Fisher's exact test was used. The association between ovarian cancer and the *CASR* polymorphism was measured using the odds ratios (OR) and clinicopathological characteristics of ovarian cancer were analyzed using logistic regression models. The Kaplan-Meier method was used to assess the association of the SNP rs17251221 genotype with ovarian cancer patient survival. Data were considered statistically significant at P<0.05. The data in our study were analyzed with SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA) software.

Results

Relationship between rs17251221 and ovarian cancer susceptibility. Ovarian cancer patients and healthy controls were all Mainland Chinese women. There was no significant difference between the two groups with respect to matching characteristics. A χ^2 test was used to confirm that subjects met HWE. The values for χ^2 for healthy control and ovarian cases were 0.02 and 0.15, respectively (P>0.05).

An allelic discrimination assay was used to analyze the rs17251221 polymorphism distribution in the ovarian cases and controls. Significant differences between ovarian cancer cases and controls are shown in Table I. AG and GG genotypes had fewer cancer cases than controls and the χ^2 results showed that the genotype distribution between the two groups was statistically significant (AA vs. AG vs. GG, P=0.001). The difference between the additive genetic model of AA and AG genotypes
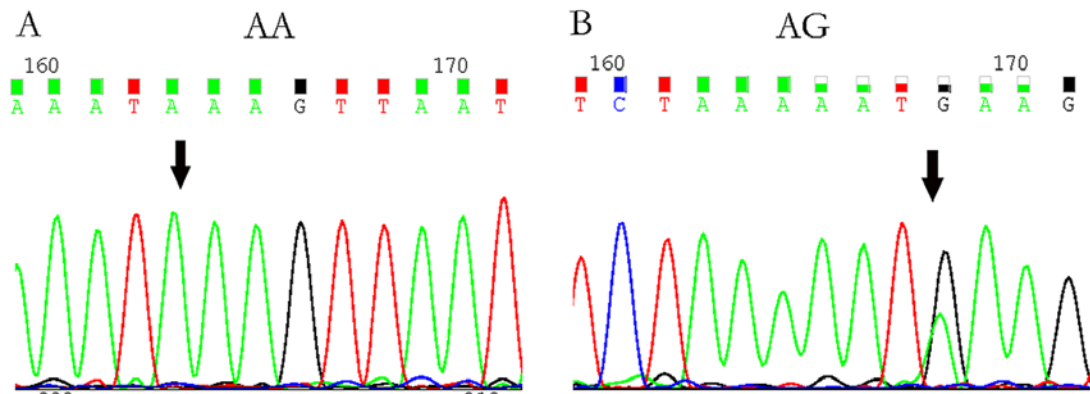
Figure 1. Sequencing chromatograms of polymorphism rs17251221. Several samples were chosen randomly for DNA sequencing to confirm genotype PCR results. Sequencing chromatogram results of the (A) AA and (B) AG genotype.
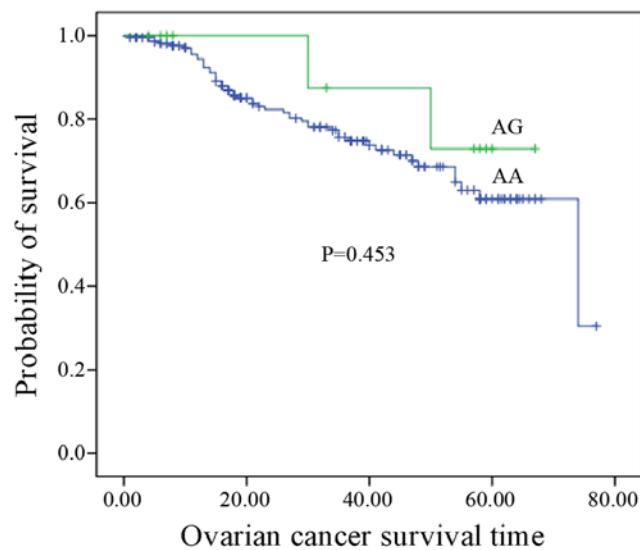

Figure 2. Association between polymorphism rs17251221 and ovarian cancer prognosis. The Kaplan-Meier method was used to evaluate the prognosis of rs17251221. Differences were considered significant at P<0.05.

was statistically significant (P=0.001). Additionally, AG patients had a low risk for ovarian cancer compared with AA patients. Few GG patients were included for the analysis. Thus, AA patients of the genotype AG+GG were assessed and the results showed that this genotype was significantly correlated with lower risk of ovarian cancer risk [P=0.001, OR=0.349, 95% CI (0.181-0.671)]. The G allele of the CaSR rs17251221 polymorphism appears to be protective against ovarian cancer [P=0.001, OR=0.345, 95% CI (0.186-0.671)]. Random DNA samples were subsequently selected for sequencing to confirm genotyping results (Fig. 1).

Association analysis between rs17251221 and clinicopathological variables. To assess the association between rs17251221 and age at diagnosis, tumor size, histologic type, pathological subtype, and lymph node metastasis, serum CA-125 expression was measured (Table II). The Kaplan-Meier method was used to assess the association between rs17251221 genotypes and ovarian cancer survival. No correlation between rs17251221

genotypes and ovarian cancer survival was identified (P=0.453, P>0.05) (Fig. 2).

Discussion

Ovarian cancer is the most deadly of the gynecologic cancers (1). Largely due to non-specific symptoms, late diagnosis, and few effective clinical examination methods and indications during early stages of ovarian cancer (23). Current screening strategies are limited by sensitivity and specificity (24,25). Thus, investigations are underway to identify ovarian cancer biomarkers, especially genetic markers for assessing cancer risk and monitoring therapeutic response (23).

CaSR is a G-protein-coupled receptor (8) that senses calcium and parathyroid hormone regulation in response to blood calcium (8,10,11). CaSR regulates homeostasis in response to the extracellular changes of polycationic small molecules (14). *In vivo*, CaSR is required for normal epidermal differentiation by mediating calcium signaling required for

Table II. Association analysis between CaSR rs17251221 and clinicopathological characteristics.

Clinicopathological data	All (%)	Genotype (%)		P-value	OR
		AA	AG		
Age (years)				0.153	
≤50	110	108 (98.18)	2 (1.82)		1 (reference)
>50	179	168 (93.85)	11 (6.15)		3.536
Tumor size (cm)				0.539	
<10	177	170 (96.05)	7 (3.95)		1 (reference)
≥10	109	103 (94.495)	6 (5.505)		1.415
Tumor histologic type				0.231	
Epithelial	252	241 (95.63)	11 (4.37)		1 (reference)
Others	29	28 (96.55)	1 (3.45)		0.782
Tumor pathological subtype				0.054	
Serous	195	189 (96.92)	6 (3.08)		1 (reference)
Others	90	84 (93.33)	6 (6.67)		2.25
Positive lymph node				0.393	
Positive	56	55 (98.21)	1 (1.79)		1 (reference)
Negative	226	215 (95.13)	11 (4.87)		2.814
CA-125 (U/ml)				0.067	
<500	155	146 (94.19)	9 (5.81)		1 (reference)
≥500	127	123 (96.85)	4 (3.15)		0.527
Clinical stage				0.091	
I, II	100	94 (94)	6 (6)		1 (reference)
III, IV	174	168 (96.55)	6 (3.45)		0.56
Degree of differentiation				0.099	
Low	189	183 (96.83)	6 (3.17)		1 (reference)
Middle, high	35	32 (91.43)	3 (8.57)		2.859

CaSR, calcium-sensing receptor; OR, odds ratio.

keratinocyte differentiation (12). CaSR has been confirmed to act as a tumor suppressor in colon and breast cancer (26,27). If normal CaSR-induced responses to extracellular calcium are lost or upregulated, neoplastic cell physiology can be altered, contributing to neoplastic progression (14). rs17251221 is a CaSR genetic variation confirmed to be a susceptibility marker of stone multiplicity in nephrolithiasis and it is associated with coronary heart disease, type 2 diabetes, HCC, and prostate and breast cancer risk (15,17-19).

In the present study, we examined CaSR expression in ovarian cancer patients and controls and identified that AG and GG genotypes were correlated with low ovarian cancer risk compared to the controls. The genotype distribution between the two groups was statistically significant. Patients with AG genotypes had less risk for ovarian cancer compared with homozygote AA. We also assessed combined AG+GG genotypes in both groups and found a significant correlation with low ovarian cancer risk. The G allele of the CaSR polymorphism, rs17251221, appears to protect against ovarian cancer. However, rs17251221 was not associated with clinicopathological variables, and a prognosis analysis showed that the genotype AG was not associated with ovarian cancer survival. These results suggested that rs17251221 may be an independent factor contributing to the progression of ovarian cancer in the Han Chinese population, but it was not a prognostic indicator for ovarian cancer survival. Enrolled subjects were from the Shandong Province of China, but we suggest these results may be extrapolated to larger samples. Our results can be used to suggest strategies for ovarian cancer prediction. This represents the first variant study of the CaSR polymorphism, rs17251221, and ovarian cancer risk and, to the best of our knowledge, this is the first report to suggest that the G allele of the CaSR polymorphism protects against ovarian cancer, thus, the homozygous GG genotype may indicate lower risk of ovarian cancer.

Acknowledgements

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