NOVEL ASSOCIATIONS BETWEEN BRCA1 VARIANTS C.181 T>G (RS28897672) AND OVARIAN CANCER RISK IN SAUDI FEMALES

NOVE VEZE IZMEĐU VARIJANTE BRCA1 C.181 T>G (RS28897672) I RIZIKA OD KARCINOMA JAJNIKA KOD SAUDIJSKIH ŽENA

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Summary

Background: Mutations in BRCA1 gene have been implicated in ovarian cancers, and BRCA testing may be conducted in high-risk women. This study was designed to determine the frequency of three single nucleotide polymorphisms (SNPs) variants in BRCA1 gene and BRCA1 expression in Saudi females with ovarian cancer.

Methods: Expression levels of mRNA of BRCA1 gene were studied in 10 ovarian cancer and 10 normal ovarian tissues, by quantitative real time polymerase chain reaction (qPCR). The study also included 28 females who had suffered from ovarian cancer and had been successfully operated upon and 90 healthy females with no history of cancer. Blood was drawn in EDTA tubes and used for extraction of DNA. The genotyping was carried out using Taqman® SNP Genotyping kit by RT-PCR. The variants investigated included c.871 T>C (rs799917), c.1040 G>A (rs4986852), c.181 T>G (rs28897672) in BRCA1 gene.

Results: The c.181 T>G (rs28897672) showed significantly different genotype and allele frequencies between the patients and the control subjects (p value = 0.002 and 0.02).
The study showed that c.181 T>G in BRCA1 genes is associated with the development of ovarian cancer in Saudis. More studies are needed to unveil other SNPs that may be associated with ovarian cancer and to understand the mechanism(s) involved in reducing the expression of BRCA1 gene in ovarian cancer tissues.

**Conclusions:** This study showed that c.181 T>G in BRCA1 genes is associated with ovarian cancer in females living in Saudi Arabia. This study was designed to examine BRCA1 gene expression levels among ovarian cancer and normal tissues, as well as to determine the role of three BRCA1 gene variants (c.871 T>C, c.1040 G>A and c.181 T>G) in the development of ovarian cancer in females living in Saudi Arabia.

Factors that prompted our interest in the study of c.871 T>C (rs799917) polymorphisms in the BRCA1 gene in ovarian cancer were that it is one of the most studied SNPs, though the results have been inconsistent in different populations, and little is known about the biological, functional, and clinical impact of this polymorphism. Since it is a missense, non-synonymous mutation (P871L) and was reported to be associated with breast cancer, we hypothesized that it could potentially alter the function or the stability of the BRCA1 protein, and may play a role in ovarian cancer development. Finally, c.181 T>G (rs28897672) in the BRCA1 gene was selected as it occurs in a highly conserved region, where the T>G transition replaces cysteine with glycine at codon 61 of the BRCA1 protein. This results in a large physicochemical difference due to the difference between cysteine and glycine. Furthermore, this variant was reported to be a common cause of ovarian cancer in individuals of Eastern European ancestry.

**Materials and Methods**

**Sample collection**

The study was approved by the Ethical Committee at King Khalid Hospital, King Saud University, Riyadh. Each participant was required to give a written informed consent. The clinical data recorded included the age of each subject, family history, clinical profile, and disease severity and recurrence risk.

For gene expression studies, a total of 20 ovarian tissue samples were received from the oncology department of King Khalid University hospital (KKUH), King Saud University (KSU), Riyadh, Saudi Arabia. Ten of these samples were ovarian cancer tissues collected at the time of surgical removal of the affected ovaries. Histological biopsies were cut into
small sections (1–2 mm) and were subjected to routine histological examination procedures. The sample was considered to be cancerous only when the biopsy showed more than 80% malignant cells. Five tissues were adjacent to the normal ovarian tissues, and another five were obtained from patients who underwent surgery for benign ovarian tumor (ovarian cyst). These ten samples were used as controls. All tissue samples were kept in RNAlater solution (Ambion® Life Technologies, USA) to avoid degradation of RNA and stored at –80 °C until used for analysis.

For genotyping, blood samples were collected in EDTA tubes (BD Vacutainer, USA) by venepuncture, from 28 ovarian cancer patients (these patients had already undergone surgical procedure, and their fresh tissues were not available for gene expression studies), and 92 normal individuals, who were attending the clinic for routine investigations and had no history of ovarian or any other cancer.

**Extraction of RNA and DNA**

The RNA was extracted from ovarian tissue samples. For RNA extraction, the tissue was homogenized using Medic tools (Switzerland) homogenizer. RNA was extracted using the AllPrep DNA/RNA Mini kit according to the manufacturer's protocol (Qiagen, Germany). Whole blood was used for the extraction of DNA, using QIAamp DNA Blood Mini Kit.

**cDNA Preparation**

Extracted RNA (approximately 1 μg) from each sample was used as a template for the reverse transcription reaction. Single stranded cDNA was synthesized from the purified RNA using random primers and the high-capacity cDNA reverse transcription kit, according to the protocol provided by the manufacturer (Applied Biosystems, USA).

The cDNA Reverse Transcription (RT) reactions were prepared by pipetting 2 X RT master mixes (10 μL) into each well of a 96-well reaction plate and adding the same amount of RNA samples. The plate was placed in the thermal cycler for which the Reverse Transcription protocol had been set as: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min and 4 °C for 5 min.

**Expression Primer Screening**

The primers were selected from already published reports. The expression primers for BRCA1-c were F-5’-CATCATTACCCTTGCCACA-3’ and R-5’-CATTTGTCCTGTCCAGGCAT-5’. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene, and the primers used were: F- 5’-TGATGACATCAAGAAGGTGGTA-3’ and R-3’-TCCTTGAGCCCCATGTGGGCAT-5’.

Quantitative real-time PCR analysis was carried out using SYBR Green (Bio-Rad Laboratories) according to the manufacturer’s instructions. The 25 μL PCR reaction contained 6 μL of template cDNA (20 ng/μL) with 1 μL of each primer set (100 ng/μL), 12.5 μL SYBER Green master mixed 4.5 μL of DNase, RNase free water. The conditions used for PCR amplification were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 20 s, 55 °C for 30 s and 72 °C for 30 s.

The relative expression levels of BRCA1 mRNA were quantified using the standard curve method. A GAPDH transcript was used for relative quantification and to normalize the amplification signal of the target in all the experimental samples. The target and reference genes were amplified under the same experimental conditions, run separately in triplex real-time PCR with the same concentration of cDNA per reaction. Cycle threshold Ct (defined as the cycle number at which the fluorescence exceeds a threshold level) values were determined for each reaction (run in triplicates) using a sequence detection software. Data were analyzed and relatively compared using the comparative Ct method [2 Ct (Livak) relative expression method] that determines the ratio between the amount of target and an endogenous reference gene.

The fold changes between disease and control samples for each gene were compared, and the data analyses were performed with normalized real-time PCR data.

**Genotyping using TaqMan assay**

Three variants (c.871 T>C, c.1040 G>A, c.181 T>G) in the BRCA1 gene were investigated using TaqMan genotype assay according to the manufacturer’s instructions (Applied Biosystems, USA).

**Statistical analysis**

Frequencies of the genotypes and alleles of each SNP were calculated, and independent sample t-test was used to compare the results in the control and patient groups. Hardy Weinberg (H-W) equilibrium tests for association (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) were applied to test if the H-W equilibrium was obeyed. Odds ratios (OR), 95% confidence intervals (CIs) and p-value were determined by logistic regression to examine the association between each genotype and risk of ovarian cancer. The common allele and genotype were used as the reference. Significance was set at p < 0.05. The obtained results from this study were compared with the results reported from other populations using specific statistical tests. The gene expression results in the cancer tissue were compared with the results in the normal tissue using Student’s t’ test.
Results

Clinical characteristics

Table I summarizes the clinical data of women from whom the ovarian tissue samples were extracted, and Table II presents the clinical data of the women from whom the blood samples were obtained. These tables also show the clinical profile, including family history, disease severity, recurrence risk and age at first diagnosis.

Tissue identification and histopathology

To confirm the diagnosis of ovarian cancer and to distinguish the cancer tissue from normal tissue, histopathological examination was carried out. The histological characteristics of the biopsy sections were recorded and stained with hematoxylin and eosin and examined under the microscope to distinguish between ovarian cancer and the adjacent normal ovarian biopsies. Figure 1 presents the histological differences between the normal and cancerous ovarian tissues.

Table I Characteristics of women from whom ovarian tissue samples were extracted.

| Age (years) | Number (%) |
|-------------|------------|
| <50         | 5 (50%)    |
| 50–59       | 3 (30%)    |
| 60          | 2 (20%)    |

Family History

| Yes         | 10 (100%) |
| No          | 0         |
| No information | 0     |

Disease Severity

| Stage I | 0 |
| Stage II| 1 (10%) |
| Stage III| 0 |
| IV      | 9 (90%) |

Table II Characteristics of ovarian cancer patients from whom blood samples were obtained (N=28) and the controls (N=98).

| Age (years) | Number of Patients | Normal Controls |
|-------------|--------------------|-----------------|
| <50         | 15 (53.6%)         | 44 (47.8%)      |
| 50–59       | 10 (35.7%)         | 34 (36.9%)      |
| 60          | 3 (10.7%)          | 13 (14.1%)      |
| No information | 0          |

Family History

| Yes | 13 (46.4) |
| No  | 15 (53.6) |
| No information | 0     |

Disease Severity

| Stage I | 15 (53.6) |
| Stage II| 0         |
| Stage III| 2 (7.1)  |
| IV      | 6 (21.4)  |
| No information | 5 (17.9) |

Figure 1 Tissue identification and histopathology. (A) Normal ovarian tissue stained by Hematoxylin and eosin stain (H & E) and visualized under a microscope with a digital zoom (micro, 200X). (B) Ovarian serous cyst adenocarcinoma tissue stained by (H&E) and visualized under a microscope with a digital zoom (micro, 200X); malignant cells are showing large and pleomorphic nuclei with areas of invasion and necrosis.
Genotype and allele frequencies of SNPs in BRCA1 gene in ovarian cancer patients and controls

The genotypes were counted, and genotype and allele frequencies were obtained. The genotype and allele frequencies of all three studied SNPs are presented in Table III. Of the three studied variants, BRCA1 c.871 T>C, c.181 T>G were polymorphic in Saudis, but c.1040 G>A was not polymorphic, and the minor ‘A’ allele was not encountered in the patients or controls. BRCA1 c.871 T>C occurred at the same frequency in the patients and controls and showed no association with ovarian cancer. BRCA1 c.181 T>G correlated significantly, where for BRCA1 c.181 T>G, the mutant G allele showed a protective effect (i.e. for TG: OR = 0.360; 95% CI = 0.146–0.889; p = 0.024).

Gene expression quantification

Gene expression of BRCA1 was studied in ten ovarian cancer tissues and ten normal tissues. Figure 2 presents the expression of BRCA1 gene in cancerous

| Table III | Genotype and allele frequency of the studied SNPs in ovarian cancer patients compared to control group. |
|-----------|----------------------------------------------------------------------------------------------------------|
| rs799917 T>C Genotype Frequency |                                                                                                         |
| TT        | 28 (100)                                                  | 90 (97.8)                                           | Ref | 1.058 | 0.042–26.70 | 0.31 | 0.577 |
| TC        | 0                                                        | 1 (1.1)                                            |     | 1.058 | 0.042–26.709 | 0.31 | 0.577 |
| CC        | 0                                                        | 1 (1.1)                                            |     | 0.635 | 0.030–13.61 | 0.62 | 0.431 |
| CT+CC     | 0                                                        | 2                                                   |     |       |              |     |       |
| Alleles Frequency |                                                                                                         |
| T         | 56 (100)                                                 | 181 (98.4)                                         | 2.179 | 0.111–42.82 | 0.92 | 0.761 |
| C         | 0                                                        | 3 (1.6)                                            | 0.459 | 0.023–9.019 | 0.92 | 0.761 |
| rs4986852 G>A Genotype Frequency |                                                                                                         |
| GG        | 28 (100)                                                 | 92 (100)                                           | Ref |       |              |     |       |
| AG        | 0                                                        | 0                                                  | 3.246 | 0.063–167.278 | 1.000 |
| AA        | 0                                                        | 0                                                  | 3.246 | 0.063–167.2 | 1.000 |
| AG+AA     | 0                                                        | 0                                                  | 3.246 | 0.063–167.2 | 1.000 |
| Alleles Frequency |                                                                                                         |
| G         | 56 (100)                                                 | 184 (100)                                          | 0.306 | 0.006–15.609 | 1.000 |
| A         | 0                                                        | 0                                                  | 3.265 | 0.064–166.442 | 1.000 |
| rs28897672 T>G Genotype Frequency |                                                                                                         |
| TT        | 15 (57.7)                                                | 27 (32.5)                                          | Ref |       |              |     |       |
| TG        | 11 (42.3)                                                | 55 (66.3)                                          | 0.360 | 0.146–0.889 | 5.09 | 0.024 |
| GG        | 0                                                        | 1 (1.2)                                            | 0.591 | 0.023–15.4 | 0.55 | 0.459 |
| TG+GG     | 11                                                       | 56                                                 | 0.354 | 0.143–0.87 | 5.29 | 0.021 |
| Alleles Frequency |                                                                                                         |
| T         | 41 (80)                                                  | 109 (70)                                           | 1.949 | 0.931–4.080 | 3.21 | 0.073 |
| G         | 11 (20)                                                  | 57 (30)                                            | 0.513 | 0.245–1.074 | 3.21 | 0.073 |
and normal tissues. The gene expression was lower in the cancerous tissue compared to the normal tissue, and the difference was statistically significant (p < 0.0001).

Discussion

Ovarian cancer is common in women and is considered as one of the most lethal malignancies. Since it is associated with non-specific symptoms, it is usually diagnosed at a late stage, and by then the disease has spread to such an extent that survival frequency is reduced, and hence ovarian cancer has gained the reputation of a silent killer (3). Early diagnosis followed by early intervention has been shown to significantly reduce the mortality associated with ovarian cancer. If such genetic markers are identified that are significantly associated with ovarian cancer, either in their expression or the associated alleles, then a closer follow-up of genetically susceptible women can play an important role in early diagnosis and, hence, will help in early intervention. It is with the aim to identify such variants in the BRCA1 gene that this study was conducted.

During this study, the collection of samples was a limiting step, since ovarian cancer is reported to occur at a frequency of 2.7/100000 (10). During our study period, only ten women were diagnosed who were undergoing surgical removal of the affected ovaries. Of these, 9 of the women were at stage IV and 8/10 women were diagnosed at age < 60 years. The adjacent normal tissue could not be obtained for 5 of these women since the entire ovaries were already affected. Hence, five normal control tissues were obtained from females who were undergoing surgical removal of ovaries for pathologies other than ovarian cancer. Results of gene expression in both groups were the same and showed significantly higher gene expression of BRCA1, both in the normal tissue adjacent to the affected ovaries and in the normal ovaries from unmatched women, compared to the ovarian cancer tissue, indicating clearly that BRCA1 gene expression is reduced in cancer tissues.

The selection of BRCA1 was based on the fact that mutations in the BRCA1 tumor suppressor gene are associated with susceptibility to both hereditary breast and ovarian cancer (11). It suppresses the development of cancer, and its reduced expression could be associated with the risk of cancer development. Approximately 5% to 15% of ovarian cancers are inherited. Germline mutations in the BRCA1 gene have been shown to account for 90% of ovarian cancer, conferring a 54% cumulative lifetime risk compared to 1.8% of the general population (12). Although somatic mutations in BRCA1 are uncommon in sporadic ovarian tumors (13), more than 72% of high-grade sporadic ovarian cancers have shown down-regulation of BRCA1, suggesting that BRCA1 may also be involved in the development of sporadic epithelial ovarian cancer (15). Epigenetic inactivation of BRCA1 is at least partially due to hyper-methylation of the BRCA1 promoter, which has been observed in up to 15% of the cases (14). Wang et al. (15) have reported a decreased expression of BRCA1 in 16% of benign tumors, 38% of borderline tumors, and 72% of carcinomas. These results suggest that the down-regulation of BRCA1 protein may play a role in the development of ovarian cancers (15).

In our study, many possible explanations can be given for the reduced mRNA expression of the BRCA1 gene. This reduced expression may be a result of an epigenetic event, such as hyper-methylation of the BRCA1 promoter, which would reduce the mRNA level by decreasing transcription of the gene, and which will be associated with amelioration in the protein level. Such a possibility can be confirmed by immunohistochemistry (IHC) techniques. Other studies have shown loss of BRCA1 protein expression, and it is suggested that such loss of expression is more common in ovarian cancer than in breast cancer (16).

Other causes of decreased expression of the BRCA1 gene could be due to certain mutations. Several studies have shown that mutations in the BRCA1 tumor suppressor gene are associated with a significant risk of breast and ovarian cancer development (17), yet over 90% of ovarian cancer is believed to arise sporadically. BRCA1 mutations are uncommon in sporadic ovarian cancer. However, BRCA1 dysfunction has frequently been observed (18, 19). Gene deletion (loss of heterozygosity) or misregulation of related genes in the BRCA1 pathway, leading to silencing of BRCA1 through decreased expression may be important in the pathogenesis of a significant proportion of sporadic tumors (20, 21). Recent clinical data suggests that tumor expression of BRCA1 has predictive value in patients without a BRCA1 mutation (19, 22). A correlative study on BRCA1 protein expression in 230 ovarian tumors, with survival data on 152 patients, suggests a role for BRCA1 in the progression
of sporadic ovarian carcinoma (19). The frequency of BRCA1 and BRCA2 mutations has been reported in several studies in breast and ovarian cancer patients. A study on Moroccan women with hereditary breast and ovarian cancer showed the frequency to be 25.64%. Other studies conducted in neighboring populations such as Tunisia and Algeria have reported frequencies of 19.4% and 11.4% (23), respectively.

During this study, we investigated three variants in the BRCA1 gene to examine a possible association with ovarian cancer and compared the results reported in studies of other populations. We identified that the variant BRCA1 c.871 T>C, which resulted in a P871L substitution in the BRCA1 protein, was one of the most studied SNPs, though the results have been found to be inconsistent in different populations. Previous studies have examined the association between the P871L polymorphism in BRCA1 gene and the risk of developing ovarian cancer. A gynecologic oncology group study found that patients with the CT or TT vs. the CC genotype in BRCA1 (P871L) show a modestly increased risk of ovarian cancer and disease recurrence (24). The P871L polymorphisms were associated with an increased risk of developing ovarian cancer, however, such a correlation was not confirmed in a large-scale study (25). In another investigation, the North Carolina ovarian cancer study group showed that BRCA1 P871L polymorphism was not associated with ovarian cancer risk (OR = 0.9, 95% CI = 0.6–1.9). The results of our study are in agreement with the results of the North Carolina study and show that there was no significant difference in the frequency of the three genotypes of BRCA1 variant c.871 T>C in ovarian cancer and normal controls.

Interestingly, BRCA1 c.871 T>C has also been studied in other cancers, and an increased association has been reported with oesophageal squamous cell carcinoma (26), cervical cancer (27), gastric cancer (29), non-Hodgkin’s lymphoma (29) and glioblastoma (31). Some studies have also shown an association with breast cancer (31, 32), but several others failed to show any association with breast cancer, chronic myeloid leukemia and ovarian cancer (33–35). A large meta-analysis involving 19878 patients failed to reveal any association with breast cancer (36). Earlier reports on breast cancer in Saudi and Omani populations also failed to reveal any such association (37, 38).

The SNP variant BRCA1 c.1040 G>A in the BRCA1 gene was recently studied in a Polish population and was shown to have an association with breast cancer (32). In the present study, we did not observe any GA or AA genotypes and the A allele was absent, suggesting that this SNP variant is not polymorphic in the Saudis. No other reports of association of this SNP to any cancer were found in the literature, and no association was seen with ovarian cancer.

A novel finding in this study was the finding of an association between variant BRCA1 c.181 T>G and ovarian cancer, where the minor allele showed a protective effect against ovarian cancer development (OR=0.513). This missense mutation located in exon 5 results in the substitution of cysteine by glycine at position 61 and occurs in a highly conserved cysteine ligating residue in the ring finger domain of BRCA1. It has previously been reported as a founder mutation among Central European populations (40). In Polish families with breast-ovarian cancer, it accounted for 20% of identified mutations (41). It was also observed

Table IV Comparison of frequency of rs799917 T>C and rs4986852 G>A in BRCA1 in Saudis with different populations.

| Populations          | No. of samples (n) | Genotype frequency (%) | Allele frequency (%) |
|----------------------|--------------------|------------------------|----------------------|
|                      |                    |                       |                      |
| rs799917 T>C         |                    |                       |                      |
| European             | 226                | TT 10.6 CT 46 CC 43.4 | T 33.6 CC 66.4       |
| Asian                | 172                | 7 40.7 52.3           | 27.3 72.7           |
| Sub-Saharan African  | 226                | 85.8 14.2             | 92.9 7.1           |
| African American     | 124                | 69.4 25.8 4.8         | 82.3 17.7          |
| Saudis (this study)  | 92                 | 97.8 1.1 1.1          | 98.3 1.6           |
| rs4986852 G>A        |                    |                       |                      |
| Asian                | 164                | GG 96.3 GA 3.7 AA 0   | G 98.2 A 1.8       |
| European             | 226                | 88.5 11.5             | 94.2 5.8           |
| Sub-Saharan African  | 222                | 99.1 0.9 0            | 99.5 0.5           |
| Saudi control        | 92                 | 100 0 0               | 100 0              |
in a study from Morocco (42). The presence of this mutation in Morocco and Algeria suggests that this mutation represents a mutational hotspot, but only the haplotype analysis can establish whether such an association exists or not in the families who had a common ancestor (42). An extensive search of the literature did not reveal any studies on any other cancer patient groups. Further studies on a larger sample size may show associations with cancer.

For two variants (BRCA1 c.871 T>C and BRCA1 c.181 T>G) for which data was available on NCBI, we compared the frequencies of the genotypes and allele in the normal Saudi population with those reported for other populations. The comparative results are summarized in Table IV. For the variant BRCA1 c.871 T>C, the results show that the homozygous genotype TT is more frequent in Sub-Saharan African and African American populations compared to the other genotypes (CT and CC). The results obtained during our study showed that Saudi results were similar to the results in Sub-Saharan Africans, but differed significantly from the results reported in Asians and Europeans. Finally, the variant BRCA1 c.181 T>G was polymorphic in the Asians and Europeans, but like in Saudis, it was not polymorphic amongst Sub-Saharan Africans (Table IV). This comparison shows the significant heterogeneity that exists between populations, making it necessary to investigate SNP frequencies in each population and to study their association with different disease states.

**Conclusion**

The results of this study showed a reduced level of expression of BRCA1 gene in the ovarian cancer samples compared to normal ovarian tissue samples. The BRCA1 SNPs variant BRCA1 c.181 T>G was significantly associated with development of ovarian cancer in Saudi females. However, SNPs variants BRCA1 c.871 T>C and BRCA1 c.1040 G>A did not show any association with ovarian cancer and the SNP variant BRCA1 c.1040 G>A was not polymorphic in this population. Our study suggests that some variants in BRCA1 genes may be associated with the development of ovarian cancer in Saudi females. Further studies are needed to explore other variants that may be associated with ovarian cancer and the mechanism(s) involved in the reduced expression of BRCA1 gene in ovarian cancer tissues.

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**Conflict of interest statement**

The authors stated that they have no conflicts of interest regarding the publication of this article.

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