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ORIGINAL ARTICLE

Granzyme B Gene Polymorphism (rs11539752) and Breast Cancer in Egyptian Women

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ABSTRACT

Background*: Breast cancer (BC) is the most common type of female cancer in Egypt. Granzyme B (GrB) is primarily found in cytotoxic granules and has traditionally been considered the most abundant granzyme. However, recent research has revealed various other crucial roles for GrB. Specifically, GrB expression in both normal epithelial cells and cancer cells affects extracellular matrix remodeling, epithelial-to-mesenchymal transition, and fibrosis.* ***Objectives****: In this study, we investigated whether a common genetic variation in the gene encoding GrB, consisting of one single nucleotide polymorphisms (rs11539752), is associated with breast cancer risk in Egyptian women.* ***Methodology:*** *This study included 195 participants, with 103 diagnosed with breast cancer and 92 serving as controls. Genotyping of the Granzyme B gene variants (rs11539752) was conducted using tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR).* ***Results****: We find that CC genotype was higher in breast cancer group than control group and GG genotype was higher in breast cancer group than control group (p>0.05) and odds ratio (95% confidence interval) in GG genotype between breast cancer and control groups 0.971 (0.939-1.004).* ***Conclusion****: The variant allele (rs11539752) couldn't be considered as a predictive factor for breast cancer development in Egyptian women.*

INTRODUCTION

Breast cancer (BC) is a leading malignancy among women globally and the second most common cause of cancer-related deaths in Egyptian women.[1] It accounts for 32% of all cancers in Egyptian women, with an overall incidence of 157 cases per 100,000. By 2050, cancer incidence in Egypt is expected to triple, with a trend toward younger patients and more advanced stages. Identifying BC risk factors is crucial to addressing this growing concern.[2]

Breast cancer is a highly complex and heterogeneous disease, characterized by diverse classification schemes, molecular subtypes with distinct etiologies and clinical management strategies, and significant variability in patient outcomes and therapeutic responses. [3]

Breast cancer classification relies on the tumor's histological appearance. [4] Breast cancer cells are also classified based on key receptors: estrogen (ER), progesterone (PR), and HER2/neu. Cells are labeled positive or negative for these receptors (e.g., ER+, PR-, HER2+). Those lacking all three are termed triple-negative or basal-like.[5]

Breast cancer is histopathologically classified into carcinoma in situ and invasive carcinoma. Carcinoma in situ, accounting for 15-30% of breast biopsies, has a favorable prognosis with 5-year survival rates of 97-99%.[6,7] Invasive carcinoma represents 70-85% of cases,[5] with invasive ductal carcinoma being the most common type (80%) and invasive lobular carcinoma comprising about 10% of invasive cases 6 and 5% of all breast cancers in the U.S.[8] The 5-years survival rate for both invasive ductal and lobular carcinomas was approximately 85% as of 2003.[9] While ductal carcinoma in situ (DCIS) is non-lethal, untreated low-grade lesions have a 60% risk of progressing to invasive cancer within 40 years.[10]

Granzymes are serine proteases found in cytotoxic T-cells and natural killer (NK) cells, playing a key role in inducing apoptosis through perforin.[11] Granzyme B (GrB) is the most abundant and can affect extracellular matrix remodeling, epithelial-to-mesenchymal transition, and fibrosis in various normal and cancer cells.[12]

The GZMB gene, encoding the GrB protein, consists of five exons with several identified single nucleotide polymorphisms (SNPs). The most studied SNPs include Q55R in exon two, P94A in exon three, and Y247H in exon five. A cohort study in newly diagnosed breast cancer patients revealed that individuals with GZMB

RAH alleles have a higher risk of developing breast cancer compared to those with QPY alleles.[13] SNP analysis is a stable and reliable screening tool, useful for identifying high-risk individuals and enabling preventive interventions before disease onset.[14] The vast SNP marker databases support association studies and mapping of disease-related loci.[15] Research focuses on identifying biomarkers for predicting malignant diseases, with SNPs, a common genetic variation, emerging as valuable markers.[16] Numerous studies link SNPs to disease risk. [17,18,19] This study aimed to examine whether a common genetic variation in the gene encoding GrB, consisting of two single nucleotide polymorphisms (rs11539752), is associated with the risk of breast cancer in Egyptian women.

METHODOLOGY

Subjects and Sampling

Five milliliters of EDTA blood samples of peripheral venous blood were collected in sterile EDTA-tubes (KemikoVacutainer, Egypt). Each sample was labeled and numbered to the correspond with the other related investigations.

The subjects included in the present study were healthy volunteer individuals (n = 92) and breast cancer patients (n = 103) from Faculty of Medicine, Menoufia University, and Hematology Unit of Baheya Foundation for Early Detection and Treatment of Breast Cancer , Egypt. Blood samples were obtained from patients who given informed consent under a protocol approved (No: 2/2019 INTM2) from 2/2019 to 11/2020 by the Faculty of Medicine Ethical Committee Review Board, Menoufia University. Controls were recruited from the same population via invitations. All participants were recruited at a designated laboratory center, where investigations were conducted free of charge.

Exclusion criteria were; any cancer except breast cancer, any treatment (chemotherapy, radiotherapy and hormonal therapy), HCV, HBV and HIV viral infection, concomitant autoimmune disease, and use of immunosuppressive drugs.

All selected patients underwent physical examinations and a routine history assessment for diagnosis, including pathology for breast biopsy and immunohistochemical analysis. One hundred and three breast cancer patients, ranging in age from 32 to 82 years and in clinical stage from I to III be randomly selected. Among the 103 patients, 44.66% were diagnosed with grade II invasive ductal carcinoma, 18.45% with grade III invasive ductal carcinoma, and 7.77% with grade I invasive ductal carcinoma. Additionally, 3.89% had non-invasive ductal carcinoma or encapsulated papillary carcinoma with grade II. Invasive lobular carcinoma grade II accounted for 6.79%, while 1.96% had invasive lobular carcinoma (grades I and III), non-invasive ductal carcinoma (grade II), or mucinous carcinoma (grade I). Mixed invasive ductal and invasive lobular carcinoma of grade II was observed in 4.85%, and 0.97% had Paget's disease of the nipple or adenoid cystic carcinoma of intermediate and grade II, respectively.

Genotyping:

Peripheral Blood Leucocytes' Isolation

Approximately 2 mL of EDTA blood samples were mixed with erythrocyte lysing buffer (1:4 v/v) within three hours of collection and incubated for 20 minutes at 30°C. The samples were then centrifuged for 5 minutes at 1500 rpm, repeating the process until a white pellet of leukocytes appeared. [20] The isolated pellets were stored at -80°C until DNA extraction.

Isolation of Total Genomic DNA

Genomic DNA was isolated from peripheral blood leukocytes using the Aljanabi and Martinez extraction method. [21.] Leukocyte pellets were lysed in a buffer (50 mM NaCl, 1 mM Na_2EDTA, 0.5% SDS, pH 8.3) for 2 hours at 45°C. Proteins and 4cellular debris were removed with 4 M NaCl, while nucleic acids were precipitated using cold isopropanol. The resulting pellets were reconstituted in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and stored at -20°C until further use.

SNP Selection and genotyping

Single nucleotide polymorphisms (SNPs) were chosen based on data published in PubMed's SNP database. In this study, SNPs were selected because of their significant association with the diseases being investigated. [13,22]

To investigate the genotyping and allele analysis of polymorphisms in the Granzyme B gene (rs11539752), a tetra primers amplification-refractory mutation system (ARMS-PCR) was conducted using a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). For rs11539752, DNA samples were first denatured at 94°C for 10 minutes, followed by 30 cycles of denaturation at 94°C, annealing at 65°C, and extension at 72°C, each for 1 minute. The primer sequences are detailed in (table 1).

Table 1: Primer sequences for rs11539752 detection using tetra primers amplification-refractory mutation system (ARMS-PCR)

Reverse	Forward	rs11539752
AAGAAAGTCCAGGTCAGCCAACGAA	TGGTTCCAGAGGTGCTGCTGAAGTA	Outer
ATTATAGGCTGGATGGGGGATCGG	CCCAGCAG TTTATCCCTGTGAAAACAG	Inner

For rs11539752, the fragment sizes were 276 bp, 396 bp, and 621 bp, corresponding to the G allele, C allele, and control band, respectively. All fragments were designed to fall within a range of 250–650 bp. Amplification was carried out in a single reaction tube using four primers simultaneously. Oligonucleotide primers were designed with the PRIMER1 software for tetra-primer ARMS-PCR (http://primer1.soton.ac.uk/primer1.html, accessed January 17, 2021). The resulting amplicons were separated on 2% agarose gels (Sigma, St. Louis, MO, USA) and visualized using a UV transilluminator.[23]

Statistical Analysis

The results were gathered, organized into tables, and analyzed statistically using SPSS version 25 (SPSS, Inc., Chicago, IL, USA). Differences in allele frequencies and genotype distribution between the breast cancer patients and control group were evaluated using Pearson's χ^2 test. Odds ratios and their confidence intervals were computed to assess the relationship between genotype and breast cancer. When the assumption of Chi-square (χ^2) was violated, Fischer exact test was performed. Statistical significance was considered when the P-value was < 0.05.

RESULTS

Total of 103 BC patients (100% were females) aged from 32 to 82 years old were included in this study. Healthy adult volunteers (n = 92, 100% were females) were included as control group with similar mean age of the patients group. The patients were sorted according to breast cancer type. Estrogen Receptor, (ER); Progesterone Receptor, (PR); Human Epidermal Growth Factor Receptor-2 (HER-2) were examined in the patients. Out of the 103 patients, 82.52% were positive for Estrogen Receptor (ER) and Progesterone Receptor (PR) but negative for Human Epidermal Growth Factor Receptor-2 (HER2). Additionally, 7.77% were positive for PR while negative for both ER and HER2, and 5.83% were positive for ER while negative for PR and HER2. Furthermore, 1.94% was positive for ER, PR, and HER2, while another 1.94% was positive for HER2 but negative for both ER and PR.

The demographic of the studied subjects are demonstrated in table (2). There was significant difference among breast cancer patients and control group in age (P<0.001)

Table 2: Demographic data of the studied subjects

Groups variables	Control	Breast cancer (BC)	$\chi 2$	P value	Odds ratio (95% confidence interval)
Number (N)	92 (47.2%)	103 (52.8)			
Age (years) (Mean±SD)	46.52±10.429	53.70±12.10	12.92	<0.001	4.047(1.89-8.67)

SD= Standard deviation, $\chi 2$= Chi-Square test, P value significant <0.05

Genotyping of Granzyme B:-

The allelic frequency of rs11539752 was assessed using tetra primer ARMS-PCR, and the products were resolved on agarose gel, figure 1. The current study revealed not statistically significant difference between breast cancer patients and control group (P>0.05).

Breast cancer group had higher frequency for the G allele compared to control group (p <0.05).

We find that CC genotype was non significantly higher in breast cancer group than control group and GG genotype was non significantly higher in breast cancer group than control group (p>0.05).

Odds ratio (95% confidence interval) in GG genotype between breast cancer and control groups 0.971 (0.939-1.004).

Moreover, there was no significant difference between the breast cancer and the control groups in rs11539752 genotypes (GG, CC) (p>0.05) as shown in table (3).

Table 3: Genotyping of rs11539752

	Control N=92 N(%)	Breast cancer (BC) N =103 N(%)	$\chi 2$	P value	Odds ratio (95% confidence interval)
Genotype rs11539752 CC GG	 0 (0.0) 92 (47.9)	 3 (100) 100 (52.1)	2.72	0.099	0.971 (0.94-1.00)
Allele frequency C G	 0 184 (47.9)	 6 (100) 200 (52.1)	0.031	<0.05	Zero

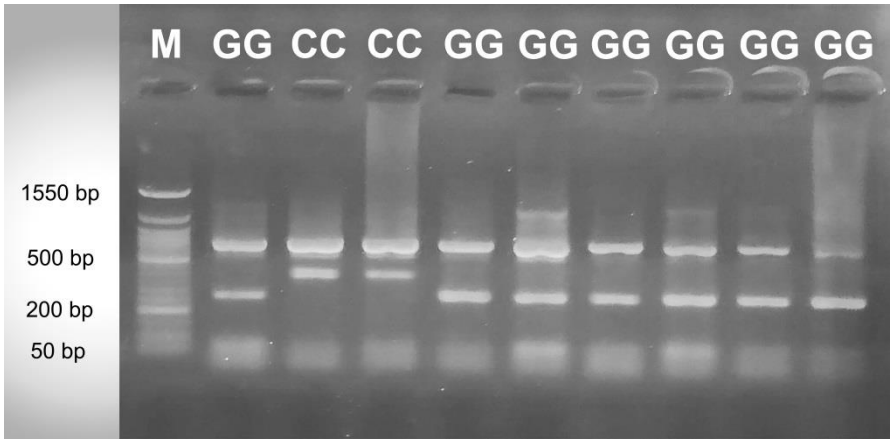

Fig. 1: Representative digital photograph of ARMS-PCR amplified products separated on 2.0% agarose gel electrophoresis showing the breast cancer (rs11539752) genotyping against Gene Ruler 50bp DNA ladder (Willowfort, Birmingham Research and Development park,Birmingham)

Hardy–Weinberg equilibrium (HWE) genotype frequencies of Granzyme B gene (rs11539752) polymorphisms are demonstrated in table (4). HWE was calculated for studied SNP.

Granzyme b (rs11539752) genotype frequencies were differed with HWE among breast cancer group.

Table 4: Hardy–Weinberg equilibrium for Granzyme b gene (rs11539752) genotype among breast cancer patients.

Groups	SNP				
	Genotype	Observed	Expected	$\chi 2$	P value
Breast cancer N=103	GG	100	97.09	103	<0.001
	GC	0	5.83		
	CC	3	0.087		

DISCUSSION

Breast cancer is a leading cause of cancer-related deaths in women worldwide. Most tumors are genetically complex and heterogeneous, involving multiple genes.[24] Single-nucleotide polymorphisms (SNPs) are the most prevalent type of variation in the human genome.[24] Genetic factors are key in breast cancer development. Since the completion of the Human Genome Project in 2001, SNPs have emerged as critical contributors to disease. The direct lytic effect on tumor cells involves the perforin/granzyme pathway, where granzyme B induces apoptosis by forming transmembrane pores and activating caspases like caspase-3. Additionally, caspase-independent mechanisms, such as DNA fragmentation, contribute to apoptosis.[٢٥] Granzyme B also plays roles in viral infections, graft rejection, graft-versus-host disease, and likely inhibits cancer growth and progression. [26-31]

The **GZMB** gene, encoding the GrB protein, consists of five exons with several identified single nucleotide polymorphisms (SNPs). Among the most studied are Q55R (exon 2), P94A (exon 3), and Y247H (exon 5). A cohort study found that breast cancer patients carrying the RAH alleles had a higher risk of developing the disease compared to those with the QPY alleles. [13]

Granzyme B gene polymorphism (rs11539752) in the current study demonstrated that the CC and the GG genotypes, in addition to C and G-allele frequency, were higher in breast cancer patients compared with control groups. A. Nizar and Mhaidat and their colleagues found similar results this study was done in Jordan and enrolled patients with colorectal cancer. Their study found a non-significant association. [22]

The present study showed that patients with breast cancer had the highest frequent C-allele while had disappeared in control group and the breast cancer group showed the highest G-allele frequency than in control.

In the current study, the CC genotype was the total frequent in breast cancer patients (100%) and lack in control group, followed by the GG (52%) in breast cancer group and the least frequent was in control group (47.9%).

These polymorphisms haven't been linked to the occurrence of breast cancer in this study. Additionally, we found that GG and CC genotypes of granzyme b (rs11539752) show high not significant percentage among breast cancer and control groups. While C and G-allele had high significantly in breast cancer group than control group, $P<0.05$.

Genotype and allele prevalence in addition to the site of polymorphisms of Granzyme B are still in doubt and further studies are needed to study this point.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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