RESEARCH ARTICLE

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Investigation of the Effects of *B7H4* (rs10754339 A>G) Gene Polymorphism and Serum Level on Breast Cancer Susceptibility

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Abstract

Background: The prevalence of breast cancer groups requires further clarification to understand the mechanisms leading to its development. *B7H4* is a novel immune checkpoint protein that negatively regulates T cell activation and function. It is overexpressed in several cancers and breast cancer is one of them. **Aim:** This case-control study aimed to examine the association of *B7H4* (rs10754339 A>G) gene polymorphism and its serum level with breast cancer among Egyptian females. **Method:** The study enrolled 100 women with breast cancer and 100 healthy cancer-free women. *B7H4* (rs10754339 A>G) genotyping was performed for all participants using Tetra-ARMS-PCR technology. Additionally, serum sB7-H4 level was measured using an ELISA method. **Results:** *B7H4* (rs10754339 A>G) gene variant indicated a substantially association with the breast cancer risk under various genetic models, including codominant model (AG genotype), dominant and over-dominant models ($p<0.001$). Also, the serum *B7H4* level of patients was significantly higher in the patients than healthy controls ($p<0.001$). **Conclusion:** The *B7H4* (rs10754339 A>G) genetic variant was considered a risk factor for breast cancer among Egyptian women and assessment of serum *B7H4* levels could be considered as a prognostic marker for breast cancer.

Keywords: Breast cancer- *B7H4* (rs10754339 A>G)- polymorphism- T-ARMS-PCR- ELISA

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Introduction

Breast cancer (BC) is the most prevalent cancer among women in the world and the first cause of mortality from malignant tumors [1]. Millions of women worldwide suffer from breast cancer. The economy has been impacted by the cost of chronic disease in the first decade of the twenty-first century [2]. Although this kind of cancer is becoming more common worldwide, developed nations have the highest incidence [3]. It is responsible for 33% of female cancer cases in Egypt, with over 22,000 new cases diagnosed annually. Due to population growth, shifts in the population pyramid, and the adoption of a western lifestyle, this is anticipated to increase dramatically during the ensuing years [4].

Even while survival rates in many developed nations have significantly improved, 5-year survival rates in Egypt remain lower, ranging from 28% to 68%, according to several studies [5]. Many factors contribute to the low survival rates, and it is believed that most patients are diagnosed at late stage [5]. Breast cancer varies widely from patient to patient and even from tumor to tumor (intra-tumor heterogeneity). The development of the illness has entailed intricate biological mechanisms, including several genes and stages [6].

The *B7-Homolog 4* (*B7H4*) belongs to the class of transmembrane glycoprotein type I and is a component of the B7/CD28 superfamily, located on chromosome 1p12/13.1. *B7H4* is encoded by the VTCN1 gene [7, 8].

The B7 family is vital for regulating the immune system and avoiding over-activation. Co-stimulatory and co-inhibitory molecules belong to the B7 family, which is one of the most significant secondary signaling pathways in T cell activation. *B7H4* is a *B7H4* member of the B7 family co-inhibitory molecules, functions as an immune checkpoint regulator that controls immunological and anti-inflammatory responses [9]

Typically, antigen-presenting cells produce *B7H4*, which is mediated by hypoxia and the synthesis of local cytokines including IL-6 and IL-10 [10]. Activated T cells express the *B7H4* receptor, this suggests that *B7H4* may play a role in controlling T cell activation and exhaustion [11]. The soluble form of *B7H4*, known as s*B7H4*, was reported to be present in cancer patients' serum, while normal tissues have very little *B7H4* expression [12]. Because *B7H4* suppresses the immune system in cancer, it is currently being extensively researched as a therapeutic target, as mainly, the cancer patients presented with *B7H4*-

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positive tumors [13-15].

According to earlier studies, it might also have a role in cancer monitoring, prognosis, and diagnosis. Because *B7H4* is primarily missing in normal cells and produced into the bloodstream by cancer cells and cancer-like tissues, it may have potential as a screening protein in addition to its therapeutic utility. However, because this gene is overexpressed in several cancer types, it cannot be used as a cancer-specific biomarker [16]. Normal cells and breast cancer cells have distinct *B7H4* expression patterns. When comparing breast cancer cells to normal cells, the surface expression of *B7H4* is noticeably higher [17].

This study aimed to examine the association of *B7H4* (rs10754339 A>G) gene polymorphism and its serum level with breast cancer susceptibility among Egyptian females through a case-control study.

Materials and Methods

Study Participants

From May 2022 to April 2023, this case-control study enrolled 100 women diagnosed with BC from the Mansoura University Oncology Center's outpatient clinics in Egypt. A control group of 100 healthy volunteers with matched ages was included attained the Oncology Center for routine checkup. Using a core needle biopsy to assess, breast cancer was diagnosed and tissue was identified [18]. Furthermore, tumor staging was carried out using the TNM staging approach, which was based on the most amended staging criteria of the AJCC and the UICC [19]. Immunohistochemical methods have been used to evaluate predictive biomarkers for breast cancer, such as ER, PR and HER2 [20]. The Ethics Committee of Mansoura University granted ethical approval (MDP.22.11.116), and all participating ladies consented to participate in this study.

Sample Collection

Every woman who took part in the study had five milliliters of blood collected, which was then split into two sections. The first section was taken into vacuum tubes with EDTA for hematological and genetic analysis, and the second section was taken into vacationer tubes devoid of any additives for biochemical and tumor marker analysis. A biochemical analyzer (Cobas c501, Roche Diagnostics, Germany) was used to perform biochemical tests, such as ALT, AST, albumin, creatinine, and uric acid. ELISA kits were used to detect the tumor markers CA 15-3 and CEA. Furthermore, a human ELISA kit (NOVA Cat. NO.In – Hu4225 China) was used to evaluate the *B7H4* level in serum.

Genomic DNA extraction

Using the extraction kit (QIA amp, catalog number: 51104 USA), DNA was extracted from all blood samples and the NanoDropTM 1000 Spectrophotometer was used to measure the concentration of DNA. Absorbance ratio (A260/A280) is accepted for pure DNA if the ratio is between 1.8 – 2.0 [21].

Amplification of B7H4 (rs10754339 A>G) gene Variant

Genotyping and amplification of the *B7H4* (rs10754339 A>G) gene variant was performed using a tetra-primer amplification refractory mutation system with polymerase chain reaction (T-ARMS-PCR) technique as described previously Elsaid et al. [22] using the primers [(forward outer: 5′- AAG GCT ATC CGA CTC TCA TTA GGA GCA C -3′, reverse outer: 5′- GAC ATC CAG CTT CTC CTG TAT GAC CCT A); while the two inner primers were (forward inner A allele: 5′- ATG ACT TTG CAT GCT TTT TTG TGG ACA -3′, and the reverse inner G allele: 5′- CCC TTA CCT GAT GCT AAA ATA ATG TGC ATC -3′)]. The reaction contained (3 µl of 100 ng/µl of DNA template, 3 µl 10 pmol/µl of each primer, 10 µl of PCR master mix). The PCR thermal cycler (Applied Biosystems Thermo Fisher) was used, with adjusting the cycling conditions as illustrated in Table S1. The resulting fragments were and identified using 2.5% agarose gel electrophoresis and visualized with the ethidium bromide staining which facilitates imaging under UV illumination.

Statistical analysis

To verify the data normality, the Kolmogorov-Smirnov test was employed. The student-t test was used to handle the parametric data, and the Mann-Whitney U-test was used to assess the non-parametric data. The association between qualitative variables was examined using the Fischer-Exact (FET) and Chi-Square (χ^2) tests. Binary logistic regression was used to estimate 95% confidence intervals (CI) for the odds ratio (OR).

Results

Basic Characteristics of the Study participants

The study included 100 breast cancer patients had a mean age of 51.95 ± 11.84 years and 100 cancer-free controls with a matched-age. Breast cancer patients had a significant decrease in lymphocyte count compared to the healthy group. The serum levels of ALT, AST, bilirubin, creatinine, lactate dehydrogenase, alkaline phosphatase and uric acid were significantly higher in BC group than controls ($p<0.05$). Furthermore, tumor markers CEA and CA15.3 were higher in BC patients compared to controls. The expression level of *B7H4* in of the patients was significantly higher compared to controls ($p<0.001$), Table 1.

Genotypic and Allelic Frequencies of B7H4 (rs10754339 A>G) gene Variant

The genotype and allele frequencies were in accordance of Hardy-Weinberg equilibrium (HWE) ($\chi^2=0.20$, $p=0.60$ for BC group and $\chi^2=0.23$, $p=0.63$ for control group). The frequency of variant (G allele) was 35.4% among BC patients, and 22 % among controls. Additionally, the frequency of wild-type (A allele) was 65.5% among BC patients, and 78% among controls. The most Dominant genotype (AA) was 31% among BC patients and 4% among controls (Table 2, Figure 1 and Figure 2).

Table 1. The Demographic, Clinicopathological, Biochemical, Hematological, Tumor Markers and Hormone Receptor Status Variables of the Study Population.

Variables		BC women (n=100)	Control (n=100)	p-value
I. Demographic data				
1. Age, Years		51.95 ± 11.84	53.78 ± 9.73	0.25
2. Age groups, n(%)	< 35 years	7 (7)	1(1)	0.14
	35–54 years	52(52)	52(52)	
	≥ 55 years	41(41)	47(47)	
3. Gender, n(%)	Female	100 (0)	100 (0)	NA
II. Clinicopathological investigations				
1.Tumor grade, n(%)	G I	0 (0)	-	NA
	G II	83 (83)	-	
	G III	17 (17)	-	
2. T stage, n(%)	T0	4 (4)	-	NA
	T1	22 (22)	-	
	T2	54 (54)	-	
	T3	20 (20)		
3. N stage, n(%)	N0	25 (25)	-	NA
	N1	35 (35)	-	
	N2	15 (15)	-	
	N3	25 (25)		
4. M stage, n(%)	M0	72 (72)	-	NA
	M1	28 (28)	-	
5. Pathological type, n(%)	IDC	94 (94)	-	NA
	ILC	6 (6)	-	
III. Biochemical parameters				
1. ALT (U/L)		22.5 (15-37)	41 (38 - 47)	<0.001**
2. AST (U/L)		30 (21-49)	23 (19-29.8)	<0.001**
3. Albumin (g/dL)		4 (3.4-4.3)	4 (3.7-4.3)	0.45
4. Total bilirubin (mg/dL)		0.5 (0.4-0.7)	0.4 (0.3-0.6)	0.02*
5. Alkaline phosphatase (IU/L)		89 (73.3-124.3)	72 (56.5-83.8)	<0.001**
6. Creatinine (mg/dL)		0.8 (0.7 - 0.98)	0.8 (0.7-0.9)	0.02*
7. Uric acid (mg/dL)		5 (3.8-6.8)	4 (3.4-5.2)	0.002*
8. Lactate Dehydrogenase (U/L)		377.5 (289.8-422.8)	178 (148.3-192)	<0.001**
9. *B7H4* (ng/mL)		38 (29 – 55)	4.5 (2.8 – 5.9)	<0.001**
IV. Hematological measurements				
1. WBCs ($\times 10^9$/L)		6.5 (4.8-8.2)	6 (4.8-7.2)	0.09
2. RBCs ($\times 10^{12}$/L)		4.1 ± 0.75	4.2 ± 0.58	0.33
3. Hemoglobin (g/dL)		11.5 ± 1.8	11.5 ± 1.7	0.98
4. Platelets ($\times 10^9$/L)		223.8 ± 93.6	311.3 ± 70.02	<0.001**
V. Tumor markers and hormone receptor status				
1. CA 15-3 (U/mL)		28.4 (16.3-67)	4.0 (3.7-4.4)	<0.001**
2. CEA (ng/mL)		7.0 (2.5-27.1)	1.4 (0.9-2.0)	<0.001**
3. Tumor status, n(%)	ER +	76 (76)	-	NA
	PR +	67 (67)	-	
	HER2 +	33 (33)	-	

Genetic association models of the B7H4 (rs10754339 A>G) variant with breast cancer risk

Breast cancer patients exhibited a significant association with the *B7H4* (rs10754339 A>G) variant compared with cancer-free controls using multiple genetic association models, including dominant model (AG+GG vs. AA, OR = 0.09, 95% CI = (0.03 - 0.26), P < 0.001), recessive model (GG vs. AA+AG, p =0. 0.4) and homozygous comparison (GG vs. AA, OR = 0.25, 95% CI = 0.08 - 0.74, p < 0.001). The variant revealed a high

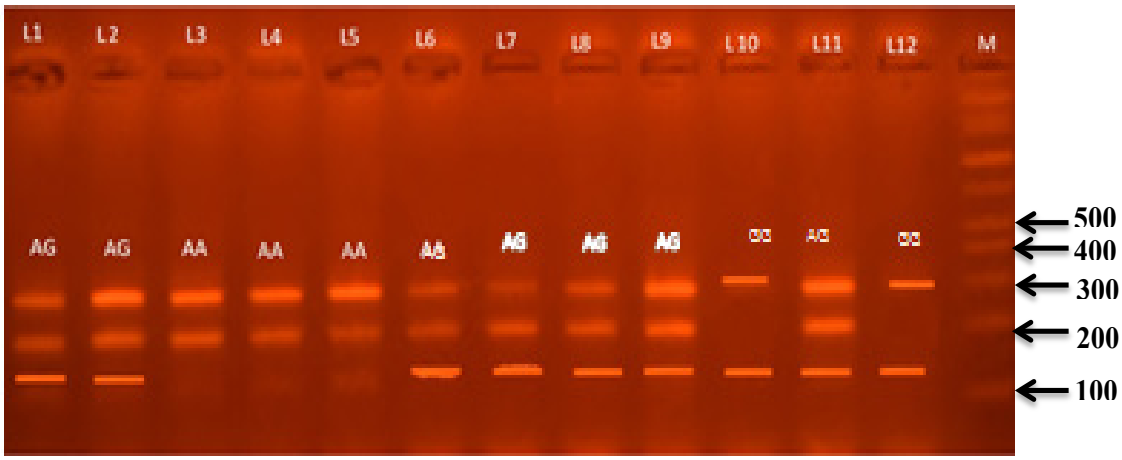

Figure 1. Shows Gel Electrophoresis of TARMS- PCR. Lanes (1, 2,6, 7, 8,9and 11) heterozygote genotyping AG bands at 142,210 and 295bp. Lanes (3,4,5) AA homozygote genotyping bands at 210 and 295 bp. Lanes (10,12) GG homozygote genotyping at 142bp, and 295 bp. M: indicate DNA ladder (100bp thermos scientific).

Table 2. The Genotypic and Allelic Frequencies of the *B7H4* (rs10754339 A>G) Variant of the Study Participants

Genetic polymorphisms	BC patients (n=100)	Controls (n=100)	OR (95% CI)	p-value
B7H4 (rs10754339 A>G)				
Genotypic frequencies	n (%)	n (%)		
AA	31 (31%)	4 (4%)	1	
AG	69 (69%)	36 (36%)	0.25 (0.08 – 0.74)	<0.001***
GG	0 (0%)	60 (60%)	-	<0.001***
HWE	χ^2=0.20, p=0.60	χ^2=0.23, p=0.63		
Allelic frequencies				
A	131 (65.5%)	156 (78%)	1	
G	69 (35.4%)	44 (22%)	0.87 (0.59 – 1.28)	0.005*

*, Significant at p < 0.05.

risk of breast cancer (AG vs. AA, P < 0.001), Table 3.

Association of B7H4 (rs10754339 A>G) variant with clinopathological investigations and lab, results

The *B7H4* (rs10754339 A>G) variant failed to show significant association with breast cancer with respect to all clinopathological, laboratory, and hematological variables (P>0.05), Table 4.

Discussion

Currently, *B7H4* is being widely studied as a

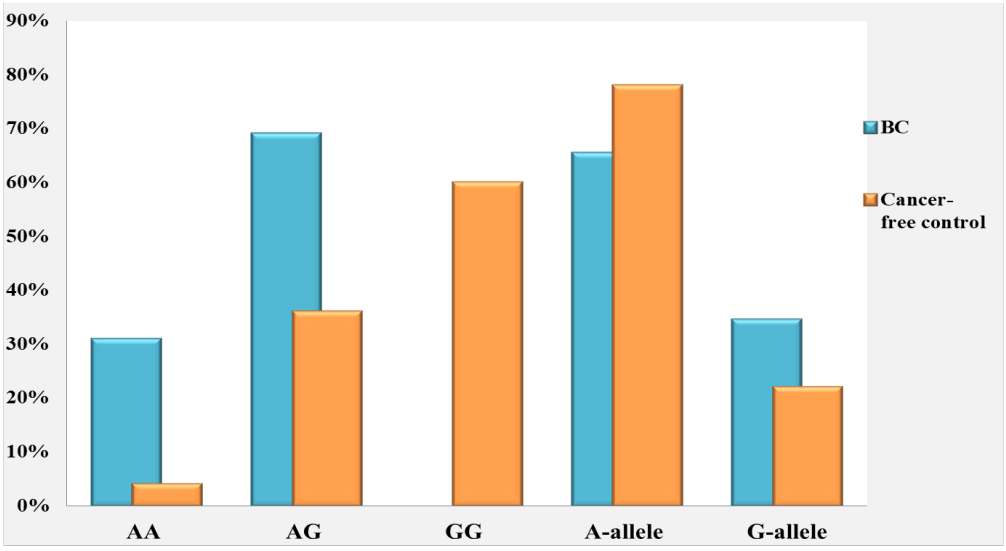

Figure 2. The Genotypic and Allelic Frequencies of the *B7H4* Variant among Patients with BC and Controls

Table 3. Genetic Association Models of *B7H4* (rs10754339 A>G) Variant and the Risk of Breast Cancer

Model	Genotypes	BC patients (n=100)	Controls (n=100)	Crude OR (95% CI)	p[a]	Adjusted OR (95% CI)	p[b]
B7H4 (rs10754339 A>G)							
Codominant	AA	60 (60%)	76 (76%)	1		1	
	AG	36 (36%)	22 (22%)	0.25 (0.08 – 0.74)	<0.001***	1.99 (1.05-3.76)	0.03*
	GG	4 (4%)	2 (2%)	-		2.74 (0.48-15.75)	
Dominant	AA	31 (31%)	4 (4%)	1		1	
	AG+GG	69 (69%)	96 (96%)	0.09 (0.03-0.26)	<0.001***	2.05 (1.11-3.79)	0.02*
Recessive	AA+AG	100 (100%)	40 (40%)	1		1	
	GG	0 (0%)	60 (2%)	-	0.4	2.30 (0.40-13.18)	0.34
Overdominant	AA+GG	64 (64%)	78 (78%)	1		1	
	AG	36 (36%)	22 (22%)	1.99 (1.07-3.73)	0.03*	1.92 (1.02-3.60)	0.04*
Log-additive	-	-	-	1.91 (1.11-3.28)	0.02*	1.88 (1.09-3.22)	0.02*

*, Significant at $p < 0.05$.

Table 4. Association of *B7H4* (rs10754339 A>G) Variant with the Demographic, Clinopathological Investigations and Lab Results of the BC Patients

Parameters	AA n=31	GG n=69	p-value
Demographic data			
Age (Years), Mean ± SD	52.77 ± 9.27	52.71 ± 14.23	0.98
Clinopathological investigations			
T-stage, n(%)	1 (3.2%)	3 (4.3%)	0.77
T0	7 (22.6%)	15 (21.7%)	
T1	15 (48.4%)	39 (56.5%)	
T2	8 (25.8%)	12 (17.5%)	
T3			
N-stage, n(%)			0.2
N0	4 (12.9%)	21 (30.4%)	
N1	11 (35.5%)	11(34.8%)	
N2	5 (16.1%)	10 (14.5%)	
N3	11 (35.5%)	14 (20.3%)	
M-stage, n(%)	20 (64.5%)	52 (75.3%)	0.26
M0	11 (35.5%)	17 (24.7%)	
M1			
Tumor grade, n(%)			0.46
G2	27 (87%)	56 (81%)	
G3	4 (13%)	13 (19%)	
Tumor size, n(%)			0.18
Small	4 (13%)	17 (24.6%)	
Large	27 (87%)	52 (75.3%)	
Pathological type, n(%)			0.23
IDC	22 (55%)	40 (66.7%)	
ILC	18 (45%)	20 (33.3%)	
Biochemical parameters			
ALT (U/L)	38.5 (26–64.5)	47 (30–90)	0.41
AST (U/L)	68 (44–168.5)	74 (52–160)	0.45
Bilirubin (mg/dl)	2.05 (1.1–6.5)	5.02 (1.4–6.5)	0.69
Albumin (g/dl)	3.1 ± 0.5	2.9 ± 0.82	0.17
Creatinine (mg/dl)	1.42 (0.8–1.3)	1.44 (0.8–1.7)	0.19
LDH (U/L)	399 (234-444)	376(292.5-412.5)	0.66

Table 4. Continued

Parameters	AA n=31	GG n=69	p-value
Demographic data			
Hematological measurements			
RBCs (× 10^{12}/L)	4.0 ± 0.99	3.6 ± 1.03	0.05
WBCs (× 10^{9}/L)	7.13 (4.7–10.2)	8.2 (5.3–12)	0.33
Hemoglobin (g/dl)	11.52 ± 2.3	10.96 ± 2.49	0.14
Platelets (× 10^{9}/L)	146.05 (89.5–212)	139 (77.26–212)	0.38
Tumor markers and hormone status			
CEA(ng/ml)	3.68 (0.69 -270)	7 (1 – 211)	0.1
CA 15.3	31 (7 – 431)	25 (4.9- 850)	0.93
ER	25 (80.6%)	51 (74%)	
PR	20 (64.5%)	47 (68%)	0.46
Her2	10 (32.2%)	23 (33.3%)	0.72

therapeutic target due to its role in suppressing the immune system in tumorigenesis [13]. For the great majority of cancer patients with *B7H4*-positive tumors, it thus offers a chance for successful treatments [15].

In addition, following therapy, the *B7H4* targeting technique may decrease the burden of metastases and tumor recurrence. These factors collectively imply that *B7H4* is a significant and effective therapeutic target [23, 24].

Moreover, decreased levels of cytotoxic T lymphocyte infiltration are linked to elevated *B7H4* expression [25, 26]. In immunocompetent cold breast tumors, inhibition of *B7H4* glycosylation has been shown to restore antitumor immunity. These findings provide credence to the theory that cancer patients' normal T cell function is restored when *B7H4* function is inhibited [27].

According to early studies, *B7H4* expression levels are linked to tumor growth, immunological suppression in the tumor environment, and a poor prognosis. These conditions include breast, cervical, colorectal, lung, ovarian, and urinary tract epithelial cancers [28].

In certain malignancies, a worse prognosis has been linked to *B7H4* expression in tumor tissue [29]. However, there is inconsistency in the statistics about breast cancer. According to Huang et al., patients with higher B7-H4 expression had a much poorer overall survival rate than those with lower expression [30]. According to Wang et al. individuals with triple-negative breast cancer who overexpressed *B7H4* had a much lower survival and recurrence time than those who underexpressed it. *B7H4* may be a possible negative prognostic sign, according to these data [31].

A prior work by Zhou et al., similarly demonstrated a negative correlation between the upregulation of CD8 T cells in tumor locations and elevated *B7H4* levels in breast cancer cells. By encouraging cell cycle progression, *B7H4* deficiency enhances breast cancer cell proliferation, migration, and metastasis [32]. Additionally, *B7H4* can disrupt the epithelial-to-mesenchymal transition (EMT), chemotherapy resistance, and the development of human breast cancer stem cells [33].

According to the current study, patients' serum *B7H4* levels were substantially greater than those of healthy controls ($p<0.001$). According to the study by Mach et al., these findings are in line with this of Ye et al. which shown that *B7H4* expression is markedly elevated in ovarian, pancreatic, and breast cancer. s*B7H4* was found in blood samples from a variety of cancer patients, including those with ovarian, gastric, renal, and bladder epithelial cell carcinomas [34, 35]. High levels of s*B7H4* were found to be a significant prognostic indicator, which is consistent with the finding of the study by Zhang et al. [36].

In blood samples from patients with ovarian cancer, renal cell carcinoma, colon cancer, breast cancer, lung cancer, and prostate cancer, s*B7H4* was found, according to our findings, which were corroborated by the studies by [37, 38]. Although the mechanism of soluble *B7H4*'s generation and function are still unknown, these investigations indicate that serum *B7H4* may be a valuable marker for diagnosis and prognosis.

A study by Xu et al. found that patients with NSCLC had significantly higher serum s*B7H4* levels than healthy controls ($P < 0.05$), and patients with HCC had significantly higher serum s*B7H4* levels than healthy controls ($P < 0.001$) [39]. Our findings were also validated by Xie et al., who demonstrated that patients' serum and lymphoma tissues had considerably greater levels of *B7H4* expression than healthy controls ($P < 0.01$) [40].

Single-nucleotide polymorphisms can either enhance or reduce the expression of several genes that affect breast cancer risk, making them one of the variables that influence hereditary breast cancer risk [18]. But *B7H4* has never been studied in this kind of setting. Moreover, nothing is known about its mechanistic function in breast cancer. In the 3'UTR of *B7H4*, the SNP Rs10754339 A>G has a significant role in the development and risk of breast cancer.

This is the first polymorphism study to examine the relationship between Egyptian women's risk of breast cancer and the *B7H4* gene's rs10754339 polymorphism. Three polymorphisms were found in the UTRs and the first intron of *B7H4*, which may be related to breast cancer risk in the Han population of China, according to findings published by Zhang et al. in the Chinese population [41].

The rs10754339 G allele may be involved in breast cancer risk, as our results of the *B7H4* (rs10754339 A>G) variant gene showed that the risk of breast cancer in women, AG genotype, and G allele were higher in the breast cancer case than in the control group ($p < 0.001$). This finding aligns with the findings of Zhang et al. and Özgöz et al. [40, 42]. Additionally, our research supported the findings of El Din et al., which found that the "G" allele is substantially linked to a 1.45-fold higher risk of breast cancer as well as increased development of breast cancer and lymph node metastases [17]. Our findings that the minor allele frequency (G allele) of the *B7H4* (rs10754339 A>G) polymorphism was 34.5% among BC patients and 22% among cancer-free control were supported also by the reports Zhang et al. and Tsai et al. [41, 43].

The current study is consistent with the findings Jin et al. (2023), results of the meta-analysis which showed that rs10754339 and rs12976445 contributed to cancer susceptibility in the Chinese population and also revealed a significant association between rs10754339 and breast cancer risk [44].

The current investigation is supported by the discovery that the *B7H4* (rs10754339 A>G) polymorphism has been linked to breast cancer [42], which revealed a correlation in breast cancer (ORs > 1 for the G allele and AG genotype) as documented in other earlier studies. As opposed to Özgöz et al., this disparity might mostly be explained by some studies' inadequate power because of their small sample sizes. In fact, 30 breast cancer patients and 30 healthy women participated in a study conducted by Ozguz et al [42].

Our investigation demonstrated that *B7H4* (rs10754339 A>G) was a genetic marker for bladder cancer, in line with the study Jin et al. (2023), who found to be strongly linked to the risk of breast cancer and to be considerably connected with total cancer risk, particularly in the Chinese population [44]. The frequencies of G-allele of *B7H4* (rs10754339 A>G) was significantly different between breast cancer cases and treatment controls, according to the current study, which is consistent with the study by Tsai et al. [43]. This suggests that immunity may play a role in the development of breast cancer, particularly in the stages of progression and metastasis. In our investigation, the *B7H4* (rs10754339 A>G) polymorphism did not significantly correlate with an elevated risk of breast cancer across all clinical, pathological and laboratory variables (P>0.05).

Our comparative study concluded that Egyptian women's vulnerability to breast cancer is linked to the *B7H4* (rs10754339 A>G) gene variant. This could be a hereditary component of Egyptian women's breast cancer. Additionally, the blood *B7H4* protein level was higher in breast cancer patients than in controls, and it may be a predictor of breast cancer outcome. Nevertheless, more research with larger sample sizes is required to validate our present conclusions.

Author Contribution Statement

Kamal Jwameer Owaid and Afaf M. ElSaid contributed to project administration, investigation, and the performance of the statistical analysis. Kamal Jwameer Owaid and Manar Refaat Abdel Khalek contributed to methodology, formal analysis, and supervision. Kamal Jwameer Owaid and Abdel-Aziz,A.F were involved in validation, writing, and editing. Kamal Jwameer Owaid and Abdel-Aziz,A.F contributed to software development, data curation, and formal analysis. Kamal Jwameer Owaid and Manar Refaat Abdel Khalek were involved in formal analysis, methodology, and visualization.

Acknowledgements

Data Availability The dataset utilized in the preparation of this study will be available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare that they have no funding, financial relationships, or potential conflicts of interest on the subject of this study.

Ethical Approval

All procedures accomplished in this work, including human subjects, were in accordance with the ethical guidelines of the institutional research committee and with the 1964 Helsinki declaration and its adjustments.

Informed Consent Informed consent was obtained from all participants in this work.

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