Polymorphisms of Heat Shock Protein70-hom (*Hsp*70-hom) Gene in Iraqi Women with Breast Cancer

Wafaa Sabri Mahood[1, a)], Salwa Ali Ganim[1, b)], Shaimaa Sabah Al Obaidy[1, c)], Lenha Qutaiba Ahmed[1, d)]

[1]*Department of Biology, College of Education for Pure Sciences (Ibn Al-Haitham), University of Baghdad, Baghdad, Iraq.*

[a)] *corresponding author: wafaa.s.m@ihcoedu.uobaghdad.edu.iq*
[b)]*salwa.a.g@ihcoedu.uobaghdad.edu.iq*
[c)]*shaima.s.m@ihcoedu.uobaghdad.edu.iq*
[d)]*linha.q@ihcoedu.uobaghdad.edu.iq*

Abstract. In the current study, we investigated the incidence of breast cancer and determined the most variable clinicopathological parameters in a cohort of female carcinoma patients from Baghdad. We also analyzed whether the heat-shock protein gene *Hsp*70-hom T/C (rs2227956) polymorphism is associated with susceptibility to developing breast cancer in Iraqi females. The *Hsp*70-hom polymorphism was analyzed using the Restriction Fragment Length Polymorphism (RFLP) technique in 90 breast cancer patients and 40 apparently healthy women as a control group. The results showed a significantly higher percentage of patients (60%) were over 50 years of age (P≤ 0.05). All patients (100%) in the study were diagnosed with Invasive Ductal Carcinoma (IND). The majority of cases (78%) were classified as Grade II (P≤ 0.05), and there was a notably higher incidence of the T2NM stage (60% or 54/90) (P≤ 0.05). No significant association was found between breast cancer and Lymph Node Metastasis (LNM). Regarding hormonal status, positive results were observed in 82% (74/90) and 70% (63/90) of patients for Estrogen Receptor (ER) and Progesterone Receptor (PR), respectively, although these findings lacked statistical significance. Furthermore, 90% of the breast cancer cases had the Hsp70-hom TT genotype in a homozygous state (compared to 80% in the control group), with an Odds Ratio (OR) of 0.85 (95% CI: 0.55-1.18) and a P-value of 0.259. These genetic findings indicate a negative risk association between the Hsp70-hom T/C (rs2227956) genotypes and the induction of breast cancer in Iraqi females.

Key Words: Heat Shock protein gene, Chaperone, Hsp70-hom gene, Breast Cancer, Iraq

INTRODUCTION

Breast cancer is a major problem concern the Iraqi females. High incidence of new cases has been documented since 2000; it was responsible for approximately one-third of all cancer cases in 2019. It exhibits the highest percentage incidence rate (18.17/100,000) among the top five cancers in Iraq (1, 2). Heat shock proteins (HSPs) are a group of proteins that can be activated response to various cellular stressors, including hypoxia, heat, ischemia, and reactive oxygen species (oxidative stress) and toxins protecting cells from environmental stresses (3,4). HSPs are classified into various families depend on molecular masses, including (HSP110, HSP90, HSP/HSC70, HSP60, HSP47, in addition to the small HSPs (HSP10–30), the main biological role of HSPs is their ability to act as chaperone molecules. The HSP70 family includes eight members that exhibit structural similarity yet display distinct biological activities and cellular localization (5, 6). HSPs play an important dual role as housekeeping genes and molecular chaperones. As chaperones, they are essential for achieving the suitable folding of newly synthesized proteins. Their functions are continuously required to prevent the accumulation of unstable or misfolded proteins and to facilitate protein transfer across cellular compartments (7).The members of the HSP70 class are encoded by three genes found at (6p21.3) inside major histocompatibility complex class III region: *Hsp70*-1, *Hsp70*-2 and *Hsp70*-hom. The heat-inducible proteins encoded by *Hsp70*-1 and *Hsp70*-2 genes with an identical domains but differ in their regulatory domains. Whereas a non-heat inducible protein encoded by HSP70-hom gene (8). Some variants of the HSP70 class polymorphic genes potentially account for differences in protein function and an individual's susceptibility to stress tolerance (9). A key change is the nonsynonymous mutation in the *Hsp*70-hom T > C polymorphism (rs2227956), which result in a Methionine to Threonine (Met→Thr) substitution at position 493 in the peptide binding domain, which may impact HSP70's chaperone activity and substrate binding selectivity (10).

Researchers have shown that genetic mutations of the HSP70-2 and/or HSP70-hom may alter a person's susceptibility to cancer; the frequency of these variant genotypes was much higher in cancer patients than in controls (11-13). It has been observed to have associations with several cancers such as cervical carcinoma (14), lung cancer (15) and breast cancer (16).

The HSP70 family acts as a chaperone molecule for antigenic peptides derived from tumor cells, inducing cytotoxic T lymphocytes (CTL) activation. This mechanism is crucial for the anti-tumor immune recognition process (17,18). However, HSP70 also assist to overcome the stressful conditions faced by the tumor cells, such as lack of oxygen, nutrient, or immune response leading to their survival (19). High levels of *Hsp*70 gene expression have been found in metastatic and non-metastatic breast cancer patients, and linked to cancer cell survival and resistance indicating to a strong association between over expression of HSP70 and advanced disease, HSP70 could be a useful biomarker for early detection, diagnosis, follow-up, and could be used as effective strategies for target therapy of cancer patients (16,20).

This study used a cohort of phenotypically well-defined Iraqi breast cancer women and normal control females to describe the incidence of breast cancer, identify the most variable clincopathological parameters, and explore the hypothesis that breast cancer is linked to the *Hsp70* hom gene polymorphism (rs2227956).

MATERIAL AND METHODS

Patients' Blood Samples

Between May 2023 and February 2024, a total of 90 blood samples were drawn in EDTA-containing tubes from females diagnosed with breast cancer. whose ages ranged between 32 and 71 years old. The mean age of the patients was 51.5 ±10.4 years. Forty blood samples were drawn from healthy women as a control group whose ages ranged between 30 and 64 years. Their mean age was 46.9 ± 9.8 years. Diagnosis for the patient cohort was confirmed by medical experts at the Medical City (Oncology Teaching Hospital), Baghdad, based on clinical findings, mammography, and histological results. All patients were identified early in their diagnosis, and none of them had undergone mastectomy, chemotherapy, or radiotherapy prior to blood sample collection. Every participant in this study provided their informed consent and agreement. The current study received approval from the Ethics Committee of the Iraqi Ministry of Health.

Genotyping

Using the ReliaPrep^{TM} Blood gDNA Miniprep System (Promega Corporation, USA), genomic DNA was isolated from EDTA blood samples. Following purity and concentration evaluation, PCR amplification was performed. The following primers were utilized to genotype the *Hsp70*-hom gene T/C (rs2227956) using the Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) technique. forward-5′GGACAAGTCTGAGAAGGTACAG-3′and reverse-5′-GTAACTTAGATTCAGGTCTGG-3′ (21). PCR amplifications were carried out using 20µl volumes that contained GoTaq Green Master Mix (2X) (10µl) , 3µl of template DNA, and 1µl of each primer (10 pmol), finally, 5µl of nuclease-free water, The following temperature program was used to carry out PCR cycling using PCR Express (Thermal Cycler, Veriti, USA): 30 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds were performed after being denatured at 95°C for 5 minutes. To halt the reactions, a final extension incubation was performed for seven minutes at o 72°C, followed by a hold at 4°C. The resulting amplicon (expected size 862 bp) was visualized in 1% agarose gel and staining the gel with ethidium bromide (10 mg/ml) . The amplified products were subjected to restriction endonuclease NcoI (New England Biolabs, UK) as restriction digestion for 2 hrs at 37°C and then resolved in 1.5% agarose gel.

Statistical Analyses

The statistical analyses were conducted using SPSS, a commercial software program (version 11.5). It was deemed significant when $P \leq 0.05$. To test the relationships between genotypes and breast cancer risk, an odds ratio (OR) with a 95% confidence interval (CI) were used too.

RESULTS

Characteristics of Patients

Table 1 refer to the features of the patients, revealing that 36 out of 90 individuals (40%) were below 50 years of the age, while a significantly higher percentage of 54 out of 90 individuals (60%) were 50 years or older ($P \leq 0.05$). This indicates a statistically significant association between advanced age and breast cancer in this cohort. All of the patients (100%) in the study were diagnosed with invasive ductal carcinoma (IND), the majority of cases (78%) classified as grade II tumors in comparison with (22%) of grade III, high statistical significant association depend on the grade ($P \leq 0.05$), there is a notably higher incidence of T2NM (54/90 or 60%) with high statistically significant association ($P \leq 0.05$),while no significant relation between breast cancer and lymph node metastasis (P 0.424). The classification based on hormonal status reveals positive results ($P \leq 0.05$) in 82% (74/90), 70% (63/90), of patients for estrogen receptor (ER), progesterone receptor (PR) respectively:

TABLE 1. Characteristics of the patients with Breast cancer

Characteristics of Patients		No.(%)	*P*-Value
Age	Less 50	36(40%)	0.046
	More than 50	54(60%)	
Tumor type	IDC	90(100%)	
Tumor Grad	II	70 (78%)	≤ 0.05
	III	20(22%)	
TNM	T1NM	22(24%)	≤ 0.05
	T2NM	54(60%)	
	T3NM	10(12%)	
	T4NM	4(4%)	
LNM	+	49(54%)	0.424
	-	41(46%)	
ER	+	74(82%)	≤ 0.05
	-	16(18%)	
PR	+	63(70%)	≤ 0.05
	-	27(30%)	
Her/2	+	36(40%)	0.046
	-	54(60%)	

Genotyping of *HSP70*-hom Gene

The Current study screened the polymorphism of the *HSP70*-hom gene using the Restriction Fragment Length Polymorphism (RFLP) technique. The initial PCR products of *Hsp* 70-hom gene, which is 878 base pairs when uncut, is shown in **figure 1**. The PCR products of the HSP70-hom gene were subjected to enzymatic digestion using Nco1 enzyme. The electrophoretic analysis demonstrated the successful separation of reaction products on a 1.5% agarose gel (**figure 2**). The identification of the *Hsp*70-hom T/T homozygous genotype was shown to be correlated with the presence of an Nco1 restriction site and was indicated by the presence of two products size 551 bp and 327 bp. Hsp70-hom C/C homozygous genotype was shown to be correlated with the absence of an Nco1 restriction site and was indicated by the presence of one band with products size 878bp.While Hsp70-hom T/C heterozygous genotype was detected in three bands of products size 551 bp, 327 bp and 878bp.

TABLE 2 presents the Hsp70-hom genotypes and allelic frequency in patients with breast cancer and the control group. The data indicates that 90% of breast cancer cases had the Hsp70-hom TT genotype (homozygous), compared to 80% in the control group. This finding did not show a statistically significant positive association, with an Odds Ratio (95% CI) : 0.85 (0.55-1.18) at (P value 0.259). Conversely, about 10% of breast cancer patients had the Hsp70-hom TC genotype (heterozygous), compared to 20% of controls. The Odds Ratio (95%CI): 0.44 (0.11-1.81) at P value (0.259).

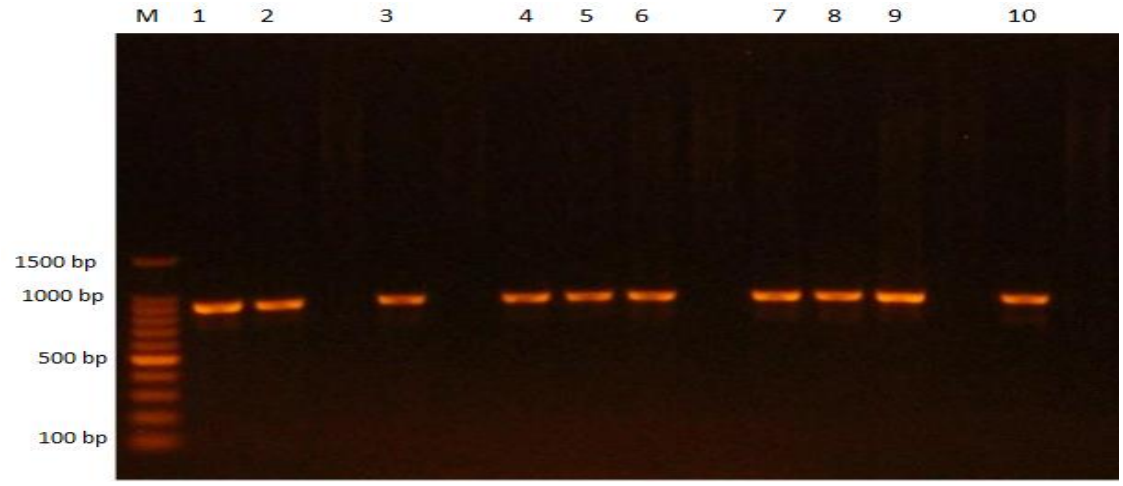

FIGURE 1. The *Hsp 7*0-hom gene amplification in breast cancer females' blood samples were separated on 1% agarose gel electrophoresis and stained with the Ethedium Bromide, M: 100 bp ladder markers, the samples had a size of 878 bp.

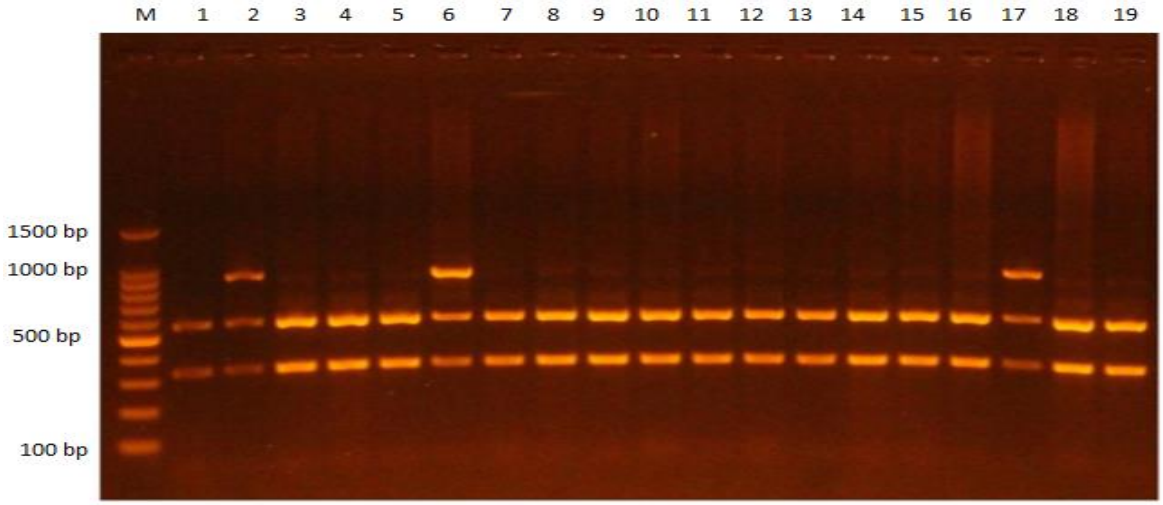

FIGURE 2. The RFLP products obtained after digestion of *Hsp70*-hom PCR product with NocI restriction enzyme visualized on 1.5% agarose gel stained with Ethedium Bromide M: 100bp ladder marker. Lanes 1-19 resemble RFLP products. The presence of the *Hsp*70-hom T/C allele was detected in Lanes 2, 6, and 17 with three fragments (551, 327 and 878) bp. The *Hsp70*-hom T/T genotype was observed in lanes 1, 3-5, 7-16, and 18,19, which included digested *Hsp70*-hom PCR products of (551 and 327) bp.

TABLE 2. Frequencies distributions of the genotypes and alleles of the *hsp*70-hom in patients with breast cancer and control groups

Genotype	Total No. of Patients =90		Total No. of Control=40		Odd's ratio (95% CI)	*P* value
	No	%	No.	%		
TT	81	90	64	80	0.85 (0.55- 1.18)	0.259
TC	9	10	16	20	0.44 (0.11-1.81)	0.259
CC	0	0	0	0	-	-
Alleles frequency						
T	86	96	72	90	0.81 (0.54-1.20)	0.276
C	4	4	8	10	0.47 (0.12-1.84)	0.276

DISCUSSION

This study aims to detect the most variable clinicopathological characteristics in breast carcinoma, and to examine the incidence of breast cancer in a cohort of females from Baghdad. The study also aims to screen the genotype and allele frequencies of a significant missense Single Nucleotide Polymorphism (T/C) rs2227956 in the *Hsp*70-hom gene in 90 females with breast cancer and 40 apparently healthy females used as a control group. The results indicated a high risk of breast cancer in females older than fifty years with a statistically significant association. A positive association was also found based on Grade II, (of the TNM classification), and hormonal status ER and PR ($P \leq 0.05$). Conversely, a negative association was found between breast cancer and HER2. Breast cancer represents one of the top five cancers in Iraq, followed by lung cancer, colorectal cancer, brain cancer, and leukemia (22). One of the most predominant causes of breast cancer is advanced age, in addition to body mass index in females. All patients in the present study were diagnosed with Invasive Ductal Carcinoma (IDC), the majority of whom (78%) were Grade II, which may suggest an advanced stage of breast cancer in Iraqi females. Similar findings were reported in Saudi Arabia (23), Iran (24), Turkey (25), and Egypt (26). The current results are inconsistent with another study reporting a lower incidence in older Iraqi Kurdish women (27). These differences may be attributed to lifestyle, social factors, genetic factors, and the use of hormonal therapy.

The current study evaluates the association between a specific *Hsp*70-hom genotypes, its allelic variants, and the risk of developing breast cancer in Iraqi females. The frequencies of the *Hsp*70-hom genotypes (TT) homozygote and (TC) heterozygote) and their corresponding allele frequencies were not significantly associated with breast cancer risk. Specifically, the TT genotype had an Odds Ratio of 0.85% (95% CI: 0.55-1.18) and the TC genotype had an Odds Ratio of 0.44 (95% CI: 0.11-1.81) with a non-significant P-value of 0.259 for both. Notably, the CC homozygote was absent in the Iraqi study population.

These results suggest that the *Hsp* 70-hom polymorphism did not show any effect on susceptibility to breast cancer in Iraqi females. These findings align with published data from Saudi Arabia, where other SNPs were studied; no significant relationship was identified between either the rs35253356 (A>G) or the rs4977219 (A>C) polymorphisms in *HSF*1 gene and breast cancer (28).

This contrasts with reports from other studies, such as one conducted in the Kashmiri population. That specific study found a high, statistically significant relative risk for breast cancer associated with the *Hsp* CC genotype (Frequency of 0.50 in patients versus 0.30 in controls). Conversely, the risk was significantly decreased with an increased frequency of the *Hsp*70-hom G allele in either the homozygous or heterozygous state (RR=0.43, P=0.005).Another Tunisian study found that females with breast cancer had a higher frequency of the G allele in the *Hsp*70-hom, whether in the homozygous or heterozygous state, (0.13) than the control group (0.05) (RR=3.4, P=0.01) (29).The variation in peptide binding specificity among *Hsp*70-hom haplotypes is linked to the (Met→Thr 493) amino acid substitution at position 493 caused by the *Hsp70*-hom polymorphism caused. This substitution has been linked in numerous reports to a number of illnesses, including multiple sclerosis (30), acute pancreatitis (31), schizophrenia (32), and insulin-dependent diabetes mellitus (33).Other studies have reported the association of the *Hsp70*-hom variation with cancer-related risks, such as inflammatory bowel disease (34) and a high relevance to the risk and prediction of hepatocellular carcinoma (35). However, another study did not confirm the association of the Hsp70-hom polymorphism with lung cancer risk (12). These contradictory results regarding the *Hsp70*-hom polymorphism may be due to several reasons, including differences in ethnic populations, genetic predisposition, and the influence of environmental factors such as diet and smoking. Furthermore, differences in the laboratory techniques used to detect the polymorphism may also contribute to the variance in results.

CONCLUSION

A high percentage of breast cancer cases were found to be associated with older patients, invasive ductal carcinoma, grade II tumors, T2NM, and positive hormonal status, ER receptor, and PR and progesterone receptor status in Iraqi women. Given these clinical correlations, it is important to implement effective health programs aimed at breast cancer early detection in Iraq. The *Hsp70-hom* gene polymorphism did not show any effect on susceptibility to breast cancer in Iraqi females. Prospective research with larger sample sizes and an investigation into diverse genetic and environmental factors is warranted.

ACKNOWLEDGMENTS

With great appreciation, we extend our thanks to all the women and their families for their invaluable participation in this study.

REFERENCES

1. M. M. Y. Al-Hashimi, Asian Pac. J. Cancer Prev. 22, 3889–3896 (2021).
2. S. Almotwaa, M. Elrobh, H. AbdulKarim, M. Alanazi, S. Aldaihan, J. Shaik, M. Arafa, and A. S. Warsy, PLoS One 13, e0193095 (2018). https://doi.org/10.1371/journal.pone.0193095
3. C. Hu, J. Yang, Z. Qi, H. Wu, B. Wang, F. Zou, H. Mei, J. Liu, W. Wang, and Q. Liu, MedComm 3, e161 (2022). https://doi.org/10.1002/mco2.161
4. J. Szyller and I. Bil-Lula, Oxid. Med. Cell. Longev. 2021, 6678457 (2021). https://doi.org/10.1155/2021/6678457
5. M. Zhang and X. Bi, Int. J. Mol. Sci. 25, 876 (2024). https://doi.org/10.3390/ijms25020876
6. M. K. Singh, Y. Shin, S. Ju, S. Han, W. Choe, K.-S. Yoon, S. S. Kim, and I. Kang, Int. J. Mol. Sci. 25, 4209 (2024). https://doi.org/10.3390/ijms25084209
7. A. J. Ambrose and E. Chapman, J. Med. Chem. 64, 7060–7082 (2021). https://doi.org/10.1021/acs.jmedchem.0c02091
8. C. Boiocchi, M. C. Monti, C. Osera, G. Mallucci, C. Pistono, O. E. Ferraro, G. Nosari, A. Romani, M. Cuccia, S. Govoni, A. Pascale, C. Montomoli, and R. Bergamaschi, J. Neuroimmunol. 289, 189–193 (2016). https://doi.org/10.1016/j.jneuroim.2016.07.011
9. A. Bakhshandeh, A. K. Dolatabadi, F. Farazmanfar, and M. Shahbaz, Am. J. Clin. Exp. Immunol. 13, 278–284 (2024). https://doi.org/10.62347/CMYA9839
10. C. M. Milner and R. D. Campbell, Immunogenetics 36, 357–362 (1992).
11. M. Ferrer-Ferrer, W. Malespín-Benda˜na, V. Ramírez, et al., Arch. Med. Res. 44, 467–474 (2013).
12. L. He, T. Deng, and H. S. Luo, Sci. World J. 2014, 540309 (2014). https://doi.org/10.1155/2014/540309
13. S. Medhi, M. P. Sarma, M. Asim, and P. Kar, J. Viral Hepat. 20, e141–e147 (2013).
14. M. Garg, D. Kanojia, S. Saini, S. Suri, A. Gupta, A. Surolia, and A. Suri, Cancer 116, 3785–3796 (2010). https://doi.org/10.1002/cncr.25218
15. Y. Wang, F. Zhou, Y. Wu, D. Xu, W. Li, and S. Liang, Clin. Chem. Lab. Med. 48, 1657–1663 (2010). https://doi.org/10.1515/CCLM.2010.304
16. N. Jagadish, S. Agarwal, N. Gupta, R. Fatima, S. Devi, V. Kumar, V. Suri, R. Kumar, V. Suri, T. C. Sadasukhi, A. Gupta, A. S. Ansari, N. K. Lohiya, and A. Suri, J. Exp. Clin. Cancer Res. 35, 150 (2016). https://doi.org/10.1186/s13046-016-0425-9
17. S. K. Calderwood, M. A. Khaleque, D. B. Sawyer, and D. R. Ciocca, Trends Biochem. Sci. 31, 164–172 (2006).
18. D. R. Ciocca and S. K. Calderwood, Cell Stress Chaperones 2, 86–103 (2005).
19. C. Soti and P. Csermely, Pathol. Oncol. Res. 4, 316–321 (1998).
20. T. L. Prince, B. J. Lang, M. E. Guerrero-Gimenez, J. M. Fernandez-Muñoz, A. Ackerman, and S. K. Calderwood, Cells 9, 1046 (2020). https://doi.org/10.3390/cells9041046
21. M. Moniruzzaman, I. Ahmed, S. Huq, M. S. All Mahmud, S. Begum, U. S. Mahzabin Amin, M. H. Rahman, P. K. Sarker, M. U. Hossain, K. C. Das, and M. Salimullah, Mol. Genet. Genomic Med. 8, e1073 (2020). https://doi.org/10.1002/mgg3.1073
22. N. A. S. Alwan, F. Lami, M. Al Nsoor, and D. Kerr, Gulf J. Oncol. 1, 47–57 (2022).
23. A. M. Alsughayer, T. Z. Dabbagh, R. H. Abdel-Razeq, G. N. Al-Jussani, S. Alhassoon, and M. A. Sughayer, JCO Glob. Oncol. 8, 1–7 (2022). https://doi.org/10.1200/GO.21.00359
24. N. Ziamajidi, N. Moini, H. Danesh, R. Abbasalipourkabir, N. Radnia, and Z. Barartabar, J. Obstet. Gynecol. Cancer Res. 9, 634–641 (2024). https://doi.org/10.30699/jogcr.9.6.634
25. V. Özmen, T. Özmen, and V. Doğru, Eur. J. Breast Health 15, 141–146 (2019). https://doi.org/10.5152/ejbh.2019.4890
26. B. O. Anderson, Breast Cancer Res. Treat. 120, 161–163 (2010).
27. S. A. Molah Karim, H. H. Ghalib, S. A. Mohammed, and F. H. Rahim Fataah, Int. J. Surg. 32, 185 (2016). https://doi.org/10.1016/j.ijsu.2016.06.037

28. S. Almotwaa, M. Elrobh, H. AbdulKarim, M. Alanazi, S. Aldaihan, J. Shaik, M. Arafa, and A. S. Warsy, PLoS One 13, e0193095 (2018). https://doi.org/10.1371/journal.pone.0193095
29. L. Chouchane, S. B. Ahmed, S. Baccouche, and S. Remadi, Cancer 80, 1489–1496 (1997). https://doi.org/10.1002/(sici)1097-0142(19971015)80:8<1489::aid-cncr17>3.0.co;2-1
30. A. Bakhshandeh and A. Dolatabad, Am. J. Clin. Exp. Immunol. 13, 278–284 (2024). https://doi.org/10.62347/CMYA9839
31. L. S. Unnikrishnan, Srinivasan Pugazhendhi, Sudipta Dhar Chowdhury, and Balakrishnan S. Ramakrishna, Gastroenterol. Hepatol. Endosc. Pract. 3, 24–28 (2023). https://doi.org/10.4103/ghep.ghep_32_22
32. L. Sepe, J. Xu, and D. Li, Eur. Neuropsychopharmacol. 75, S212 (2023). https://doi.org/10.1016/j.euroneuro.2023.08.377
33. M. Moniruzzaman, I. Ahmed, S. Huq, M. S. All Mahmud, S. Begum, U. S. Mahzabin Amin, M. H. Rahman, P. K. Sarker, M. U. Hossain, K. C. Das, and M. Salimullah, Mol. Genet. Genomic Med. 8, e1073 (2020). https://doi.org/10.1002/mgg3.1073
34. F. Scalia, F. Carini, S. David, M. Giammanco, M. Mazzola, F. Rappa, N. I. Bressan, G. Maida, and G. Tomasello, Int. J. Mol. Sci. 24, 12129 (2023). https://doi.org/10.3390/ijms241512129
35. K. Su, Y. Liu, P. Wang, K. He, F. Wang, H. Chi, M. Rao, X. Li, L. Wen, Y. Song, J. Zhang, T. Gu, K. Xu, Q. Li, J. Chen, Z. Wu, H. Li, W. Huang, L. Chen, J. Tong, … Y. Han, Hepatology Int. 16, 1208–1219 (2022). https://doi.org/10.1007/s12072-022-10391-y