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Abstract

Breast cancer is the most prevalent malignancy among women and one of the leading causes of cancer-related deaths globally. Many individuals are still diagnosed at advanced stages, and its incidence continues to rise due to lifestyle, hormonal, and hereditary factors.

Evaluation of FOXP3 Treg and their Correlation with Heat Shock Protein 90 in Breast Cancer Patients

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The disease is biologically diverse, with molecular subtypes (luminal, HER2-positive, triple-negative) that influence prognosis and therapy. Despite progress in screening and targeted treatments, recurrence and therapy resistance remain major challenges, highlighting the need for new prognostic markers and improved therapeutic strategies.

The study aims to evaluate immunological markers to assess the prognostic value of Forkhead Box P3 (FOXP3). Positive Tregs and their correlation with Heat Shock Protein 90 (HSP-90) in breast cancer. This case-control study, conducted over three months (November 2024 to February 2025), included 140 females from the Baghdad Medical City (Oncology Educational Hospital) who underwent a three-milliliter blood aspiration. Participants were divided into two groups; one group consisted of 70 patients with breast cancer, while the other group consisted of 70 apparently healthy people (control group). ELISA measured serum levels of FOXP3 and HSP-90. Participants provided demographic information, including age, sex, family history, and medical history. The study samples ranged in age from 19 to 85 years, with a mean age of 49.86 ± 12.71 years. The majority of the sample (46.4%) was in the 46-60 age range. Women with breast cancer had significantly higher mean values of HSP-90 than the control group ($P < 0.001$). In contrast, women with breast cancer had significantly higher mean levels of the immune protein Forkhead Box P3 (FOXP3) than the control group ($P < 0.001$).

Immunological markers can be used for the analytical evaluation of FOXP3 and HSP-90 in breast cancer patients and are proposed as indicators for early diagnosis.

Keywords: Breast Cancer, FOXP3 Treg, HSP-90.



Introduction

Breast cancer is the most prevalent cancer in women globally and a leading cause of cancer-related fatalities (1). According to WHO, there are about 685 thousand deaths and 2.3 million new cases per year. Although it primarily affects women, men are also affected and make up less than 1% of cases (2). It is a diverse illness with a range of molecular characteristics, histology, and clinical manifestations. Breast cancer occurs when breast cells grow out of control, forming tumors that, if left untreated, can spread to other organs (3).

Breast cancer is a diverse disease with several subtypes and distinct epidemiological traits. It accounts for over one-third of all cancers in women and 15% of cancer-related deaths globally. Incidence rates are influenced by a combination of lifestyle, environmental, and genetic factors (4).

Higher incidence but lower mortality are typically reported in high-income nations, primarily due to extensive surveillance and sophisticated treatment. In contrast, as populations grow and Western lifestyles proliferate, the number of cases appears to be steadily rising in many emerging nations (5). However, as early identification, preventive programs, and modern medicines become more widely available, mortality was expected to decline over time. Earlier views suggested that breast tumors provoke only a limited immune response. Still, recent evidence shows that disease progression can be restrained by anti-tumor immune activity or, conversely, promoted by pro-inflammatory cytokines secreted primarily by immune cells (6).

Recent research has shown that tumor-infiltrating lymphocytes (TILs) are important players in the biology of cancer and in its response to therapy. Because of their critical

roles in immunological regulation and outcomes, regulatory T cells (Tregs), identified by the expression of the transcription factor FOXP3, have drawn more attention among these cells (7).

FOXP3⁺ Tregs are immunosuppressive cells that can inhibit antitumor immune responses (8). The prognostic significance of FOXP3⁺ Tregs in breast cancer is complex. It may vary by molecular subtype and tumor location. A systematic review and meta-analysis of 28 studies found that higher levels of FOXP3⁺ TILs were associated with improved pathological complete response (PCR) and OS in HER2-positive breast cancer patients (9).

The majority of solid human malignancies harbor large numbers of CD4⁺ effector regulatory T cells (eTregs) in the tumor microenvironment. These cells exhibit a more activated phenotype, with strong FOXP3 expression; intratumoral eTregs are associated with poor clinicopathological characteristics and significantly impair the antitumor immune response (10).

It is well established that breast cancer and other malignancies overexpress heat shock protein 90 (HSP90), a molecular chaperone that stabilizes numerous oncogenic proteins. Moreover, evidence indicates that HSP90 can modulate immunological activity within the tumor microenvironment, thereby affecting the prevalence and functional behavior of infiltrating immune cells (11).

Heat shock protein 90 (HSP90) is a molecular chaperone that helps many client proteins, many of which are involved in oncogenic signaling, fold and remain stable. Elevated expression of HSP90 has been found in several cancers, including breast cancer, and is linked to more aggressive disease and treatment resistance (12).



In addition to its intracellular role, HSP90 influences tumor-immune interactions. HSP90 can be released by tumor cells into the extracellular space, where it alters cytokine production, activates dendritic cells, and promotes the development of antigen-presenting cells, all of which help shape the immunological milieu. By modifying regulatory T-cell activity or stabilizing checkpoint molecules, HSP90 may simultaneously promote immune escape (13).

Material and Methods:

Sample collections and isolation of bacteria:

This case-control study investigated the association between immune biomarker levels and breast cancer. The study was conducted at the Teaching Oncology Hospital in Baghdad Medical City over 16 weeks, from November 2024 to February 2025.

The target population was women with breast cancer. 140 women donated blood samples. The total sample was divided into control and case groups, comprising 70 women recently diagnosed with breast cancer at various stages and 70 healthy women.

Three milliliters of venous blood were promptly transferred to a plain gel tube and allowed to coagulate at room temperature (20–25 °C) for 15 minutes. Following a 10-minute centrifugation at 3000 rpm to separate the serum, the serum was transferred to five Eppendorf tubes and stored in a cooling box at -80 °C until analysis.

Data Collection:

Demographic data were obtained from patients, including age, sex, social status, Family history,

and medical history, through face-to-face interviews, depending on the study's feasibility and scope, with participants' consent.

Inclusion Criteria

Women of all ages who have been newly diagnosed with breast cancer at any stage of the disease.

Exclusion Criteria

For the group of cases, women with breast cancer who had received any treatment, including chemotherapy, hormone therapy, or radiation, were excluded.

Evaluation of HSP-90 and FOXP3 levels in the serum of the study groups:

To assess the amounts of each marker in the Human FOXP3 ELISA kit/Elabscince/ U.S.A. and Human HSP-90 ELISA kit/Elabscince/U.S.A., two serological tests (HSP-90, FOXP3) were conducted using two ELISA kits that employ the Sandwich-ELISA principle. A Combiwash HS / Germany (Automated ELISA washer) and an Hs-human reader / Germany (Automated ELISA reader) were used to record the data (figures 1 and 2).

Statistical Analysis:

Computer applications STATISTICA version 9 and the Statistical Package for the Social Sciences (SPSS) version 26 were used to enter, verify, and analyze the data. For qualitative data, descriptive statistics such as frequency distributions, counts, and percentages were used; for quantitative data, descriptive statistics included the mean, standard deviation, and range.



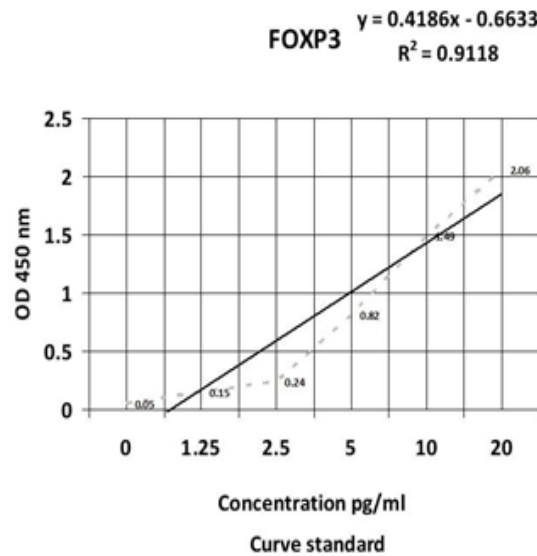


Figure (1): Standard Curve of FOXP3

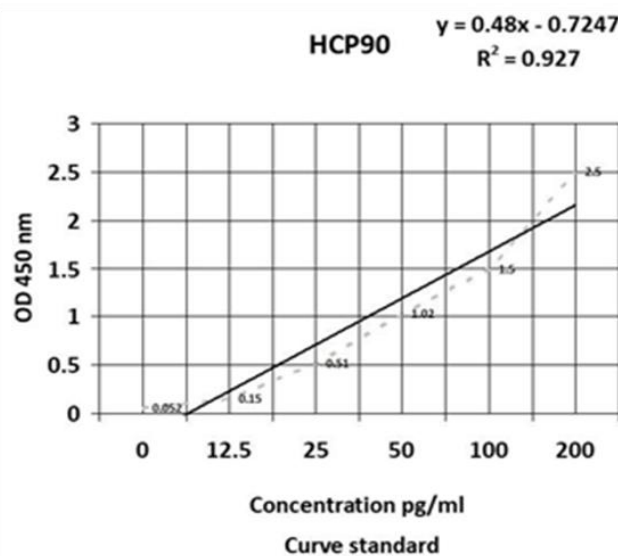


Figure (2): Standard Curve of HSP-90

Results:

A total of 140 samples—70 controls and 70 cases—were examined. The mean age of the breast cancer case group was 52.74 ± 12.454 years, with the majority of cases occurring in the 46–60 age range (47.1%); the control group's mean age was 46.97 ± 12.393 , with a significant mean difference ($P = 0.007$).

Nonetheless, 89.3% of the women in the study were married (Table 1).

However, except for the control group, women with breast cancer diagnoses had a duration of less than three months (25.7%) compared to those with a diagnosis of three months or more (24.3%) (Figure 3).



In addition, stage II was the most common stage (35.7%), followed by stage III (34.3%), stage I among women in the breast cancer group (24.3%), and stage IV (5.7%) (Figure 4).

Table 1: the main characteristics of the study's sample (n=140)

Characteristics	Study groups		
	Cases (n=70)	Control (n=70)	Significancy
Age (years)			
Mean ± SD	52.74 ± 12.454	46.97 ± 12.393	$t = -2.748$, df: 138 $P = 0.007^a$
Age (In groups)			
< 30	2 (2.9)	9 (12.9)	$\chi^2: 6.396$, df: 4, $P = 0.171^b$
30-45	18 (25.7)	18 (25.7)	
46-60	33 (47.1)	32 (45.7)	
61-75	16 (22.9)	11 (15.7)	
> 75	1 (1.4)	-	
Marital status			
Single	6 (8.6)	1 (1.4)	<i>Likelihood Ratio:</i> 4.162, df: 2, $P = 0.125^c$
Married	60 (85.7)	65 (92.9)	
Others	4 (5.7)	4 (5.7)	

^a: Unpaired T-Test, ^b: Chi-Square Test, *Likelihood Ratio*

However, except for the control group, women with breast cancer diagnoses had a duration of less than three months (36; 25.7%) compared to those with a diagnosis of 3 months or more (34; 24.3%) (Figure 3).

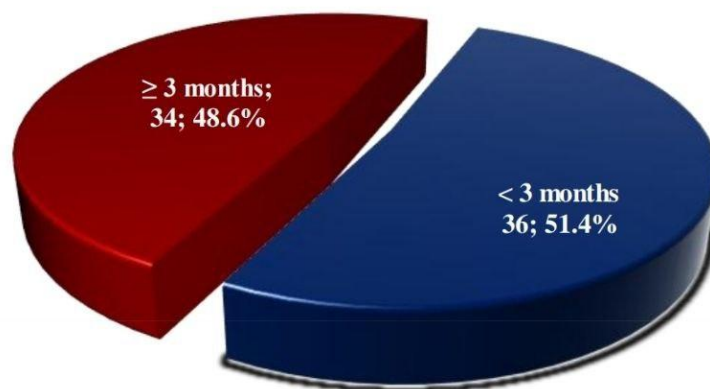


Figure 3: Distribution of breast cancer cases according to duration of diagnosis (n= 70)



Immunohistochemistry parameters

According to immunohistochemistry (IHC) parameters for the estrogen and progesterone receptors (ER, PR) and the expression of the human epidermal growth factor receptor 2 (HER2) protein.

The cases group's ER and PR receptors were significantly more positive than those of the HER2 group (46; 56.7% vs. 23; 32.9%) (Figure 5).

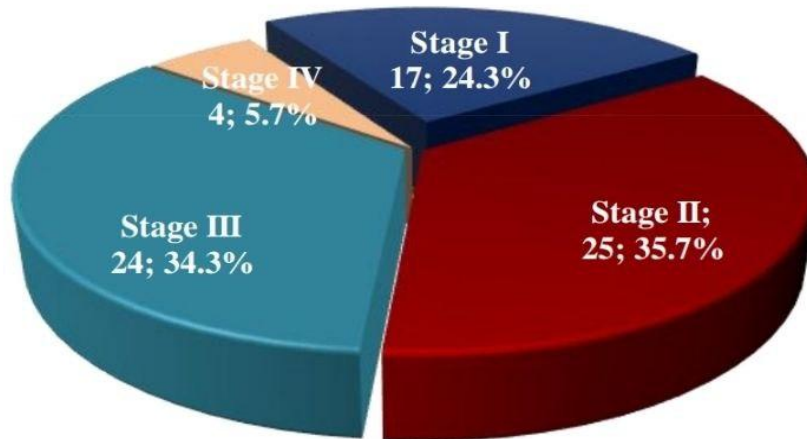


Figure 4: Distribution of breast cancer cases according to stage of cancer (n= 70)

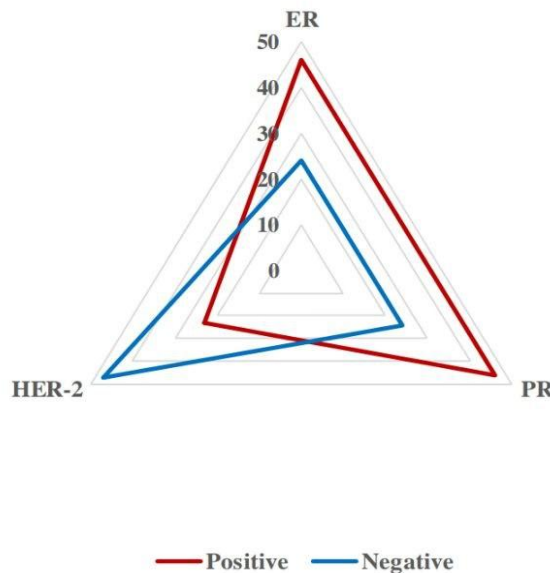


Figure 5: Distribution of immunohistochemistry (IHC) parameters (positivity expression) among the case group of study samples (n=70)



The immunological parameter Heat Shock Protein-90 (HSP-90) was compared between the study groups. The mean HSP-90 level was significantly higher among women with breast cancer than in the control group (47.85257 ± 3.89 vs. 14.57260 ± 5.18), with a significant mean difference ($P < 0.001$) for the cases group (Table 2) (Figure 6).

Likewise, women with breast cancer had significantly higher mean levels of the immune protein forkhead box P3 (FOXP-3) than the control group (4.67081 ± 0.9 vs. 1.18077 ± 0.7), with a significant mean difference ($P < 0.001$) (Table 3) (Figure 7).

Table 2: Mean comparison of immunological parameters of Heat shock protein-90 (HSP-90) among the study's groups (n=140)

Immunological Parameters (Mean \pm SD)	Study groups (n=140)		Significance ^a
	Cases (n=70)	Control (n=70)	
Heat shock protein 90 (HSP-90)	47.85257 ± 3.8893877	14.57260 ± 5.176012	$t = -27.058$, df: 138, $P < 0.001$

^a: Unpaired T- Test.

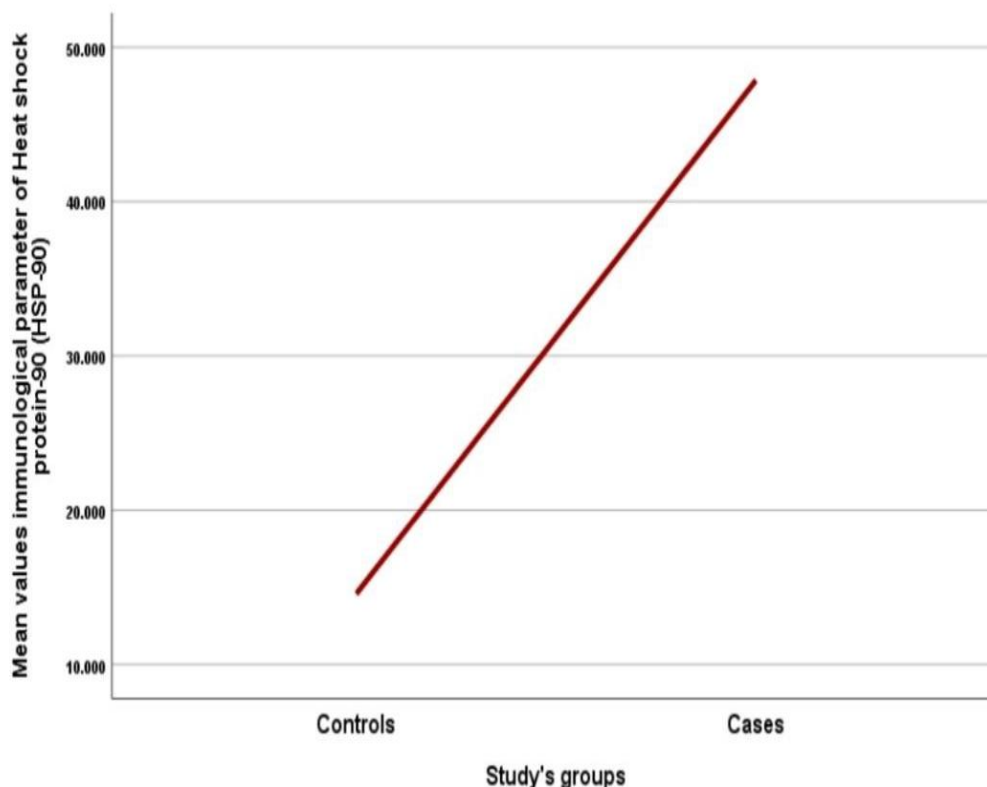


Figure 6: Comparison of immunological parameters of Heat shock protein 90 (HSP-90) among the study's groups (n=140)



Table (3): Mean comparison of immunological parameters of forkhead box P3 (FOXP-3) among the study's groups (n=140)

Immunological Parameters (Mean ± SD)	Study groups (n=140)		Significance ^a
	Cases (n=70)	Control (n=70)	
Forkhead box P3 (FOXP-3)	4.67081 ± 0.879961	1.18077 ± 0.690959	t = -26.099, df: 138, P<0.001

a: Unpaired T- Test.

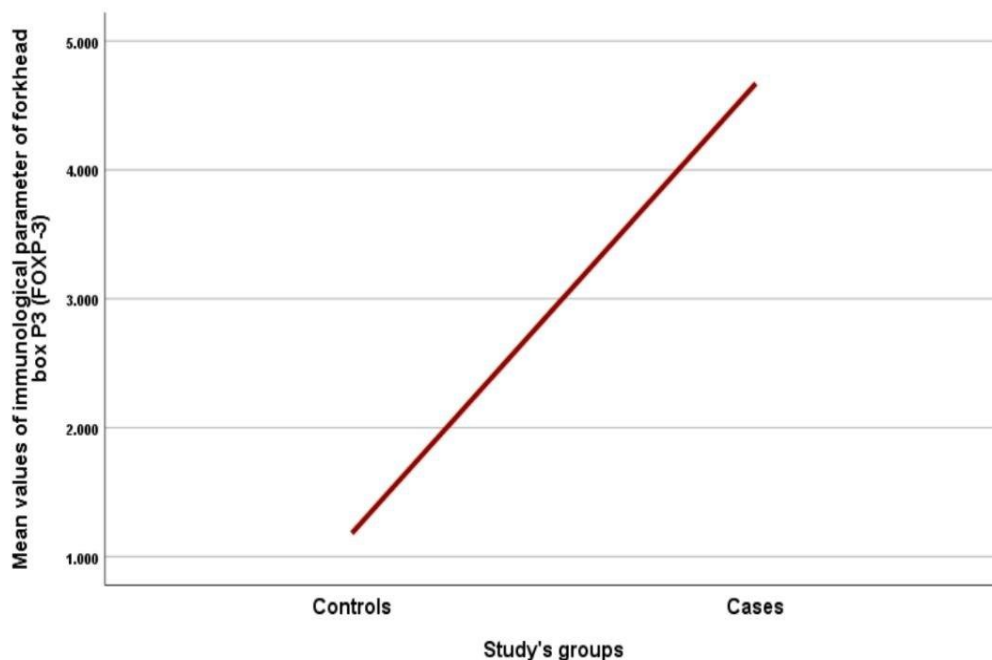


Figure (7): Comparison of immunological parameters of forkhead box P3 (FOXP-3) among the study's groups (n=140)

Heat shock protein-90 (HSP-90) as a predictive diagnostic marker for the developing risk of breast cancer

Among 140 study samples, the optimal cutoff for Heat Shock Protein-90 (HSP-90) was 30.63100, with a sensitivity of 97.1% and a specificity of 100%. The regression model predicted this cutoff with 98.6% accuracy, yielding an excellent area under the ROC curve (AUC) of 0.989± 0.008 (P<0.001) (Table 4, Figure 8).

Forkhead box P3 (FOXP-3) as a predictive diagnostic marker for the developing risk of breast cancer

Nearly, the regression model accurately predicted the optimal cutoff value of forkhead box P3 (FOXP-3) to be 2.72850, with a sensitivity of 100% and a specificity of 98.6%, and an excellent area under the ROC curve (AUC) of 1.000 ± 0.000 (P<0.001) (Table 5, Figure 9).



Table (4) Predictive value of Heat shock protein-90 (HSP-90) as a marker for developing breast cancer (n=140)

Paramter	Validity of the model				
	Sensivity (Sn)	Specificity (Sp)	Accuracy	Area Under the curve (AUC)	Significance (P-value)
Heat shock protein-90 (HSP-90)	97.1	100	98.6	0.989	P< 0.001

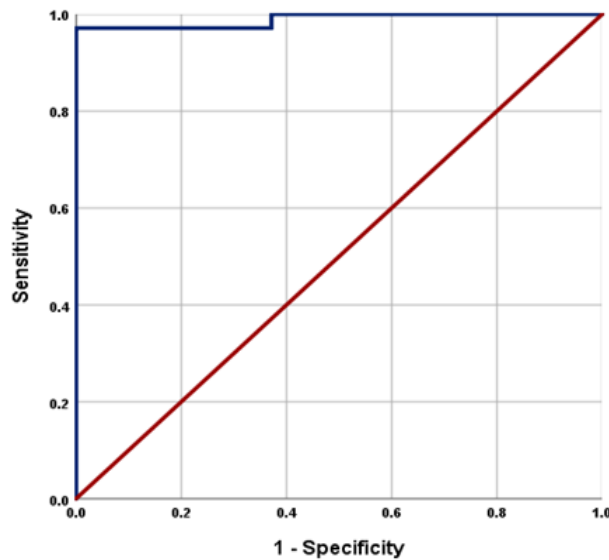


Figure (8): ROC Curve of breast cancer risk development predicted by immunological parameter of Heat shock protein-90 (HSP-90) among study samples (n=140)

Table (5): Predictive value of forkhead box P3 (FOXP-3) as a marker for developing breast cancer (n=140)

Paramter	Validity of the model				
	Sensivity (Sn)	Specificity (Sp)	Accuracy	Area Under the curve (AUC)	Significance (P-value)
Forkhead box P3 (FOXP-3)	100	98.6	100	1.000	P< 0.001



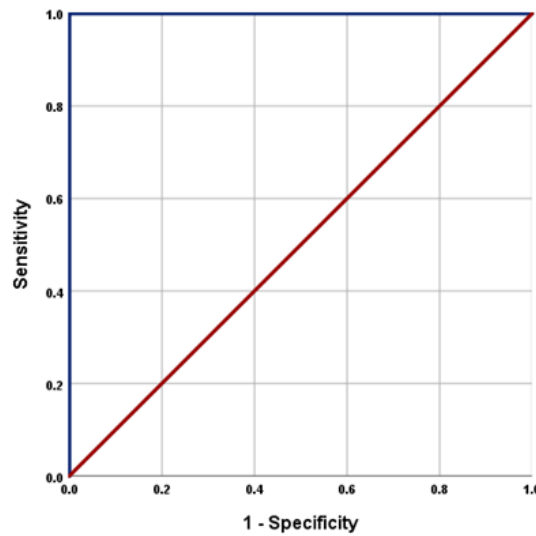


Figure (9): ROC Curve of breast cancer risk development predicted by immunological parameter of forkhead box P3 (FOXP-3) among study samples (n=140)

Discussion

The findings indicated that the most commonly affected age group with breast cancer was 46–60 years (46.4%). The results are consistent with regional statistics from the Middle East and North Africa, where women aged 45–59 years had the highest incidence and mortality from breast cancer across 21 nations and territories (1990–2019) (14). Global statistics are also generally consistent: according to U.S. surveillance data from 2017–2021, the highest prevalence is observed in women aged 65–74 years (27.1%) and 55–64 years (25.1%), findings similar to those of the current study (15).

Immune cells may act in opposing ways within the tumor microenvironment: they can foster tumor destruction through cytotoxic activity and surveillance, or facilitate tumor progression and metastasis by sustaining chronic inflammation and inducing immunosuppression. Key immune

components, such as FOXP3⁺ regulatory T cells, and proteins, such as heat shock protein-90 (HSP90), are increasingly being investigated for their prognostic value and potential as therapeutic targets in breast cancer (16).

Heat shock protein-90 and Forkhead Box P3 (FOXP3) were assessed in this investigation. The study demonstrated that breast cancer was associated with a significant elevation in serum FOXP3 and HSP90 levels. High levels of these markers indicate stronger anti-tumor immune responses and can also be used as biomarkers to predict how well immune checkpoint blockade treatments will work.

In breast cancer, FOXP3 is an essential tumor suppressor. It acts by downregulating oncogenic targets, such as metastasis-associated protein 1 (MTA1), through transcriptional repression. This regulatory function inhibits tumor cell invasion and metastatic potential. Furthermore,



FOXP3 expression in tumor cells has been linked to decreased proliferative activity and slowed malignant growth, confirming its role as a protective factor and a positive prognostic signal in patients with breast cancer (17).

On the other hand, regulatory T cells (Tregs), which frequently infiltrate breast tumor tissue, are primarily transcriptionally regulated by FOXP3. Increased FOXP3⁺ Treg frequencies and numbers create an immunosuppressive environment that suppresses cytotoxic T-cell responses, hinders tumor surveillance, and is associated with a worse prognosis (18).

Recent clinical research has provided additional evidence for these compounds' multiple activities. In a breast cancer study of 313 patients, immunofluorescence found that reduced FOXP3 expression was associated with prolonged disease-free and overall survival compared with high FOXP3 levels (19). Additionally, patients with breast cancer had higher FOXP3⁺ Treg frequencies; higher levels are linked to more advanced disease and a poorer prognosis. All of these findings point to FOXP3 as a valuable biomarker for prognosis in breast cancer (20).

In this study, mean forkhead box P3 (FOXP-3) levels were substantially higher in the case group than in the control group ($P<0.001$). This finding is consistent with previously reported studies. In breast cancer, HSP90 is a crucial molecular chaperone that stabilizes and activates several oncoproteins, including ER, HER2, AKT, and HIF-1 α , which promote tumor cell development, survival, and proliferation (21). Because HSP90 is often overexpressed in breast cancer, it may be a promising therapeutic target. Numerous researchers have reported its upregulation across various cancer types, further supporting this possibility (22).

Clinical data have also confirmed this, showing that HSP90 plays a crucial role in targeted therapy. Coworkers found that triple therapy (trastuzumab, pertuzumab, and docetaxel) prolonged progression-free survival more than chemotherapy alone in a trial of 72 patients with HER2-positive breast cancer, particularly in tumors with higher HSP90 expression (23).

Higher serum HSP90 levels were associated with increased mortality and progression risk in patients, suggesting that the protein may be employed as a stand-alone predictor of outcome. These results demonstrate HSP90 α 's potential as a therapeutic target for the treatment of breast cancer as well as a biomarker of a bad prognosis (24).

This is in agreement with this study. Women with breast cancer in the case group had a significantly higher mean value of Heat Shock Protein-90 (HSP-90) than those in the control group ($P<0.001$).

Conclusion

Women with breast cancer in the case group had significantly higher mean levels of Heat Shock Protein-90 (HSP-90) than women without breast cancer in the control group. Additionally, women with breast cancer in the case group had significantly higher mean levels of the immune protein Forkhead Box P3 (FOXP-3) than those in the control group, indicating that these biomarkers may be used to predict and prognosticate.

RECOMMENDATION:

Although FOXP3 and HSP90 appear to have clear prognostic potential based on their biological roles and observed associations, the



current study's case-control design limits definitive conclusions about their prognostic value. Consequently, further prospective studies with long-term patient follow-up are warranted to clarify and confirm the true prognostic significance of these markers.

Ethical considerations could include:

Ensuring that the study is conducted in a manner that respects the rights and dignity of participants, including obtaining informed consent and maintaining confidentiality. The Ethical Approval Committee of the College of Medicine / Al-Iraqia University permitted this study, with an emphasis on fulfilling all ethical requirements of the research.

Conflict of Interest: Non

Funding: Nil

Author contributions

All authors contributed to sample collection and the preparation of the original draft, and read and approved the final version of the manuscript.

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