Enhanced Detection of Breast Carcinoma by Fork head Box Protein p3, Vascular Endothelial Growth Factor and Transforming Growth factor Beta

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Abstract: Background and Objective: This study examined mRNA are performed using conventional RT-PCR expression of VEGF, TGFβ and Foxp3 in benign breast lesions and breast carcinoma and protein expression in blood samples of the same patients by ELISA. The current study also evaluate whether the ELISA or RT-PCR detection is more sensitive and effective in diagnosis of benign breast lesions and breast cancer. **Materials and methods:** This study was conducted on three groups; invasive breast carcinoma (grade II) group (n=30), high risk patients with benign breast lesions (neoplastic fibrocystic atypical hyperplasia disease) group (n=30) and control group (n=30). The females included in this study were aged from 36-48 years old. Detection of VEGF, TGFβ. and Foxp3 mRNA are performed using conventional RT-PCR while protein expression was assessed by ELISA. **Result:** Breast cancer patients recorded a highly significant increase in the mean value of serum VEGF, TGF-β and Foxp3 protein level (3.65 ± 0.11) when compared to the high risk and the control groups ($p < 0.001$) by using ELISA technique. Meanwhile, the high risk VEGF, TGF-β and Foxp3levels were significantly elevated from the control group levels ($p < 0.001$). A highly significant increase in the mean value of VEGF, TGF-β and Foxp3 gene expression in breast cancer group when compared to control and high risk groups ($p<0.001$). The high risk group recorded non-significant change in the VEGF, TGF-β and Foxp3 compared to the control group ($p<0.001$). **Conclusion**: Sera levels of VEGF, TGF-β and Foxp3 ELISA could be used as a sensitive biomarker for the early detection of breast cancer especially in high risk patients. And qRT-PCR has a lower limit of detection for VEGF, TGF-β and Foxp3 expression than ELISA technique. Therefore ELISA provides a sensitive, quantitative, accurate, and robust assay for measurement of VEGF, TGF-β and Foxp3. It is potentially a valuable tool for patient selection in clinical investigations.

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1. Introduction:

Benign breast disease is more prevalent than malignancy of the breast and Prevalence rate is 68% among all breast disease and 6.9% among all diseases of women. Majority of them require treatment in their life time **(Vijayalakshmi *et al., 2016*).** Women with severe atypical epithelial hyperplasia have four to five times' higher risk of developing breast cancer than women who do not have any proliferative changes in their breast. Women with this change and a family history of breast cancer (first degree relative) have a nine fold increase in risk. Women with palpable cysts, complex fibro adenomas, duct papilloma's, sclerosis adenosis, and moderate or florid epithelial hyperplasia have a slightly higher risk of breast cancer (1.5-3 times) in comparison with women with nonproliferative breast lesions **(McPherson *et al., 2000;* Collins *et al., 2016* and Oh *et al., 2017).**

Epidemiologic studies have shown that women with proliferative epithelial disorders affecting the small ducts and terminal ductal lobular units of the breast are at increased risk of subsequent breast cancer, particularly when the epithelial proliferation is accompanied by evidence of atypia. Risk is increased approximately 1.5–2 fold for those with epithelial proliferation without atypia (risk level 2) and 4–5 fold for those with proliferative disease with atypia (risk level 3) **(Marshall *et al.*, 1997; Schnitt, 2003; Hartmann *et al.*, 2005; Worsham *et al.*, 2007a, 2007b)**. The risk of breast cancer is multifactorial and is an interaction between environmental, lifestyle, hormonal, and genetic factors **(Spaeth, *2018)*.**

Hereditary cancers are often characterized by

gene mutations associated with a high probability of cancer development, vertical transmission through either the mother or father, and an association with other tumor types **(Pharoah *et al., 1997)*.**

The alterations or mutations in genes which coding by the various TGF-β signaling components will be cause the tumors. In addition, when the tumors developed or progressed, the effects of TGF-β are often lost and it's signaling off to promote the progression, invasion and metastasis of tumors cancer **(Lebrun*, 2012 and* Busch *et al., 2015).*** Anumber of circulating tumor proteins have been suggested as prognostic and predictive biomarkers that may be used to assess patients with BC at any stage of the disease, one of which is transforming growth factor-β (TGF-β) **(Kashiwagi *et al.,* 2010).**

The level of serum vascular endothelial growth factor increased in patients which have loco-regional ductal cancers compared with those which have benign breast tumors. Inhibition of angiogenesis to attenuate cancer growth is becoming desirable strategy for breast cancer administration **(Sanguanraksa*, 2012 and* Dewangan *et al., 2018).***

VEGF were greater in breast cancer patients than controls. The levels increased with advanced tumor, nodes, metastasis (TNM) staging, thus correlating with the patients' prognoses. VEGF levels can be used as diagnostic tools and prognostic factors in breast cancer **(Sahana *et al., 2017).***

Foxp3 expressing regulatorycells attenuate autoimmunity as well as immunity against cancer and infection. Some studies demonstrated that Foxp3 is an epithelial cell-intrinsic tumor suppressor for breast, prostate, ovary and other cancers. Corresponding to its broad function, Foxp3 regulates a broad spectrum of target genes. While it is now well established that Foxp3 binds and regulates thousands of target genes in human genomes, the fundamental mechanisms of its broad impact on gene expression remain to be established. Foxp3 is known to both activate and repress target genes by epigenetically regulating histone modifications of target promoters **(Hori *et al., 2003*; Fontenot *et al., 2003 and* Katoh *et al., 2013)***. The prognostic role of Foxp3Tregs was highly influenced by tumor site, and was also correlated with the molecular subtype and tumor stage ***(*Shang *et al., 2015).***

The current study also evaluate whether the ELISA or RT-PCR detection is more sensitive and effective in diagnosis of benign breast lesions and breast cancer.

2. Patients and Methods:

Patients of study were categorized into: Group of patients A: Breast cancer group (invasive ductal carcinoma grade II) (n=30) who were aged from 36-48 year sold, and had 24 patients with Axillaries lymphadenopathy and 6 patients free lymphnode. Group of patients B: High risk patients group with benign breast lesions (fibrocystic atypical ductal hyperplasia disease) (n=30) who aged from 36 to 48 yrs. old Group of healthy control C: Individuals healthy control group (n=30) who aged from 36-48 yrs.

Sample collection and processing:

Blood samples were obtained by venipuncture from peripheral blood of breast cancer, high risk patients and healthy control. All specimens were collected in heparinized and a sterile plastic Falcon tubes for lymphocytes and serum separation.

Samples preparation:

Serum preparation for ELISA and Spectrometer assay: five mL of venous blood were collected in a sterile 15mL plastic Falcon tube, allowed to maximally clot (30–60 min) and then centrifuged at 12000 rpm for 15 min at room temperature (22–24°C). Serum samples were then stored at −80°C till the time of assay.

ELISA technique:

1. Estimation of head Box Protein p3 (Foxp3) level in the serum:

The enzyme-linked immune-sorbent assay was used for the quantitative detection of human Foxp3 using kit manufactured by ***Glory Science co., China.***

2. Estimation transforming growth factor – beta (TGF-β) level in serum.

Human TGF-β was determined by enzyme linked immunoassay technique using quantitative kit manufactured by ***DRG Instruments GmbH, Germany Division of DRG International, and Inc-Frauenbergstr.***

3. Estimation of Vascular endothelial growth factor (VEGF) level in serum.

Human VEGF was determined by enzyme linked immunoassay technique using quantitative kit manufactured by ***eBioscience Bender Med systems GmbH, Austria***.

Quantitative real-time polymerase chain reaction (RT- PCR) for analysis of TGF-β, VEGF and foxp3 genes expression

Principle

Quantitative real-time polymerase chain reaction (Real-Time PCR) is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end and is used to qualitatively detect gene expression through the creation of complementary DNA (cDNA) transcripts from RNA; qPCR is used to quantitatively measure the amplification of DNA using fluorescent dyes.

RNA was purified from samples using The SV Total RNA Isolation System (Promega) and cDNA was produced using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Both were following manufacturer's instructions. The primer sequences are provided in Supplementary Table 1.

Table (1): the primer sequence used for RT- PCR

Gene name	Primer sequence	Annealing Temp.
Foxp3	forward: 5'-ACTGACCAAGGCTTCATCTGTG-3' 5'-reverse: 5'-GGAACTCTGGGAATGTGCTGT-3'	60.0 °C
TGF-β	forward:5'-GAGGCCCTCCTACCTTTTG-3' reverse:5'-GCAGCTTGGACAGGATCT-3'	60.0 °C
VEGF	forward: 5′GCCAGCACATAGGAGAGATGAGC-3 reverse: 5′ CGGCTTGTCACATTTTTCTGG-3	60.0 °C
Beta-actin	forward: 5'-ATGATATCGCCGCGCTCA-3' reverse: 5'-CGCTCGGTGAGGATCTTCA-3'	60.0 °C

Calculation of data:

Analysis of data was performed by using the **ΔΔ**Ct method (**Livak and Schmittgen *2001)***. Values were normalized to beta- actin and were expressed as relative expression levels.

ΔΔCT= [(CT gene of interest– CT beta actin) in breast cancer patients] – [(CT gene of interest– CT beta actin) in normal healthy volunteers].

Statistical analysis

All data were presented as a mean ± standard error. Data analysis was performed with one-way ANOVA using SPSS (Version 23). Post hoc test was used to assess differences between means. A significant difference for all statistical analysis in this study was considered at the level of $P \leq 0.05$ and high significantly different at the level of $P \leq 0.001$ as compared to the control group.

3. Result:

1- Evaluation of Foxp3 levels using ELISA, PCR

The serum Foxp3 level in the three studied groups by using the different techniques were demonstrated in **table (2)** and **fig. (1)**. Breast cancer patients recorded a highly significant increase in the mean value of serum Foxp3 protein level (3.65 ± 0.11) when compared to the high risk and the control groups ($p < 0.001$) by using ELISA technique. Meanwhile, the high risk Foxp3 levels were significantly elevated (1.73 ± 0.05) from the control group levels ($p < 0.001$).

The fold change of Foxp3 gene expression (ΔΔCT of Foxp3) by using RT-PCR quantitative analysis were also investigated. Breast cancer group recorded a highly significant increase in the mean value of the fold change of Foxp3 gene expression when compared to both of the control and high risk groups ($p<0.001$). The high risk group recorded non significant change in the Foxp3 fold change compared to the control group ($p<0.001$) as shown in **Fig (1).**

Table (2): Foxp3 levels using ELISA and PCR in the different studied groups.

Foxp3 level		
Groups	**ELISA**	**PCR**
Control	0.73 ±0.06	4.1 ± 0.19
High risk	1.73 ± 0.05 **	4.02 ± 0.4
Breast cancer	3.65 ± 0.11 **	8.57 ± 0.22 **

Values are expressed as mean ± SE of 30 female per group. * means significantly different as compared to the control group at P≤ 0.05. ** means high significantly different as compared to the control group value of P ≤ 0.001.

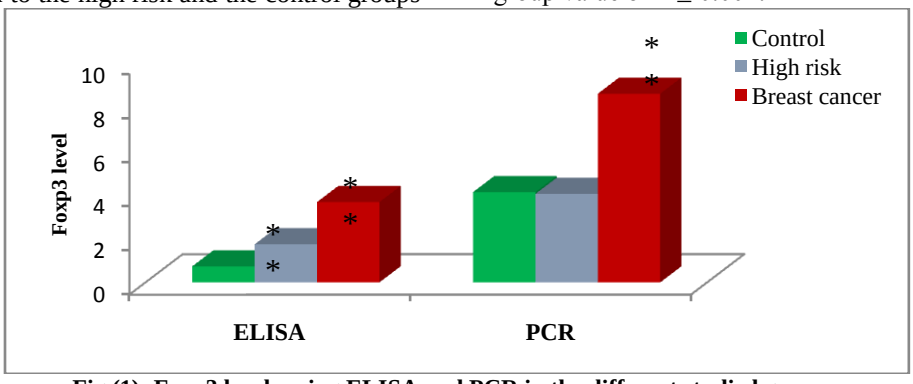

Fig (1): Foxp3 levels using ELISA and PCR in the different studied groups.

* means significantly different as compared to the control group at P≤ 0.05. ** means high significantly different as compared to the control group value of P ≤ 0.001

2- Evaluation of TGF- β levels using ELISA and PCR techniques

By using ELISA technique, breast cancer patients recorded a highly significant increase in the mean value of serum TGF-β protein level (407.4 ± 4.3) as compared to the control group (p< 0.001). Meanwhile, A highly significant elevation (p < 0.001) in the mean value of serum TGF-β protein level was also recorded in the high risk group as compared to the control group (p < 0.001) as revealed in **table (3) and Fig (2)**.

Table (3) Serum TGF-β level in the studied groupsby using ELISA and PCR

TGF-β level		
Groups	**ELISA**	**PCR**
Control	64.81± 4.4	3 ± 0.32
High risk	191.6± 3.9**	3.2 ± 0.69
Breast cancer	407.4 ± 4.3 **	9.22 ± 1.38**

Values are expressed as mean ± SE of 30 female per group. * means significantly different as compared to the control group at P≤ 0.05. ** means high significantly different as compared to the control group value of P ≤ 0.001.

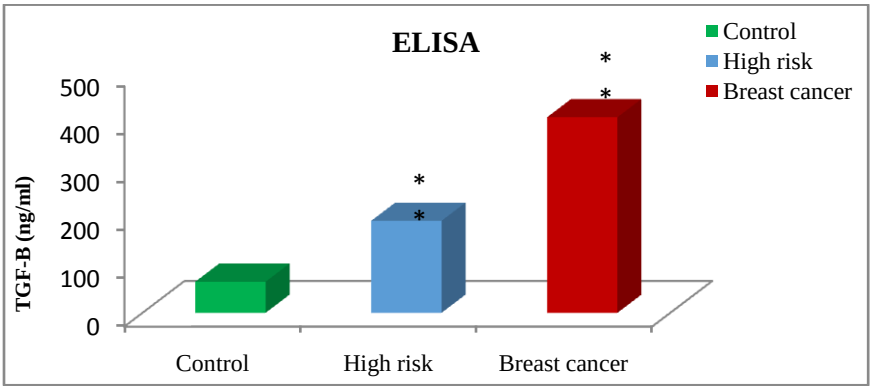

Fig (2): TGF-β level using ELISA technique in the different studied groups.

* means significantly different as compared to the control group at P≤ 0.05. ** means high significantly different as compared to the control group value of P ≤ 0.001

The fold change of TGF-β gene expression (2-ΔΔCT) by using RT-PCR quantitative analysis, recorded a highly significant increase (p<0.001) in the breast cancer group when compared to the control group (**table3and Fig 3**). On the other hand, no change in the fold change of TGF-β gene expression in the high risk group when compared to the control group as shown in Fig (3).

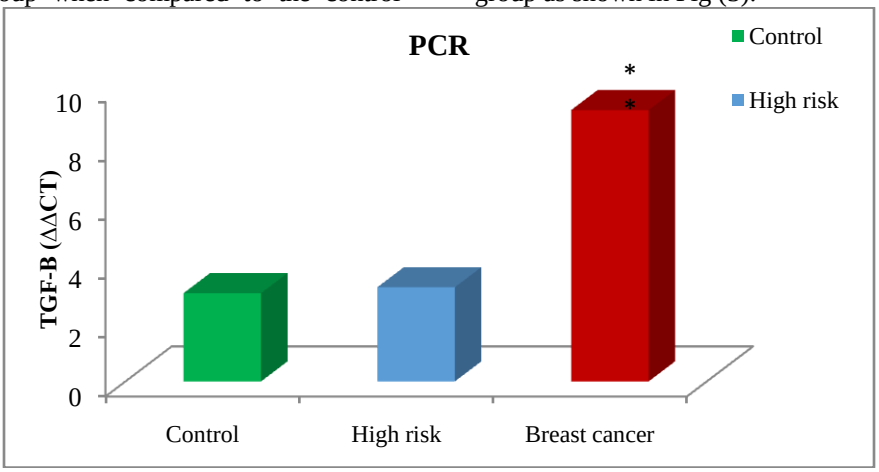

Fig (3): TGF-β level using PCR technique in the different studied groups.

* means significantly different as compared to the control group at P≤ 0.05. ** means high significantly different as compared to the control group value of P ≤ 0.001

3- Evaluation of VEGF levels using ELISA and PCR techniques

Breast cancer patients recorded a highly significant increase ($p< 0.001$) in the mean value of serum VEGF protein level (1013.2 ± 134.7) when compared to the control group (75.3 ± 0.97). Moreover, the high risk patients also recorded a highly significant increase ($p < 0.001$) in the mean value of serum VEGF protein level (292.6 ± 3.7) when comparedto the control group **(Table 4** and **Fig. 4)**.

Table (4): VEGF level in the studied groups by using ELISA and PCR

VEGF level		
Groups	**ELISA**	**PCR**
control	75.3 ± 0.97	4± 0.053
High risk	292.6 ± 3.7**	4.4 ± 0.14
Breast Cancer	1013.2 ± 134.7**	13.8 ± 1.38**

Values are expressed as mean ± SE of 30 female per group. * means significantly different as compared to the control group at P≤ 0.05. ** means high significantly different as compared to the control group value of P ≤ 0.001.

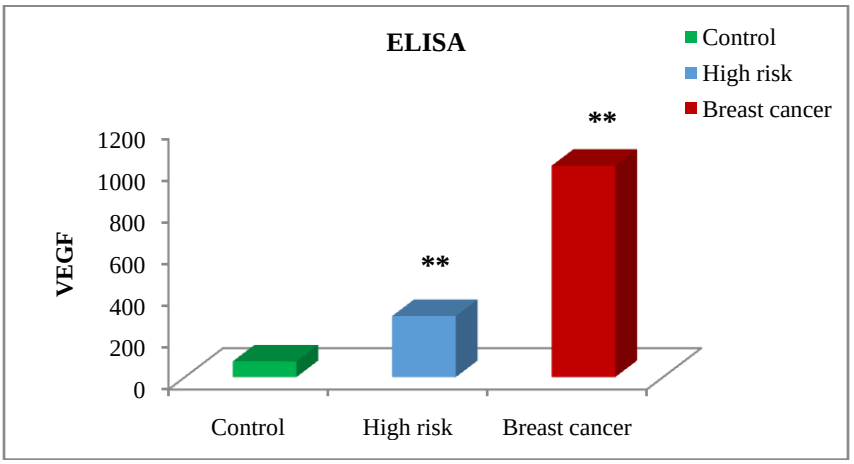

Fig (4): VEGF levels using ELISA technique in the different studied groups.

* means significantly different as compared to the control group at P≤ 0.05. ** means high significantly different as compared to the control group value of P ≤ 0.001.

By using RT-PCR quantitative analysis, the breast cancer group recorded a highly significant increase ($p<0.001$) in the fold change of VEGF gene expression ($2^{-\Delta\Delta CT}$) when compared to the control group, while the high risk group recorded a non significant increase in the fold change of the gene expression as compared to the control group (**Table 4** and **Fig. 5**).

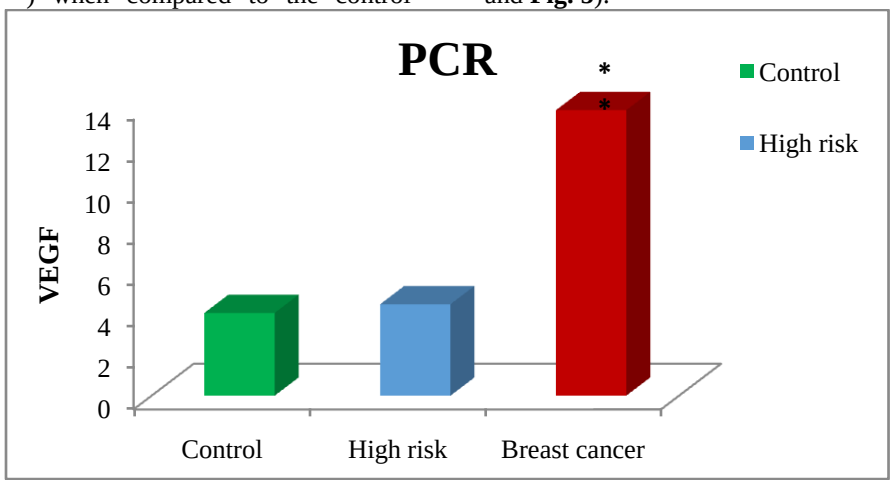

Fig (5): VEGF levels using PCR technique in the different studied groups.

* means significantly different as compared to the control group at P≤ 0.05. ** means high significantly different as compared to the control group value of P ≤ 0.001

A multiple regression analysis was carried out to investigate whether PCR or ELISA techniques for estimation of TGF-β, VEGF and Foxp3 could significantly predict participants' diagnosis. The results of the regressions indicated that the models explained less degree of the variances and that the different models were not significant predictors of participants' diagnosis (table 5).

Table (5): ELISA and PCR regression equations

Parameter	Group	Regression Equation
ELISA/Foxp3	**Control**	1.092 - 0.000083 ELISA/TGF-β + 0.001164 ELISA/VEGF - 0.0242 PCR /Foxp3 + 0.1303 PCR/TGF-β - 0.0037 PCR /VEGF
	High Risk	1.22 - 0.00350 ELISA/TGF-β + 0.00093 ELISA/VEGF + 0.0278 PCR/Foxp3 + 0.222 PCR/TGF-β - 0.342 PCR/VEGF
	Cancer	3.37 - 0.000172 ELISA/TGF-β + 0.000092 ELISA/VEGF + 0.007 PCR/Foxp3 + 0.028 PCR/TGF-β + 0.0040 PCR/VEGF
ELISA/TGF-β	**Control**	58.1 - 14.2 ELISA/Foxp3 + 0.188 ELISA /VEGF + 4.04 PCR/Foxp3 + 5.6 PCR/TGF-β - 10.6 PCR/VEGF
	High Risk	211 - 4.4 ELISA/Foxp3 - 0.004 ELISA/VEGF - 6.13 PCR/Foxp3 + 13.5 PCR/TGF-β - 7.4 PCR/VEGF
	Cancer	732 - 63 ELISA/Foxp3 - 0.0272 ELISA /VEGF + 47.8 PCR/Foxp3 - 109 PCR/TGF-β + 9.5 PCR/VEGF
ELISA/VEGF	**Control**	-47 + 8.3 ELISA/Foxp3 + 0.417 ELISA/TGF-β + 5.97 PCR/Foxp3 + 16.3 PCR/TGF-β + 0.8 PCR/VEGF
	High Risk	212 + 118.2 ELISA/Foxp3 - 0.008 ELISA /TGF-β + 3.53 PCR/Foxp3 - 28.1 PCR/TGF-β - 10.3 PCR/VEGF
	Cancer	2733 + 921 ELISA/Foxp3 - 0.74 ELISA /TGF-β - 150 PCR/Foxp3 - 847 PCR/TGF-β + 48.8 PCR/VEGF

Foxp3 and VEGF showed similar sensitivity in PCR technique for the patient diagnosis, as it form a clade with the TGF-B levels detected by ELISA representing a great homology. However, the others parameters formed clades with lesser degree of similarity (fig.6).

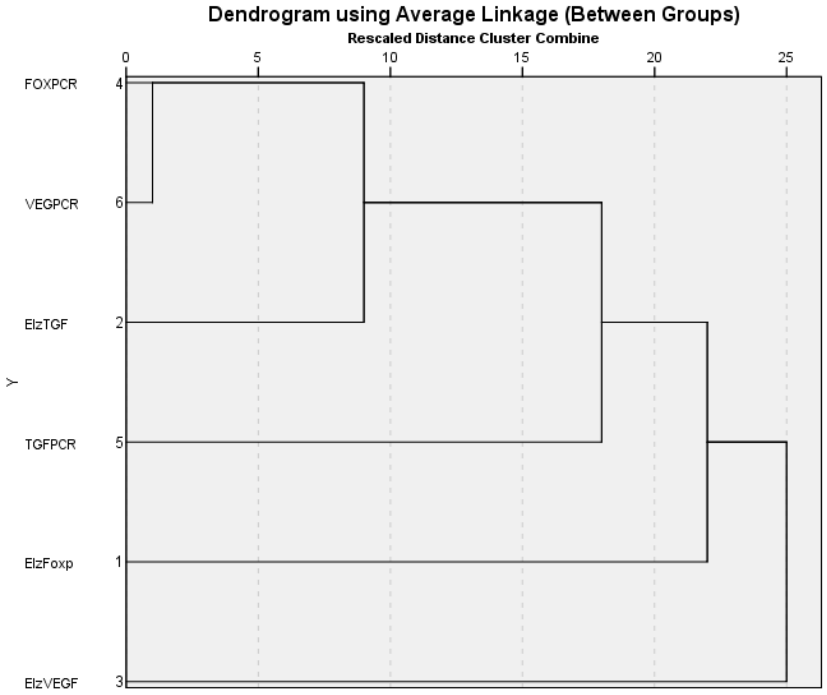

Fig (6): Dendrogram with homologous using Minitab constructed with PCR and ELISA techniques for VEGF, TGF-B and Foxp3 based on groups.

4. Discussion:

Our results suggest that some biomarkers can used as indicator of prognostic in patients with risk of breast cancer.

Genetic analyses in both mice and humans revealed that Foxp3 is an important X-linked tumor suppressor in breast and in prostate cancer **(Katoh *et al.*, 2010; Ladoire *et al.*, 2011; Li *et al.*, 2011; Zuo *et al.*, 2007a, 2007b).** Micewith germline FoxP3 mutations are substantially more proneto developing both spontaneous and carcinogen-induced mammary carcinomas **(Zuo *et al.*, 2007b).** Frequent chromosomal deletions and somatic mutations of the FoxP3 gene were detected in human cancer samples including cutaneous melanomas **(Karanikas *et al.*, 2008).**

The present study, showed a highly significant increase in the mean value of Foxp3 gene expression in breast cancer group when compared to control and high risk groups ($p<0.001$). The high risk group recorded non significant change in the Foxp3 fold change compared to the control group ($p<0.001$).

These results agreement with **Lin *et al.* (2018)** evaluated that the value of Foxp3 mRNA expression in the peripheral blood for breast cancer patients was higher than patients with benign breast tumors significantly ($P<0.001$). **Merlo *et al.* (2009)** suggested that Foxp3 expression was scored positive in breast tumor specimens. Foxp3 expression can also be detected in the epithelial as well as the stromal component of tumors in breast cancers.

Zou *et al.* (2007b) showed that functional somatic mutations, and down-regulation of the FOXP3 gene, were commonly found in human breast cancer samples and although this also correlated with HER-2/ErbB2 overexpression it was clearly lower than that of normal breast tissue. **Ladoire *et al.* (2011)** demonstrated that the influence of FoxP3 was dependent on the molecular sub-type of breast cancer. Indeed, FoxP3 expression in cancer cells may be a marker of good prognosis in HER2-overexpressing tumors and of poor prognosis in other molecular sub-types of breast cancer.

The present data revealed that breast cancer patients recorded a highly significant increase in the mean value of serum Foxp3 protein level (3.65 ± 0.11) when compared to the high risk and the control groups ($p < 0.001$) by using ELISA technique. Meanwhile, the high risk Foxp3 levels were significantly elevated (1.73 ± 0.05) from the control group levels ($p < 0.001$).

These results similar to the result that reported by **Karanikas *et al.*, *(2008)*** who found that *Foxp3* mRNA as well as Foxp3 protein was detected in all tumor cell lines, albeit in variable levels, not related to the tissue of origin.

Foxp3 expression in tumors was associated with worse overall survival probability and the risk increased with increasing Foxp3 immune-staining intensity. Foxp3 was also a strong prognostic factor for distant metastases-free survival but not for local recurrence risk **(Bates *et al.*, *2006*** and **Merlo *et al.*, *2009)***.

Results obtained in the present study demonstrate that TGF-β gene expression recorded a highly significant increase ($p<0.001$) in the breast cancer group when compared to the control group. On the other hand, no change in the fold change of TGF-β gene expression in the high risk group when compared to the control group.

In benign epithelial cells, TGF-β is generally considered an antiproliferative.

And proapoptotic signal **(Shi *et al.*, 2003).** A key step in TGF-β dysregulation is the loss of this response. In advanced disease, TGF-β can have prosurvival/antiapoptotic effects **(Pasche *et al.*, 2001; Shin *et al.*, 2001; Massague *et al.*, 2008; Padua *et al.*, 2009 and Wendt *et al.*, 2012).**

In the current studyhigh risk and breast cancer patients recorded a highly significant increase ($p< 0.001$) in the mean value of serum TGF-β protein level when compared to the control group.

TGF-β expression has been studied in nearly all epithelial cancers, including, prostate, breast, lung, colorectal, pancreatic, and skin cancers **(Padua *et al.*, 2009).** Through these studies, it has become clear that TGF-β can function as both a tumor suppressor and a tumor promoter **(Akhurst *et al.*, 2001; Inman *et al.*, 2011 and Wendt *et al.*, 2012).** In benign epithelia and many early-stage tumors, TGF-β is a potent inducer of growth arrest. However, in advanced tumors, TGF-β signaling pathways are severely dysregulated. Rather than inhibiting carcinogenesis, TGF-β promotes tumor growth and progression at late stages **(Akhurst *et al.*, 2001; Pasche *et al.*, 2001; Massague *et al.*, 2008; Langenskiold *et al.*, 2008; Padua *et al.*, 2009; Inman *et al.*, 2011 and Zhao *et al.*, 2012).** This functional switch is known as the TGF-β paradox **(Wendt *et al.*, 2012).**

This paradox is reflected in the clinic, where in early stage cancers, levels of TGF-β are positively associated with a favorable prognosis. Yet in advanced tumors, levels of TGF-β in the tumor microenvironment are positively associated with tumor size, invasiveness, and dedifferentiation, making TGF-β a useful prognostic biomarker and predictor of recurrence after initial or failed therapy. **(Shariat *et al.*, 2001; Padua *et al.*, 2009; Langenskiold *et al.*, 2008 and Zhao *et al.*, 2012).**

Our data in the present study pointed out to the fold change of VEGF gene expression in the breast cancer group recorded a highly significant increase

($p<0.001$) when compared to the control group, while the high risk group recorded a non-significant increase in the fold change of the gene expression as compared to the control group.

The difference was significant between cancer cases and control cases regarding tissue expression of VEGF. This finding was expected as VEGF expression has no or limited role in the benign lesions. This was matched with other studies which were also performed on breast carcinoma **(Jacobs *et al.*, 2006; Cimpean *et al.*, 2008; Al-Harris *et al.*, 2008 and Ali *et al.*, 2011).** This result was inconsistent with others who reported expression of VEGF not only in carcinoma cells but also in inflammatory cells, endothelial cells and fibroblast **(Cimpean *et al.*, 2008 and Valkovic *et al.*, 2002).**

The present study indicated that a highly significant increase ($p< 0.001$) in the mean value of serum VEGF protein level in breast cancer patients and high risk patients when compared to the control group. These results agreement with **Ragab *et al.* (2016)** showed that statistical difference between serum concentration of VEGF in benign breast lesions and primary breast cancer patient. This may raise the possibility of using VEGF in differentiating between patients with malignant and benign breast tumors. On the other hand no correlation was found between concentrations of VEGF and the patient's age, size of the primary tumor, metastasis to lymph nodes, histological type and grade.

This can confirm the concept that this growth factor is involved in the breast carcinoma development and thus can be used to differentiate between malignant and benign breast cases **(Cimpean *et al.*, 2008).** VEGF expression in breast carcinoma cases and concluded that serum marker might be a biologically and clinically useful marker in diagnosing breast cancer and identifying high risk group **(Ali *et al.*, 2011).**

5. Conclusion:

Serum levels of VEGF, TGF-β and Foxp3 ELISA could be used as a sensitive biomarker for the early detection of breast cancer especially in high risk patients. And qRT-PCR has a lower limit of detection for VEGF, TGF-β and Foxp3expression than ELISA technique. Therefore ELISA provides a sensitive, quantitative, accurate, and robust assay for measurement of VEGF, TGF-β and Foxp3. It is potentially a valuable tool for patient selection in clinical investigations.

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