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The Diagnostic and Prognostic Values of FOXP3 Gene in Iraqi Breast Cancer Women

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Abstract:

Background: The transcription factor forkhead-box protein P3 (FOXP3) have been identified to counteract the anti-tumor immune responses during tumor progression. In addition, by expressing FOXP3, tumor cells may evade effector T-cell responses, resulting in a survival benefit of the tumor.

Objectives: Evaluation of the diagnostic and/or prognostic values of FOXP3 gene in breast cancer Iraqi female patients by initially comparing the expression concentrations of this gene between breast cancer patients and control group, then, comparing the expression levels of FOXP3 with certain clinical features among breast cancer patients (ages of patients, tumor grade, tumor stage and the presence or absence of metastasis).

Material and Methods: The Foxp3 levels were determined in tissue samples (Formalin Fixed Paraffin Embedded Tissue "FFPE") derived from 51 Invasive Ductal Carcinoma women and 33 benign breast tumor women (control group) were attended to the Medical City and Al-Yarmouk teaching laboratories / Baghdad – Iraq. The patients' samples were subjected to total RNA extraction, and then to molecular study by using reverse transcription and quantitative real time PCR at Molecular Oncology Unit in Guy´s Hospital – Kings College / London – UK.

Results: The FOXP3 gene expression was detected in 45 (88.23%) of breast cancer patients, also, the expression levels of this gene showed high significant increase in breast cancer patients compared to control group. Furthermore, there were a gradual increase in the FOXP3 expression concentrations with disease grades (highly significant) and stages (significant) progression in patients with primary breast cancer, moreover, the metastatic breast cancer patients showed high significant increase in FOXP3 levels compared to primary breast cancer patients. There were no significant differences in the levels of FOXP3 among the age groups of patients.

Conclusions: The present study results reflect the potential utility of FOXP3 as noninvasive marker for detecting breast cancer even in the earliest cancer stages, also, they suggest that possibility of using this gene as an efficient molecular signature for detecting breast cancer disease progression, discrimination between different stages and grades of breast tumors, and it might be of value as a prognostic marker.

keywords: *FOXP3 Gene, Breast Cancer*

Introduction:

Breast cancer is the most common cancer among women worldwide (1), and the second leading cause of females' deaths after lung cancer. Although substantial improvement in survival from this disease has been reported in high-income countries such as the USA, the risk continues to increase and survival rates in middle-and low-income countries remain low (2). In Iraq, breast cancer is the most common type of malignancy among the population in general; responsible for about one third of the registered female cancers and almost one quarter of females' deaths from the disease (3, 4). Forkhead box protein 3 (FOXP3) is a member of the forkhead / winged-helix family of transcription factors (5). It plays a crucial role in the generation of immunosuppressive regulatory T cells (Tregs), which induce immune tolerance to antigens (6). Loss of FOXP3 function leads to Treg deficiency, resulting in lethal autoimmune disease, whereas FOXP3 overexpression leads to severe immunodeficiency (7). FOXP3-expressing Tregs are reportedly abundant in the tumor infiltrates and peripheral blood of cancer patients (8). They are also involved in

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the immune evasion mechanisms promoted by cancer (9). The aims of the present study were to evaluate the diagnostic and/or prognostic values of FOXP3 gene in breast cancer Iraqi female patients by initially comparing the expression concentrations of this gene between breast cancer patients and control group, then, comparing the expression levels of FOXP3 with certain clinical features among breast cancer patients (ages of patients, tumor grade, tumor stage and the presence or absence of metastasis).

Materials and Methods:

Patients and clinical samples

This study was conducted from July 2013 to July 2014 on a total number of 84 subjects including 51 women of different ages (21-76 years) experiencing different stages and grades of Invasive Ductal Carcinoma and 33 women with fibroadenoma (which were chosen as a control group) with ages range of (15-53 years). These patients attended to the Medical City and Al-Yarmouk teaching laboratories / Baghdad – Iraq. Then, after surgery, Formalin Fixed Paraffin Embedded (FFPE) tissues derived from these patients and the control group were collected, and the required informations about the patients and the histopathological properties of the tumors were recorded from the patients' files. The RNA extraction, reverse transcription and quantitative real time PCR (qRT-PCR) were done at Molecular Oncology Unit in Guy´s Hospital – Kings College / London - United Kingdom.

RNA Extraction, Reverse Transcription and Quantitative Real-Time PCR (qRT-PCR) Assay

The total RNA of breast cancer patients and control group samples was extracted using Qiagen RNA extraction kit, according to the manufacturer's instructions. Then, RNA was reversely transcribed using High-Capacity cDNA Reverse Transcription Kit. The procedures were carried out in a reaction volume of 20 µl following the protocol provided by the manufacturer (Ambion, USA). After that, the cDNA was stored at -80 °C until use. Expression of FOXP3 gene was assayed by using specific primers (Table 1). In this assay, the PGK1 gene was used as an endogenous control to normalize the variations in the integrity and the total amount of cDNA. Quantitative real-time PCR (qRT-PCR) assays were performed in duplicate for each sample by using SYBR Green master mix (Applied Biosystems, USA) in 20 µl reaction volume containing 10 µl of master mix, 1 µl of primers mix, 6µl of RNase free water and 3µl of cDNA template on the 7900 HT Fast Real-time PCR system (Applied Biosystem/ USA). Real-Time PCR protocol was as follows; stage 1: 50 ºC for 2 minutes, stage 2: 95 ºC for 10 min, stage 3 included two-steps cycle procedures (denaturation 95 ºC for 15 Sec. and annealing 60 ºC for 1 min) repeated for 50 cycles. The expression levels of FOXP3 gene from the cDNA were measured by quantitative real-time PCR using the relative quantification method (2-ΔΔCt method) (10). The fold-change in gene expression was normalized to a PGK1 gene and relative to a control group samples.

Table 1. Primers sequences

Primer	Sequence	Tm/°C
FOXP3-F 5′	CAAGTTCCACAACATGCGAC	60.0°C
FOXP3-R 5′	ATTGAGTGTCCGCTGCTTCT	60.0°C
PGK1-F 5′	GGGAAAAGATGCTTCTGGGAA	47.3°C
PGK1-R 5′	TTGGAAAGTGAAGCTCGGAAA	45.3°C

Results:

The patients' age range was 21-76 years and the mean was 47.37 years with high frequency of patients in the age range of 50-59 years. The number of breast cancer patients that gave positive FOXP3 gene expression was very high; 45 patients (88.23%) out of 51 breast cancer patients were FOXP3 positive (Figure 1), moreover, the FOXP3 gene expression levels aggressively increased in breast cancer patients compared to control group (highly significant, p value = 0.0072, p <0.01) (Figure 2). There were a gradual increase in the expression levels of FOXP3 gene with disease grades (grade I to III) and stages (stage I to III) progression in patients with primary breast cancer, (highly significant for disease grade progression, $p<0.01$ and significant for disease stage progression $p<0.05$) (Figures 3). Furthermore, the FOXP3 expression concentrations sharply increased in metastatic breast cancer patients compared to primary breast cancer patients (highly significant, p value = 0.004, p <0.01) (Figure 4). In correlation with age groups of patients, the results revealed that there were no significant differences (ns) in the levels of FOXP3 gene expression with the ages of patients.

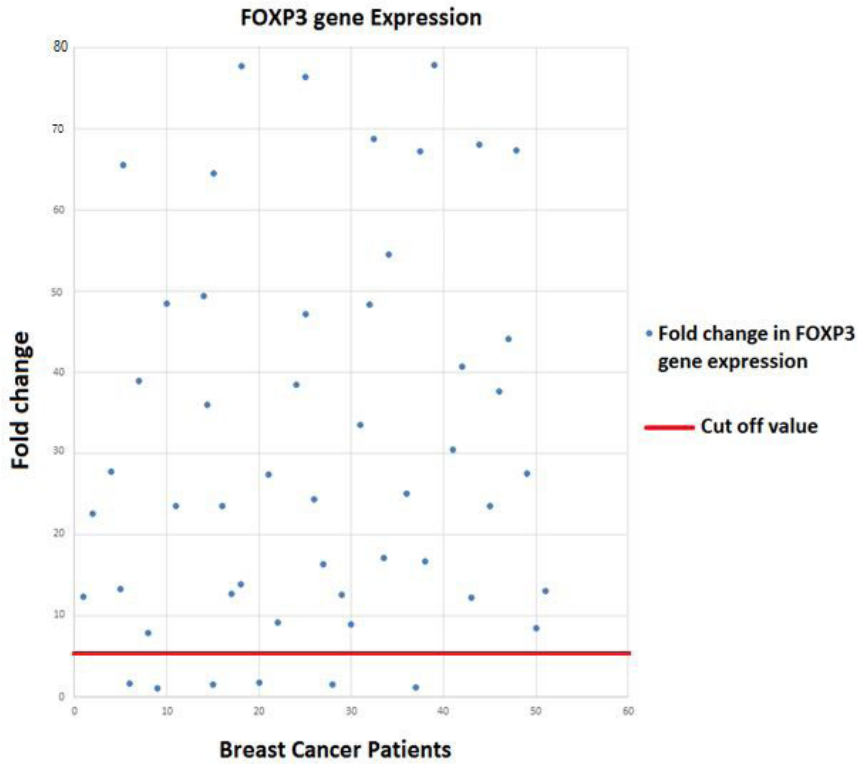

Figure 1. Breast cancer patients that gave positive FOXP3 gene expression.

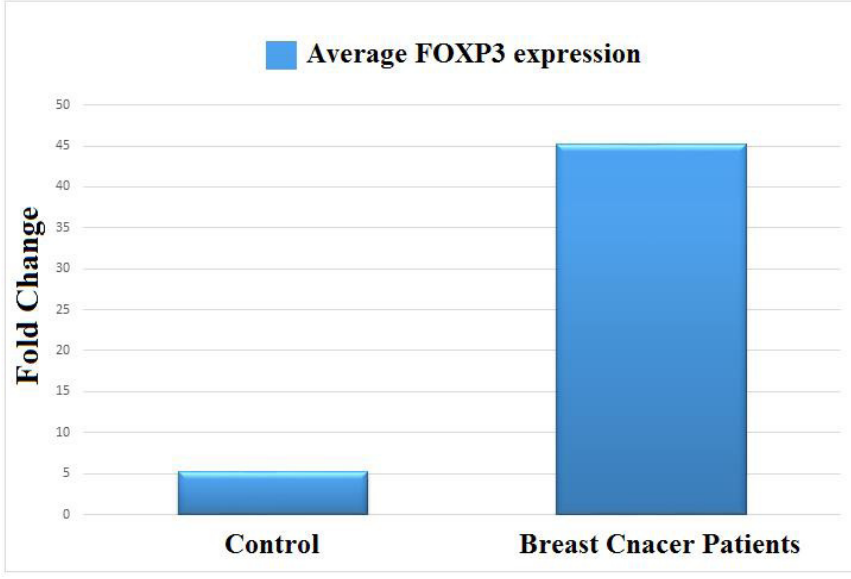

Figure 2. Comparison in the FOXP3 gene expression between breast cancer patients and control group.

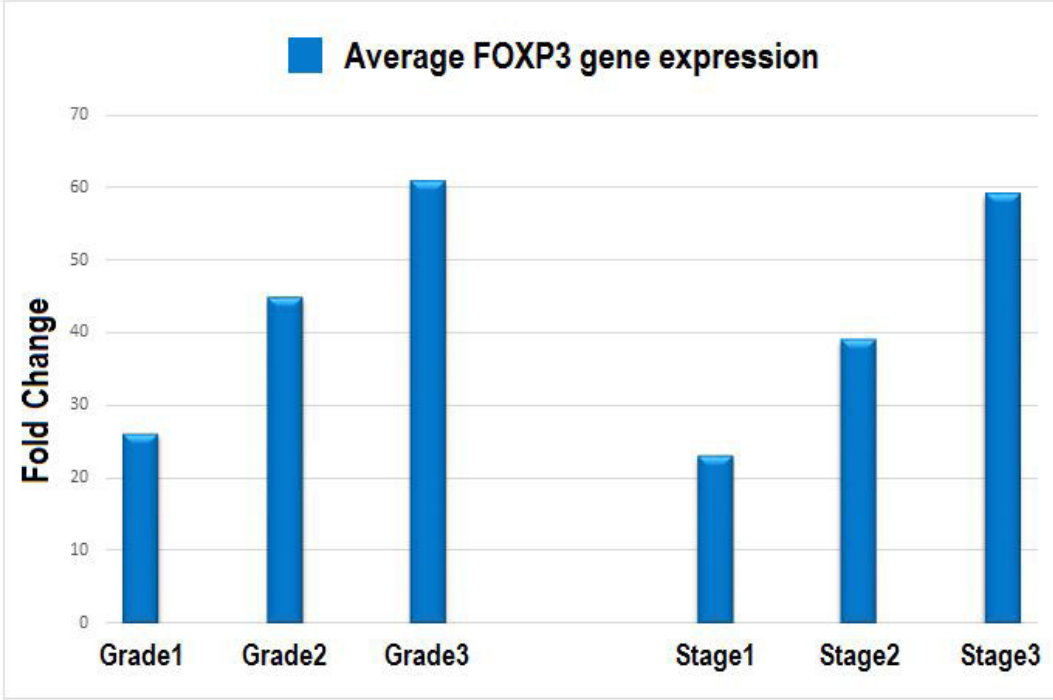

Figure 3. The effect of disease grades and stages progression on FOXP3 gene expression in primary breast cancer patients.

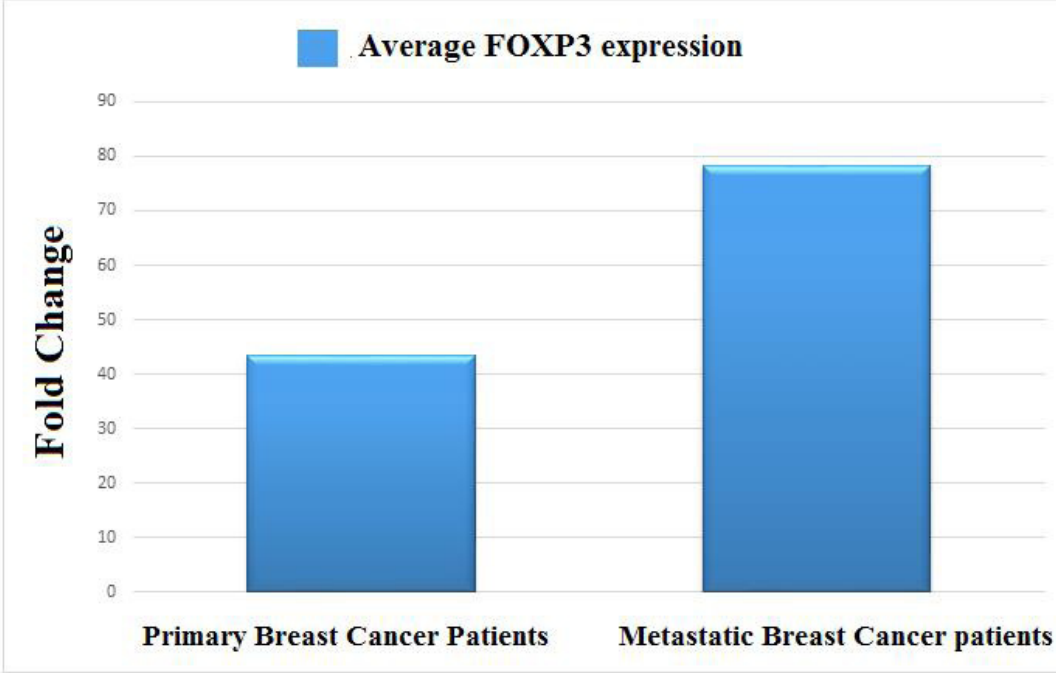

Figure 4. Comparison in the FOXP3 gene expression between primary and metastatic breast cancer patients.

Discussion:

Forkhead box protein 3 gene expression was initially thought to be restricted to hematopoietic cells and tissues, however, it has been demonstrated that the tumor cell itself can express FOXP3 (11). The present study revealed that most of the breast cancer patients were FOXP3 positive (88.23% of breast cancer patients were FOXP3 positive), also, the expression levels of this gene aggressively increased in breast cancer patients compared to control group. The expression of FOXP3 gene in tumor cells raises the possibility that tumor cells themselves may enhanced the development and the stimulation of regulatory T cells (Tregs) function (12), which in turn suppress the antitumor immunity as the Tregs have the ability to hinder the induction of immune responses against cancer. The precise mechanisms by which Tregs suppress immune cells functions remain unclear, with reports of both direct inhibition through cell-cell contact and indirect inhibition through the secretion of anti-inflammatory mediators such as interleukin 10- (IL-10) and transforming growth factor (13, 14). There are many studies reported an elevation in the expression levels of FOXP3 gene in breast cancer patients, for example; Zuo et al., (2007) reported the expression of FOXP3 in carcinoma breast tissue (15), also, by using immunohistochemical staining, the investigators characterized FOXP3 expression in 397 primary breast carcinoma specimens and FOXP3 stained positive in the majority of the breast cancer tissues examined (11). Moreover, Ohara et al., (2009) evaluated the expression of FOXP3 mRNA in 136 breast cancer patients; the total RNA was extracted from frozen breast cancer and normal tissues, they found that FOXP3 transcripts were significantly increased in cancer tissues, not only at late stages but also at the early stages of the disease (16).

The FOXP3 expression levels in the current study have been evaluated according to the grades and stages of breast cancer disease and the result showed that there were a gradual increase in the expression levels with disease grades (grade I to III) and stages (stage I to III) progression in patients with primary breast cancer. The progression in tumor growth might cause a further up regulation of FOXP3 gene to increase FOXP3 protein production which in turn cause a sever reduction in the anti-tumor immune response by stimulating the Tregs generation and function which finally lead to provide better environment for tumor progression.

Indeed, many studies declared that there was an increase in FOXP3 expression with breast cancer disease progression. Lal et al., (2013) evaluated quantitative FOXP3 expression in lymphocytes as well as in epithelial cells in a set of thirty-two breast tumors with synchronous normal epithelium, ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC) components. Tumors were stained for FOXP3 expression, the median proportion of FOXP3-expression significantly increased with malignant progression from normal to DCIS to IDC components, and the expression was higher in breast cancer patients of grade III. (17). In another study, tissue microarrays were used, and the expression of FOXP3 was determined in a series of 1445 cases of well-characterized primary invasive breast carcinoma cases with long-term follow up; FOXP3 levels were counted in tumor nests, tumor-adjacent stroma, and distant stroma, the FOXP3 expression was significantly correlated with higher tumor grade (18). Hamidinia et al., (2013) estimated FOXP3 transcripts as acceptable indicators of Tregs in the peripheral blood from women with different stages of breast cancer and found that there was a correlated significant increment in these transcripts concentration with the disease stage progression (19). Additional analysis revealed that FOXP3 expression in tumors was associated with worse overall survival, and the risk increased with increasing FOXP3 immunostaining intensity (11).

Considering the presence or absence of metastasis, the present data revealed that FOXP3 expression sharply increased in metastatic breast cancer patients compared to primary breast cancer patients. Likewise, by using quantitative real-time RT PCR, Matsuura et al., (2006) found significantly higher levels of FOXP3 transcripts in metastatic breast cancer patients compared with control cases (20), as well as, Merlo et al., (2009) found that FOXP3 expression was associated with distant metastasis; therefore, these authors suggest that FOXP3 expression might be related to the metastatic potential of the tumor (21). Additionally, Hamidinia et al., (2013) studied the FOXP3 mRNA concentrations in breast cancer patients with different stage and found that FOXP3 expression reached its highest values in patients with stage IV (metastatic breast cancer patients)(19). It had been found that FOXP3 binds to the gene region upstream of the transcriptional start site of CCR7 and CXCR4 (22); two chemokine receptors reported to play an important role in cancer invasion and metastasis (23). Thus, FOXP3 expressed in breast cancer cells might influence the development of metastasis by modulating the expression of these chemokine receptors or of other genes encoding cell surface or secreted molecules that alter tumor cell response to the environment (21). Additionally, the abundance of FOXP3 in tumors has been associated with worse prognosis (24, 25), for example, Bates et al., (2006) reported that elevation of FOXP3 expression were identified in patients with ductal carcinoma in situ at increased risk of relapse (26).

In summary, the results of present study reflect the potential utility of FOXP3 as noninvasive marker for detecting breast cancer even in the earliest cancer stages, also, they suggest that possibility of using this gene as an efficient molecular signature for detecting breast cancer disease progression, discrimination between different stages and grades of breast tumors, and it might be of value as a prognostic marker.

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أهمية ألجين FOXP3 في ألكشف عن و ألتنبؤ بتقدم سرطان ألثدي لدى ألمريضات ألعراقيات

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الخلاصه:

أجريت ألدراسة ألحالية للكشف عن مستويات ألتعبير للجين (FOXP3) وذلك لتقييم دوره في ألكشف عن وألتنبؤ بتطور سرطان ألثدي لدى ألنساء ألعراقيات عن طريق مقارنة مستويات تعبيره بين مجموعة من ألنساء ألمصابات بسرطان ألثدي ومجموعة من ألنساء ألمصابات بورم ألثدي ألحميد (مجموعة سيطرة)، وكذلك عن طريق مقارنة مستويات ألتعبير لهذا ألجين بين ألنساء ألمصابات بسرطان ألثدي أنفسهن بالاعتماد على ألاختلاف في بعض ألمظاهر ألسريرية (ألمجاميع ألعمرية، درجة ألمرض، مرحلة ألمرض، وجود أو عدم وجود ألسرطان من ألنوع ألمنتشر). إشتملت ألدراسة على عينات نسيجية تم إستحصالها من 51 مريضة مصابة بسرطان ألثدي (لديهن مراحل مختلفة من مرض سرطان ألثدي) بعد تشخيصها من قبل بعض ألمستشفيات ألعراقية، إضافة إلى عينات نسيجية أخرى ماخوذة من 33 إمرأة مصابة بورم ألثدي ألحميد. تم معاملة ألعينات لغرض إستخلاص ألحمض ألنووي ألريبي ألكلي، ثم أجريت ألدراسة ألجزيئية باستخدام تقنية (Quantitative Real Time - PCR) في وحدة علم ألأورام ألجزيئي في مستشفى GUYS / ألكلية ألملكية – لندن/ بريطانيا. أظهرت نتائج ألتعبير للجين (FOXP3) بأن هناك 45 مريضة (88.23%) من ألمريضات ألمصابات بسرطان ألثدي كانت نتائجهن إيجابية لهذا ألجين، كما وأكدت ألنتائج بأن هناك إرتفاعا كبيرا في مستويات تعبيره لدى مريضات سرطان ألثدي بالمقارنة مع مجموعة ألسيطرة. وقد وجد أن هناك زيادة ملحوظة في مستويات ألتعبير للجين (FOXP3) مع تطور حالة (درجة و مرحلة) ألمرض في مريضات سرطان ألثدي غير ألمنتشر. من ألناحية ألاخرى، أثبتت ألنتائج بأن هناك إرتفاعا كبيرا في مستويات ألتعبير لهذا ألجين في مريضات سرطان ألثدي ألمنتشر مقارنة بالأخريات ألمصابات بالنوع غير ألمنتشر. إضافة إلى ذلك فقد أظهرت ألدراسة ألحالية عدم وجود فروقات إحصائية في مستويات ألتعبير لهذا ألجين مع ألتقدم في ألسن.