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The Diagnostic and Prognostic Values of NCR1 and NCR3 Genes in Iraqi Breast Cancer Women

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Abstract:

Background: The Natural cytotoxicity receptors (NCR1 and NCR3) have been classically defined as activating receptors delivering potent signals to Natural Killer (NK) cells in order to lyse harmful cells and to produce inflammatory cytokines. Indeed, the elicitation of NK cells effector functions after engagement of NCRs with their ligands on tumor cells without the need for prior antigen recognition is one of the main mechanisms that allow a rapid clearance of tumor cells.

Objectives: Evaluation of the diagnostic and/or prognostic values of NCR1 and NCR3 genes in breast cancer Iraqi females patients by initially comparing the expression concentrations of these genes between breast cancer patients and control group, then, comparing the expression levels of these genes 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 NCR1 and NCR3 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 expression of NCR1 and NCR3 genes were detected in 43 (84.31%) and 41 (80.39%) of breast cancer patients respectively, also, the levels of these genes showed high significant increase in breast cancer patients compared to control group. Furthermore, there were a gradual increase in the NCR1 and NCR3 expression concentrations with disease grades and stages progression (significant for both genes) in patients with primary breast cancer, moreover, the metastatic breast cancer patients showed significant decrease in NCR1 and NCR3 levels compared to primary breast cancer patients. There were no significant differences in the levels of NCR1 and NCR3 genes among the age groups of patients.

Conclusions: The present study results reflect the potential utility of NCR1 and NCR3 as noninvasive markers for detecting breast cancer even in the earliest cancer stages, also, they suggest the possibility of using these genes as an efficient molecular signatures for detecting breast cancer disease progression, discrimination between different stages and grades of breast tumors, and its might be of value as a prognostic markers.

keywords: *NCR1 Gene, NCR3 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). Natural Cytotoxicity Receptors (NCRs), are proteins have the ability to induce NK cells to kill the tumor-transformed cells (5). Their density on the NK cells surface varies with individuals, and it has been found that there is a direct correlation between NCRs expression and NK capacity to kill tumor cells (6). The aims of the present study were to evaluate the diagnostic and/or prognostic values of NCR1 and NCR3

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genes in breast cancer Iraqi females patients by initially comparing the expression concentrations of these genes between breast cancer patients and control group, then, comparing the gene expression levels of NCR1 and NCR3 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:

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 age 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 and control samples was extracted by using Qiagen RNA extraction kit, according to the manufacturer's instructions. Then, RNA was reversely transcribed by 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 NCR1 and NCR3 genes were 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 NCR1 and NCR3 genes from the cDNA were measured by quantitative real-time PCR using the relative quantification method (2-ΔΔCt method) (7). The fold-change in genes expression were normalized to a PGK1 gene and relative to a control group samples.

Table 1. Primers sequences

Primer	Sequence	Tm/°C
NCR1-F 5′	TGGATCTGGTGGTAACAGAAA	**45.3**°C
NCR1-R 5′	TCTCTCCCGAGATCACTTCG	**62.0**°C
NCR3-F 5′	GGGCTGGATTCTATGCTGTC	**62.0**°C
NCR3-R 5′	CCTTTCCAGGTCAGACATTTG	**47.3**°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 NCRs genes expression was very high; out of 51 patients, 43 patients (84.31%) and 41 patients (80.39%) were positive for NCR1 and NCR3 genes respectively (Figure 1), moreover, the expression levels of these genes were increased sharply (highly significant) in breast cancer patients compared to control group (for NCR1 the p value =0.0048, $p < 0.01$; and for NCR3 the p value =0.0035, $p < 0.01$) (Figure 2). The NCRs concentration were steadily increased with disease grades and stages progression in patients with primary breast cancer (statistically significant for both genes, $p < 0.05$) (Figures 3), furthermore, there were a sharp decline (significant) in these genes concentrations in metastatic breast cancer patients compared to primary breast cancer patients, (for NCR1 the p value =0.0281, $p < 0.05$; and for NCR3 the p value =0.0352, $p < 0.05$) (Figures 4). In correlation with age groups of patients, the results revealed that there were no significant differences (ns) in the levels of NCRs genes expression with the ages of patients.

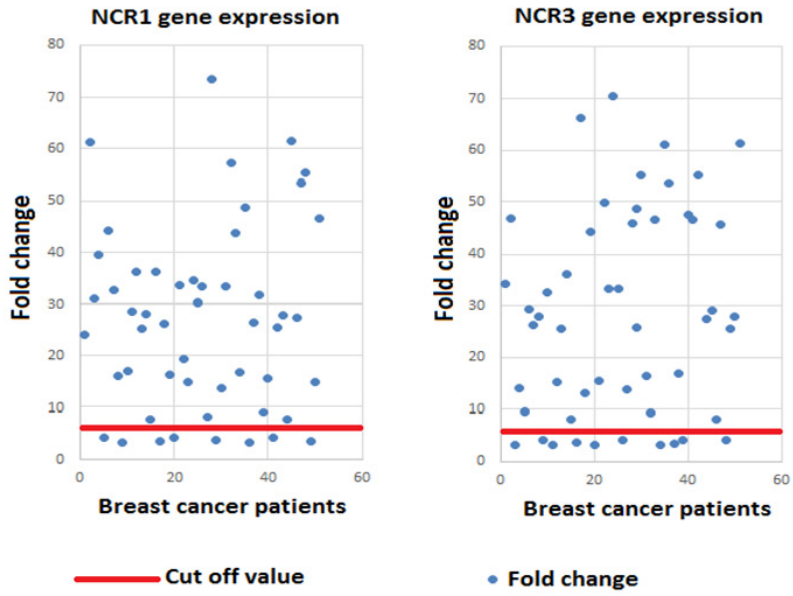

Figure 1. Breast cancer patients that gave positive NCR1 and NCR3 genes expression.

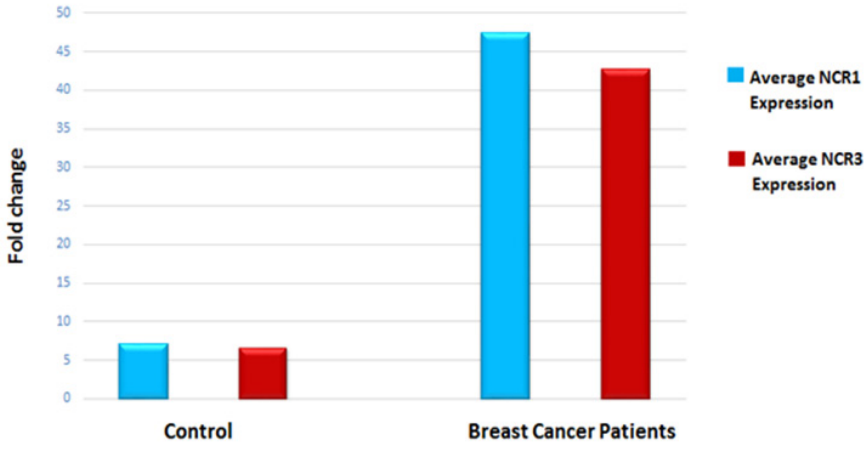

Figure 2. Comparison in the NCR1 and NCR3 genes expression between breast cancer patients and control group.

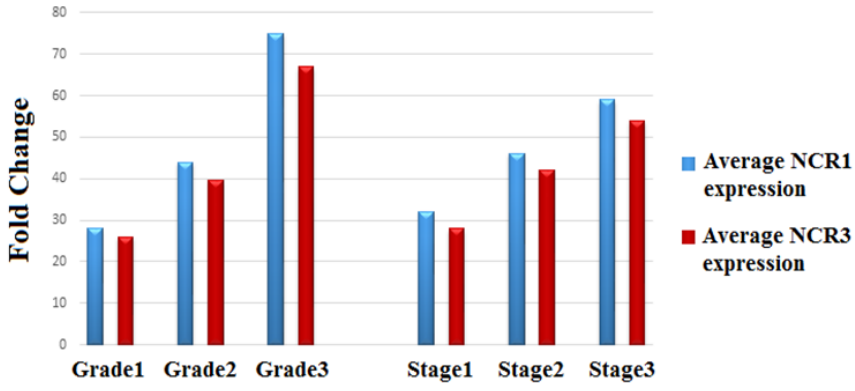

Figure 3. The effect of disease grades and stages progression on NCR1 and NCR3 genes expression in primary breast cancer patients.

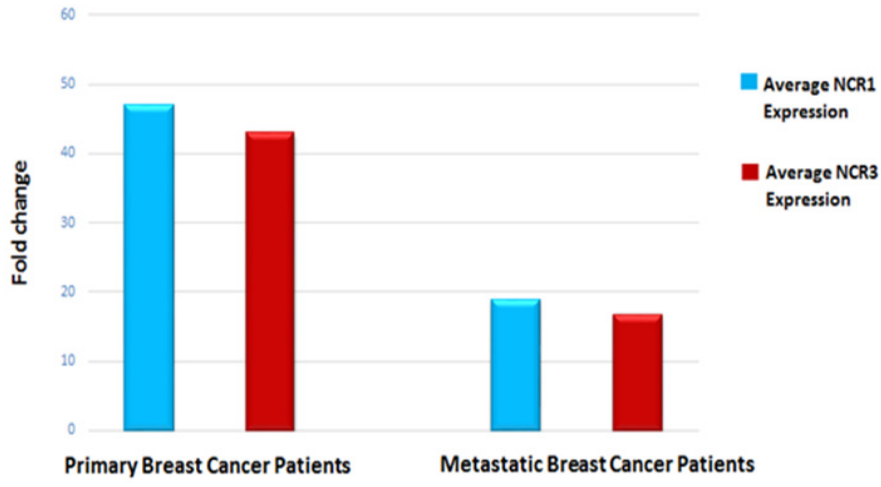

Figure 4. Comparison in the NCR1 and NCR3 genes expression between primary and metastatic breast cancer patients.

Discussion:

Historically, the name of natural killer (NK) cells came from their natural ability to kill tumor cells in vitro; moreover, accumulating data highlighted the importance of NK cells in host immune response against cancer and in therapy-induced antitumor response (8), Natural cytotoxicity receptors (NCR1 and NCR3) consider the main activator of NK cell function.

The present study revealed that most of the breast cancer patients were NCRs positive (84.31% and 80.39% of breast cancer patients were positive for NCR1 and NCR3 genes respectively), also, the expression levels of these genes aggressively increased in breast cancer patients compared to control group. Indeed, NK cell triggering is the result of a complex balance between inhibitory and activating signals and require not only a deficient MHC-I expression on target cells but also the expression of inducible ligands of activating NK cell receptors (9). Consequently, these cells have the ability to recognize and destroy a wide range of abnormal cells (including tumor cells) without damaging the healthy and normal "self" cells (10). The most important activating receptors involved in tumor lysis are NCR1 and NCR3, and its activation leads to the development of cytolytic activity and cytokine production (11). This could explain why there was an aggressive elevation in NCRs concentrations in breast cancer patients included in this study, which might be to induce a strong activation of NK cells against invading tumor cells in those patients.

The results of present data revealed that NCR1 and NCR3 expression were variable among breast cancer patients, and when the expression values analyzed among patients according to disease grades or stages, the result revealed that the NCRs concentration were steadily increased with disease grades (grade I to III) and stages (stage I to III) progression in patients with primary breast cancer. This might be as a result of further stimulation of the immune system which induced by further growth of the tumor which led to progressive up regulation of the NCR1 and NCR3 genes to stimulate aggressive NK cell activity in an attempt to eradicate tumor and prevent its further growth and progression. Indeed, it soon became clear that NCRs are able to arm NK cells with the ability to respond robustly and with high efficiency against tumor-transformed cells (12). While the killing of certain tumor cells by NK cells sometimes involves more than one NK cell receptor, the lysis of other tumor cells can be entirely mediated by either NCR1 or NCR3; thus they highlight¬ the unique ability of each one of these two NCRs to individually trigger NK cell cytotoxicity (13). Furthermore, the expression of NCR3 is correlated with NCR1; they cooperate with each other to induce NK cell cytotoxicity against tumor cells (14). Also, there are other factors that play a crucial role in determining NK cell-mediated lysis of tumor cells, such as the pres¬ence of NCR ligands on the surface of tumor cells (15) as well as the surface levels of NCRs on NK cells surface (16).

When the results of the current data are evaluated according to the presence or absence of metastasis, the results revealed that there were a sharp decline in the NCR1 and NCR3 concentrations in metastatic breast cancer patients (stage IV) compared to primary breast cancer patients. Although NK cells can kill target cells spontaneously without prior stimulation, a delicate balance between inhibitory and activating signals tightly regulates their activation (17). In the context of cancer, this balance is often deregulated through various mechanisms (18). One of these mechanism, cancer cells are able to induce a down-regulation of activating receptors (notably NCRs), which may contribute to metastatic potential of tumor by ensuring there is less or no NK cell activity (19). Indeed, this was noted in the present study as there was an observable downregulation in NCR1 and NCR3 genes in metastatic breast cancer patients. However, a number of cytokines,

growth factors, and enzymes may synthesized by tumor have reported to exert suppressive effects on NK cell activity. For example, transforming TGF-b, interleukin-10 (IL-10) and the activity of the tryptophan metabolizing enzyme indoleamine 2,3-dioxygenase (IDO) may contribute to the establishment of immune tolerance within the tumor microenvironment. (20). Regarding the age groups of patients in the present study, the results revealed that there were no significant differences (ns) in the levels of NCR1 and NCR3 genes expression among the age groups of patients.

In summary, the results of present study reflect the potential utility of NCR1 and NCR3 as noninvasive markers for detecting breast cancer even in the earliest cancer stages, also, they suggest the possibility of using these genes as an efficient molecular signatures for detecting breast cancer disease progression, discrimination between different stages and grades of breast tumors, and its might be of value as a prognostic markers.

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أهمية ألجينين (NCR1 و NCR3) في ألكشف عن و ألتنبؤ بتقدم سرطان ألثدي لدى ألمريضات ألعراقيات

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الخلاصه:

أجريت ألدراسة ألحالية للكشف عن مستويات ألتعبير للجينين (NCR1 و NCR3) وذلك لتقييم دورهما في ألكشف عن وألتنبؤ بتطور سرطان ألثدي لدى ألنساء ألعراقيات عن طريق مقارنة مستويات تعبيرهما بين مجموعة من ألنساء ألمصابات بسرطان ألثدي ومجموعة من ألنساء ألمصابات بورم ألثدي ألحميد (مجموعة سيطرة)، وكذلك عن طريق مقارنة مستويات ألتعبير لهذين ألجينين 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). اشتملت 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 ألاورام ألجزيئي في مستشفى GUYS / ألكلية ألملكية – لندن/ بريطانيا. أظهرت ألنتائج بأن 43 (84.31%) من ألمريضات كانت نتائجهن إيجابية لجين (NCR1) و 41 (80.39%) من ألمريضات كانت نتائجهم إيجابية لجين (NCR3)، كما وأكدت نتائج ألتعبير ألجيني لهذين ألجينين أن هناك زيادة كبيرة في مستويات تعبيرهما لدى مريضات سرطان ألثدي بالمقارنة مع مجموعة ألسيطرة، وقد وجد أن هناك زيادة ملحوظة في مستويات ألتعبير للجينين (NCR1 و NCR3) مع تطور حالة (درجة و مرحلة) ألمرض في مريضات سرطان ألثدي غير ألمنتشر. من ألناحية ألاخرى، أثبتت ألنتائج بأن هناك إنخفاضا كبيرا في مستويات ألتعبير لهذين ألجينين في مريضات سرطان ألثدي ألمنتشر مقارنة بالاخريات ألمصابات بالنوع غير ألمنتشر. إضافة إلى ذلك فقد أظهرت ألدراسة ألحالية عدم وجود فروقات إحصائية في مستويات ألتعبير للجينين ألمذكورين مع ألتقدم في ألسن.