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Original Research

Detection of gene expression in sentinel lymph node of primary breast cancer patients

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Abstract: Sentinel lymph node (SLN) micrometstasis detection improves outcome for breast cancer follow up procedure. The aim of the present study was to identify gene profiles that accurately predicted the outcome of breast cancer patients. Fifty tumor sample from breast cancer patients were analyzed for the expression of 3 genes using quantitative-PCR. Also clinical verification for recurrence to distant organs was performed. Three gene signature were confirmed based on tumor's stage, grade, ER status, using conditional logistic regression. Based on this findings, the negative reported lymph nodes for metastasis, had micro metastasis in significant values. There was a significant difference between normal and cancer samples in 3 gene expression marker and also there was meaningful relationship between three gene expression with tumor's grade, stage according to progression of tumor. A novel gene expression signature predictive of micro metastatic patients was evaluated. In this assessment, relationship between this gene with tumor's features that finding clear role for these genes with tumor's outcome, needs to be established.

Key words: Breast cancer; Gene signature; Sentinel lymph node; Iran.

Introduction

Cancer is called as a group of diseases involving abnormal cell growth with the potential to attack or spread to other parts of the body (1-6). Breast cancer is a heterogeneous disease with a highly variable clinical course, presenting a great challenge to prognosis and therapeutic decisions (7,8). Positive receptor status is not sufficient to ensure a therapeutic response because additional molecular alterations have effect on metastasis outcome (9). Axillary lymph node (ALN) metastasis is one of the most important prognostic factors in breast cancer (10). Sentinel lymph node (SLN) biopsy was shown to accurately predict the involvement of the remaining axilla it quickly became the standard clinical practice (11). Sentinel lymph node biopsy is commonly used to clinical staging of patients with breast cancer.

Mammaglobin (MGB1) is the gene of the uteroglobin gene family that is overexpressed in breast tissue and metastatic lymph nodes from patients with breast cancer (12). Genetic markers such as MUC1, CK19, and carcinoembryonic antigen (CEA) have been shown to increase the number of positive lymph nodes compared with immunohistochemical staining in human breast cancer cell lines and metastatic lymph nodes with an absence in noncancerous tissues (13).

A homeo domain–containing protein (HOXB13), interleukin 17 receptor B (IL17BR) and choline dehydrogenase (CHDH), GenBank accession number AI240933, are significantly associated with clinical outcome of breast cancer. It has been revealed that a two-gene expression index (HOXB13:IL17BR) might be a novel biomarker for predicting treatment outcome in tamoxifen monotherapy (14). Thus, this study aimed to investigate identifying gene profiles that accurately predicted the outcome of breast cancer patients in Iranian patients.

Materials and Methods

Tumor Samples and Patient Clinical Data

The samples in this study was obtained from a biobank of Pathology Department of Imam Khomeini Referral and Teaching Hospital, Tehran, Iran. Tumor samples were archived in the form of frozen fresh tissue in liquid nitrogen. Fifty sentinel lymph node samples and 50 normal breast tissue from the same patient were originally stored until the time of analysis. At the time of RNA extraction, patients were diagnosed with stage I or II in primary breast cancer with no distant metastasis, treated with mastectomy or lumpectomy plus axillary dissection, with or without postoperative radiation therapy and with or without adjuvant tamoxifen monotherapy. Fifty tumor specimens, and 50 normal breast tissue from the same patient were selected on the basis of having more than 10% tumor content for RNA extraction.

Table 1. Primer PAIRS/AMPLICONS Analyzed By Real-Time RT PCR.

Gene	Accession No.	Sequence of selected primer pairs	length of amplicon	Primer Tm (℃)
HOXB13	DQ158059	F: TGG AGA ACC GCG ACA TGA CT R: GAC GAA AGG CGC AGG CGT C	178bp	F: 62 R: 64
IL17BR	NG_028042	F:GCATTAACTAACGATTGGAAACTACATT R: GGAAGATGCTTTATTGTTGCATTATC	121bp	F:74 R:70
MGB (Mammaglobin)	AF015224	F: CGGATGAAACTCTGAGCAATGT R: CTGCAGTTCTGTGAGCCAAAG	108bp	F:64 R:64

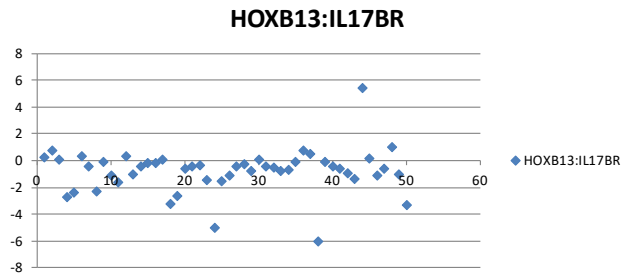

Figure 1. Joint distribution of the DDCt values of HOXB13 and IL17BR. The diagonal line corresponds to the rule determined by conditional logistic regression. Pairs to the top of the line are correctly classified with respect to their outcome (recurrence/non-recurrence) whereas pairs under the line are classified incorrectly.

Her2 receptor status of samples had been determined by Immunohistochemistry (IHC) technique. Study and patients were assigned on the basis of national/international breast cancer protocols and approved according to local law and regulations, by the Institutional Review Boards of each participating referral hospital.

The study was performed at Shahrekord Medical Academy, in adherence to the guidelines of the Declaration of Helsinki, and was approved by the ethics committee of Shahrekord Islamic Azad University.

Laboratory procedures

Total cellular RNA was isolated from breast cancer sentinel lymph node, normal lymph nodes, using a guanidium thiocynate-phenol-chloroform solution (Qiagen RNeasy Plus Mini Kit Qiagen, Germantown, MD). Briefly, a single lymph node specimen was removed from liquid nitrogen tank (- 70°C) storage and weighed as quickly as possible without allowing the tissue to thaw. Tissue (0.15 gm) was then homogenized in 1.5 mL tube with 0.5 mL Tripure (Bio-Rad, Hercules, CA). Total RNA was isolated as per the manufacturer's instructions. Final RNA pellets were dissolved in 50 mL of DEPC-treated water. RNA yield was determined by spectroscopy. Complementary DNA was made from 5mg of total RNA using M-MLV reverse transcriptase (Viva 2 - steps RT-PCR Kit (Mastercycler gradient, Eppendrof, Germany) with M-MuLV RT/Taq DNA Polymerase, 100 app. Cat no: RTPL12) and oligo d(T) 12 – 16. Real-time RT-PCR was performed on a (Rotor gene 6000 Corbett). Standard reaction volume was 10ml and contained 1X SYBR RT-PCR buffer, 3 mM MgC l2, 0.2 mM each of dATP, dCTP, dGTP, 0.4 mM dUTP, 0.005 U Ampli Taq Gold, 0.002 U Amp Erase UNG erase enzyme, 0.35 ml cDNA template and 50 – 900 nM of oligo- nucleotide primer. Initial steps of RT PCR were 1.5 min at 56°C for UNG erase activation, followed by a 10 min hold at 90°C. Cycles (n = 40) consisted of a 20 second melting at 90°C, followed by a 30 second annealing/extension at 65°C. The final step was a 60°C incubation for 1 min. All reactions were performed in triplicate. Threshold for cycle of threshold (Ct) analysis of all samples was set at 0.5 relative fluorescence units. Primer design Primers were designed according to previously designed primer sets and confirmed using Primer3 NCBI software. All primers were use from previous study (Table 1). To normalize relative levels of expression, b2-micro-Globin was used as an internal reference control. Typically, genes such as b2-microglobin, b - actin, GADPH or the 18S rRNA Gene have been used as reference genes. The18S rRNA gene was discarded from consideration since it lacks a poly A tail. Poly A tails are required for the oligo d(T)-primed cDNA synthesis step. Used in the RT PCR described in this article. b2 - microglobinis ubiquitously expressed and is considered to be a reliable reference standard and was used as such for this analysis.

Prognostic vs. Predictive Value of HOXB13/IL17B and mamaglobin gene signature

In this study, included patients were all > 40 years of age at diagnosis. This finding is supported by univariate and multivariate Cox regression analysis (both P < 0.0001), with a RR of 9.09 (CI: 3.1-26.5) and 64.5 (CI: 10.6-390.7), respectively, in the micrometastatic patients. The 3-gene signature did not reach significance in either univariate or multivariate analysis of the non-micrometastatic patients.

Analysis of the HOXB13:IL17BR ratio as predictive score

It has been shown that the ratio of HOXB13: IL-17BR can be use to predict outcome in early breast cancer patients previously , in this study the predictive value of this ratio adding the mamaglobin in obtained data set using the same approach as previously reported is evaluted. As found by Ma et al., (2004) (15), HOXB13 showed higer expression in micrometastatic patients sample and IL17BR had higher expression in tumor samples from the patients without micrometastasis (Figure 1). The HOXB13: IL17BR ratio correctly calssified 64%, and the Wilcoxon signed rank sum test, applied to the ratio values, and the P - value of 0.02 has obtained. The performed study by Harrell, J. C was mainly based on early stage cancers with few tumor-infiltrated lymph nodes without micrometastasis, while in current study, the population type was mainly based on having neither tumor-infiltrated lymph nodes at the time of diagnosis (average was 2, and only 5/50 pairs had tumor-infiltrated lymph nodes). Indeed, the predictive value of the ratio was higher in 21 pairs with a maximum of 3 affected lymph nodes (71% correctly classified, P = 0.03) compared to 21 pairs with >3 affected lymph nodes (57% P = 0.29).

Results

RT PCR analysis of 50 node negative breast cancer patients, and 50 adjacent normal tissue performed for three genes, HOXB 13, IL 17BR and mammaglobin, that these genes expression levels, are predictive for clinical outcome (16).

In comparison between tumor and normal samples for IL 17BR gene, using t-student-paired sample analytical test, there was significant difference (P = 0.000) , and the IL17BR showed meaningful decresed expression in sentinel lymph node samples

Same results obtained for mamaglobin gene expression in tumor samples (P = 0.000). In evaluation of tumor features with gene markers status, according to ΔΔCt values, these results obtained: IL17BR gene was not expressed in 9.3% (n:4) of stage 1 and 90.7% (n:39) of stage 2 and in 100% (n:7) in stage 1, 0% (n:0) in stage 2, had expression in tumor samples, in analysis of results with pearson chi-square regression test, there was meaningful difference between stage 1 and 2 for expression of IL17BR (P = 0.000), such that, in higher stage of disease, the IL17BR expression showed reduction. In assessment of tumor's grade, there was reverse relationship (P = 0.000), and was not correlation in ER status and IL17BR expression level (P = 0.100). As for HOXB 13, using ΔΔCt values, there was direct accordance (P = 0.000), that is, with highe stages, the HOXB 13 expression was invreased (Table 2).

Also in tumor's grade evaluation, there was significant difference (P = 0.000), such that, the higher grade, the higher expression of HOXB13 level. The same results obtained for ER status and in ER positive, node negative sentinel lymph nodes, the HOXB 13 expression level showes significant diffferncе compare to ER negative samples (P = 0.000).

In mammaglobin gene expression status, the very meaningful difference has seen according the tumor's stage (P = 0.000), tumor's grade (P = 0.000) but ER not correlate with positive samples (P = 0.100).

We next investigated correlation of the HOXB 13/ IL17BR index with standard prognostic factors in ER + patients. In assessment of HOXB 13/IL17BR index with ER status, 65.9% (n: 27) were ER negative and 34.1 (n: 14) were ER positive, had index value belowe 0.06 and 22.2% (n: 2) were ER negative, and 77.8% (n:7) were ER positive, had index value above 0.06, that significant difference, was seen between HOXB 13:IL 17BR and ER status (P = 0.021) (Figure 2).

Discussion

In previous studies, has been shown that, HOXB13, IL17BR and mamaglobin genes, specially HOXB 13:IL 17BR index, can predict the distant metastasis in breast cancer patients that underwent chemotherapy (17). Howerver, in recent study, we showed that these genes can predict relapse in node-negative patients. But the direct comparison of node-negative and node-positive with and without chemotherapy was not possible because of limited samples and type of study (retrospective), that the follow-up the patients was not possible. In this RT PCR assay study, the 0.06 cut-point resulted in meaningful difference in node negative and node positive or normal breast tissue regarding to metastatic prognosis, but some reasearchers failed to showing a predictive value of the HOXB 13:IL 17BR index in a mostly node negative cohort (n = 58) (18).

The mechanism of this index for prognosis is unknown, but it seems the higher values of this index is present in node-positive patients with metastasis (19). Regarding to published studies, HOXB 13:IL 17BR, and some other genes are suitable merkers for prognosis of breast cancer metastasis (20,21). As in this study, we demonstrated that HOXB 13 gene is increased according to higher tumor stage and grade, the prognostic value for HOXB 13 is supported by other studies indicating that its overexpression is related to tumor growth and invasion (22-24). Also we showed that IL 17BR as defense mediator of immune response is decreased in higher stages and grades of tumor, as in ER status that mRNA of receptor is strongly related to clinical outcome and HOXB 13 expression (P = 0.000). In this study we hypothesized that triple gene marker HoxB 13, IL 17BR and mamaglobin maybe the more accurate metastatic indicator in sentinel lymph node during the intraoperative assessment in breast cancer patients (25-27).

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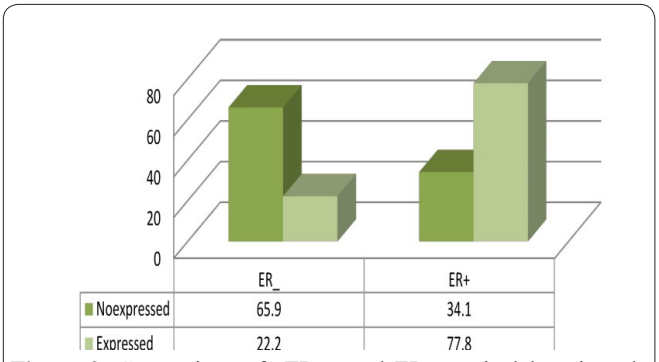

Figure 2. Comparison of ER + and ER- sentinel lymph node breast cancer samples and HOXB13:IL17BR index (Cutpoint value: 0.06).

Table 2. Assessment of HOXB13 expression in different stages of sentinel lymph node sample in breast cancer patients.Chi-Square Tests.

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	20.261[a]	1	0.000		
Continuity Correction[b]	16.983	1	0.000		
Likelihood Ratio	18.993	1	0.000		
Fisher's Exact Test				0.000	0.000
Linear-by-Linear Association	19.856	1	0.000		
No: of Valid Case [b]	50				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 3.08. b. Computed only for a 2×2 table.

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Conflict of interest

All the authors declare no conflict of interest included in the study.

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