ORIGINAL ARTICLE

Received: August 2016
Accepted: September 2016

Type I EMT Inducer Eomesodermin Has a Significant Expression in Breast Cancer

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ABSTRACT

Background: Epithelial-Mesenchymal Transition (EMT) is a fundamental stage in cancer metastasis, and the presence of vascular invasion is a strong indication of the spread of the malignant tumor cells. Regulation of the EMT is a complex process in which several different transcription factors are involved. Eomesodermin (Eomes) as a member of the T-box gene family is an important component in the induction of the germ-line layer during gastrulation. However, its role in cancer is not well understood. Thereby, in this project, we aimed to assess the Eomes gene expression in tumor tissues and to find out its possible relevance to vascular invasion.

Methods: Seventy-one breast cancer tumors were obtained from the Tumor Bank of Cancer Institute, Imam Khomeini Hospital. Quantitative real-time PCR (qRT-PCR) was conducted to evaluate Eomes gene expression at RNA level.

Results: Results have shown that Eomes has been remarkably expressed in the majority of tumor samples. High levels of Eomes expression were concomitant with the presence of vascular invasion in 72% of samples. However, no significant association was found. To the best of our knowledge, this is the first time that Eomes expression is reported in breast cancer tumors.

Conclusion: This important finding suggests that Eomes has the potential to be used as a biomarker in breast cancer.

Keywords: Breast Cancer, Epithelial-Mesenchymal Transition, Gene Expression, Neoplasm Metastasis, Transcription Factors

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BCCR
2016; 8(3): 17-22
www.bccrjournal.com

Introduction

Metastasis is the major cause of cancer death in the world. During cancer metastasis, cells at the primary site detach from their neighboring cells, invade the surrounding tissues, intravasate into the blood vessels/lymph nodes, migrate throughout the body, dwell in distant organs, survive, and finally proliferate at the secondary site[1-4]. The initiation of this well-organized process requires alterations in cells' phenotype in which epithelial cells lose their apicobasal polarity and cell-cell junctions and gain mesenchymal characteristics such as invasiveness and motility. This procedure is commonly known as Epithelial-Mesenchymal Transition (EMT)[5]. There are 3 classes of EMT and each happens by a completely distinct biological process[1, 2, 6]; Type I of EMT commonly occurs during gastrulation and embryonic development. Type II generally happens during inflammation and tissue fibrosis, and Type III takes place during cancer progression and metastasis in neoplastic cells[1, 2, 6-10]. Studies have shown that EMT has a critical role in the development of malignancy in cancers of epithelial origin, including breast cancer[10, 11].

Several transcription factors including T-box genes family have been shown to have regulatory roles on EMT process during cancer metastasis. Eomesodermin (Eomes) is a transcription factor of T-box gene family which has a critical role in the inducing and patterning of the germ-line layer during gastrulation[12-18]. Recent studies have shown that Eomes has a critical regulatory role in the differentiation and activation of CD8 T lymphocytes in colorectal cancer and is needed for antitumor activity of memory T-cells[19-22]. Eomes is highly expressed during gastrulation and embryonic development, and its expression decreases after this stage. Therefore, it is thought that a high expression of this transcription factor might be a sign of cancer.

Vascular invasion-entrance of tumor cells into blood channels is a phenomenon commonly used as an indicator of metastatic spread of tumor cells. Vascular invasion is strongly used as a prognostic factor in malignancies such as breast cancer. Studies have shown that vascular involvement within the primary tumor is associated with EMT process and differential gene expression[23]. Therefore, in this project we aimed to find out whether there is any difference in Eomes expression level in breast cancer tumor tissues compared to normal tissues, and also investigate whether there is any association between vascular invasion and the expression of Eomes in our tumor samples.

Methods

Sample collection

Seventy-one tumor tissues of breast cancer patients were randomly selected and obtained from the Tumor Bank of Cancer Institute, Imam Khomeini Hospital, Tehran, Iran. Four normal breast tissues were also included in this study. The presence or absence of vascular invasion for each patient was collected from pathology reports. All the patients included in this study gave their informed consent. This study was approved by the Ethical Committee of Tehran University of Medical Sciences.

RNA extraction and cDNA synthesis

RNAs were extracted from normal and tumor tissue samples using Easy Blue RNA Extraction kit (INtron). The quality of the extracted RNAs was checked by running the samples on 1.5% agarose gel electrophoresis, and their quantity was evaluated using NanoDrop spectrophotometer (ND-2000). One µg of RNA was used for cDNA synthesis using Prime Script tmRT Reagent kit (TAKARA-Japan) according to manufacturer's instructions. Afterwards, Polymerase Chain Reaction (PCR) was per-

formed to evaluate the accuracy of the synthesized cDNAs using GAPDH as a housekeeping gene.

Measurement of EOMES expression level by Quantitative Real-Time PCR

The level of expression of Eomes relative to GAPDH was measured by performing quantitative Real-Time PCR using SYBR®Premix ex Taq^{tm}II (Tli RNaseH plus) (TAKARA-Japan). Each reaction mixture contained 2 µL of cDNA, 10 µL of SYBR mixtures, and 1 µL of forward and reverse primers. Each reaction reached the final volume of 20 µL by adding 7 µL of H_2O. Eomes and GAPDH primer sequences were obtained from Primer3web 4.0 and their sequences were as follows: Eomes F: 5'-AAACGGACTCAATCCCACTG-3', Eomes R: 5'-TTCCCGAATGAAATCTCCTG-3', GAPDH F: 5′-GAAGGTGAAGGTCGGAGTCA-3′ and GAPDH R: 5′-AATGAAGGGGTCATTGATGG-3′. The resulting data were analyzed using $2^{-\Delta\Delta ct}$.

Statistical analysis

Student t-test was run to assess the possible association between Eomes expression level and the presence of vascular invasion in tumor tissues of breast cancer patients. $p<0.05$ was considered significant.

Results

Analyzing Eomes expression at the RNA level, we found interestingly high levels of expression for this transcription factor in the majority of samples (**Figure 1**). The presence or absence of vascular invasion was based on pathology reports and was available for 67 tumors out of 71 samples. Vascular invasion was observed in 72% (48 samples) of patients. All of the aforementioned samples had remarkably high levels of expression of Eomes. No sign of vascular invasion was observed in the remaining 19 samples (28%) (**Figure 2**), and no significant association was found between Eomes expression and the presence of vascular invasion.

Discussion

T-box genes encode transcription factors that belong to a large family containing more than 20 members in humans. These transcription factors are all evolutionarily conserved. Homologues of many of these transcription factors have been identified in other organisms such as mice and zebrafish. These transcription factors have critical roles in different developmental processes, and several disorders have been identified and linked to T-box gene family, showing their medical significance[24].

The T-box transcription factor Eomes has a pivotal role in EMT process in mesoderm formation during gastrulation and embryonic development. However, its role in EMT process during cancer and metastasis is not well understood.

In the present study, we checked Eomes expression at the RNA level for 71 tumor samples of breast cancer and found interestingly high levels of Eomes expression in almost all of the breast tumor samples; a fact that, to the best of our knowledge, had not been reported elsewhere before. Previously, Arnold and his co-workers established an experiment in mice embryo in which they performed a conditional Eomes inactivation. Their results showed that cells in Eomes-deficient mice failed to down-regulate E-cadherin, and EMT was blocked[25-28]. In addition to what has been previously reported in regards with the roles of Eomes during type I EMT process, our findings indicate that Eomes may have an important role in cancer as well; however, further investigation is needed to find out its specific roles during metastasis.

Numerous studies have also shown that TBX2, a well-known T-box gene family member, has elevated levels of expression in malignancies including breast, ovarian, and liver cancers[29]. The overexpression of another T-box transcription factor known as Tbx3 has also been reported in specific breast cancer cell lines. Brachyury, as a known member controls

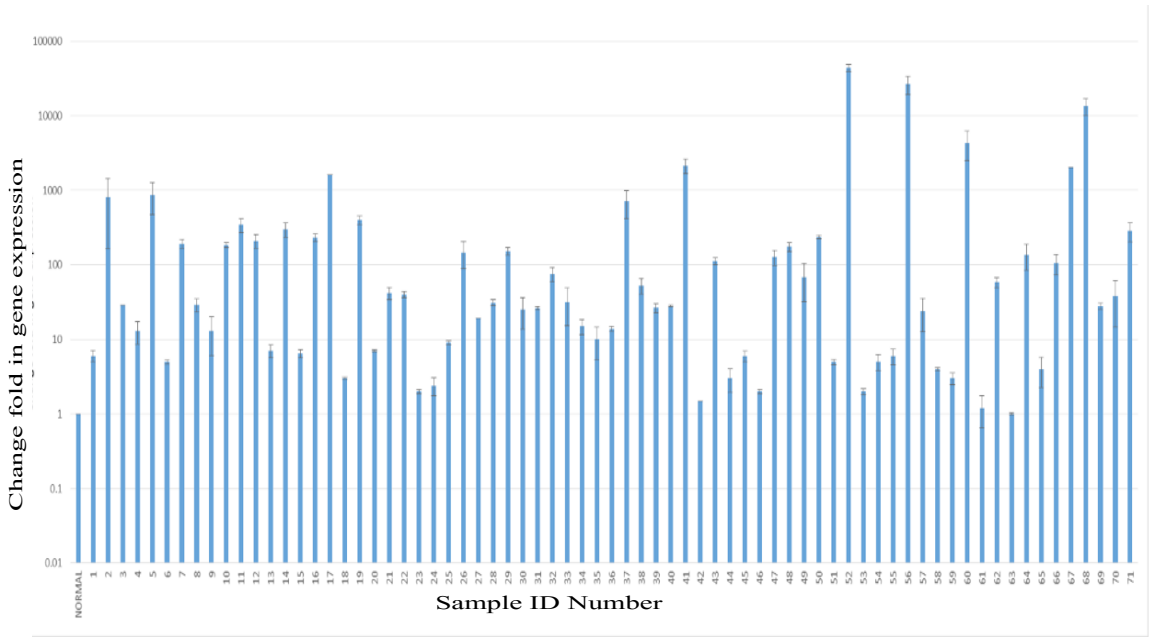

Figure 1. Ecomes gene expression at RNA level in 71 breast cancer tumor tissues

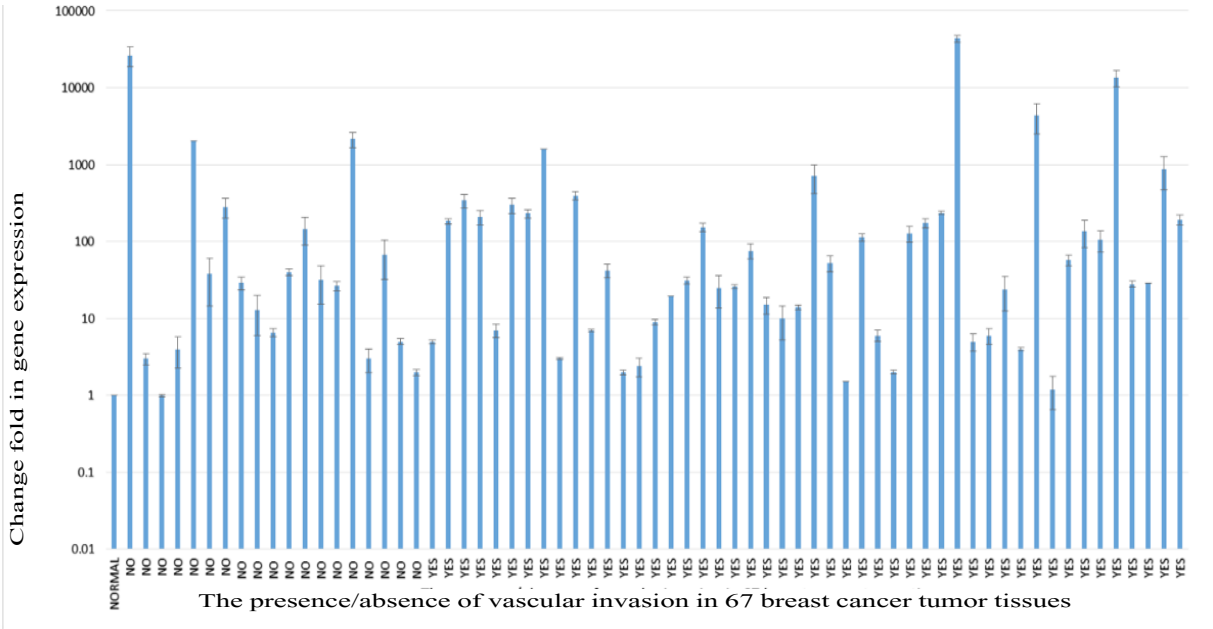

Figure 2. Comparison of Ecomes expression with the presence/absence of vascular invasion in 67 breast cancer tumor tissues

of the T-box family, has been reported to have elevated levels of expression in different cancers such as ovaries and small intestine[29, 30]. Elevated levels of expression of T-bet, another T-box family member, has also been reported in hormone-dependent cancers[29].

In the present study, the concomitant incident of vascular invasion and high levels of Eomes expression was observed in 72% of samples. This result suggests that Eomes may contribute to the dissemination of tumor cells and metastasis. A recent study by Yu et al. had also reported a significant association between Tbx2 and vascular invasion, suggesting that this transcription factor may contribute to the dissemination of tumor cells and metastasis[31]. However, no data is currently available on Eomes, and further investigation with larger sample sizes in addition to complementary tests is needed to confirm its role in cancer metastasis.

In the present study, we found high levels of Eomes expression in tumor tissues, reported for the first time in breast cancer. T-box gene family has recently been the focus of many studies representing them as interesting EMT biomarkers in cancer and, in the next step, as therapeutical targets. Therefore, targeting these transcription factors, their signaling pathways or their co-factors is worth pursuing.

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