Concurrent Down-regulation of the *EAF1* and the *EAF2* Genes in the Invasive Ductal Carcinoma Type of Breast Cancer

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Abstract

In mammals, the EAF1 and EAF2 genes build an active transcription module with other components. These genes code for factors acting as potent inhibitor of the Wnt-β catenin pathway, which might be due to their function as tumor suppressor gene. Recently, the involvement of both above-mentioned factors was described in some human tumors, but not yet in breast cancer. Breast cancer is one of the most cancer cases in Iran after colon and stomach carcinoma. We aimed therefore to investigate for the first time a possible correlation between breast cancer and the EAF1 and the EAF2 gene expression.

We collected invasive ductal carcinoma tumor grading (grade 1 to 3) with marginal normal tissue from forty women diagnosed with breast cancer with the average age of 50 years old. All patients underwent triple marker test (ER/PR and Her2/neu), indeed the most of them were triple positive with few triple negative individuals. After RNA extraction, cDNA was synthesized for subsequent real-time polymerase chain reaction. The humanRPL27 gene wasusedas endogenous control.

Analysis of real-time PCR results showeda significant down-regulation of EAF1 (p-value: 0.028), and of EAF2 (p-value: 0.0134) in tumor tissue samples in comparison to normal one.There was no correlation between clinical parameters and the target genes.

We could find significant connection between both tumor suppressor genes in triple positive and triple negative breast cancer patients, which deserves more attention.

Key-words: Breast Cancer, Invasive Ductal Carcinoma, Elongation Factor, *EAF1*, *EAF2*.

1. Introduction

The Elongation Factor for RNA Polymerase II (ELL) is a protein coding gene identified firstly in myeloid leukemia as a fusion partner gene in the 11;19 chromosome translocation (1). The

ELL also plays an early role before its assembly into the Elongation factor component of the super elongation complex (SEC) by stabilizing RNA polymerase II recruitment/initiation and entry into the pause site (2). In addition to ELL, researchers have identified several other factors as regulator of mRNA synthesis during the elongation phase (3). The function of a group of these factors including Elongin, TFIIF, and ELL, is to facilitate the processivity of transcription by suppressing transient pausing by Pol II (4).

Investigation by yeast two hybrid screen using ELL as the bait resulted in discovery of two ELL associated partners named as EAF1 & EAF2, respectively (5).

The *EAF1* (ELL Associated Factor 1) is a protein coding gene that is associated with Eosinophilic angiocentric fibrosis (EAF) disease, also known as the IgG4-related systemic disease of unknown etiology (6).

EAF2 as the paralog gene to the *EAF1* gene (ELL Associated Factor 2) is associated with prostate cancer (7). Both human *EAF1* and *EAF2* genes are strongly homologue to the vertebrates with evolutionary conserved domains (8). Recently, Jing-Xia et al. postulated a novel role for the EAF1 and the EAF2 factors as potent repressor of β-catenin in the Wnt signaling pathway. They also suggested the two EAF1 and EAF2 factors as tumor suppressor genes (8) that in this aspect might have distinct impact on the cell surveillance and/or apoptosis. As we know, the dysregulation of the Wnt signaling pathway, with accumulation of β-catenin in the cytosol as the consequence, is the main highlight in the pathogenicity of colon cancer in human. In this context, the dysfunction of upstream regulation factors including the EAF1 and the EAF2 would dramatically change the balance between oncogenes and tumor suppressor genes in the cell.

Based on the novel suggested function of the EAF1 and the EAF2, and because of unknown role of these factors in breast cancer (BC), their expression profile would provide useful information for better prognosis.

In the present study we aimed for the first time to analyze the *EAF1* and the *EAF2* gene expression in the invasive ductal carcinoma (IDC), as the most common aggressive type of BC in Iranian women (9,10). After colon and stomach cancer, BC is the most common type of cancers with higher mortality rate in Iran (11,12).

2. Methods

Sample collection: we selected forty patients with BC diagnosis for this study after informed consent. All samples have been collected from the Saadie's specialized Oncology hospital in Shiraz

city locating in south Iran. Their age ranged between 38-60 years old with negative history of BC in family members. All patients underwent triple test for ER-PR-HER2 (hormone receptor for Estrogen, Progesterone, and Her2/neu). Fresh tumor and marginal normal tissue biopsies obtained from each patient that split in two parts: one part covered immediately with RNA-later (GeneAll ® Company, South Korea) and the other part used for pathologic diagnosis of breast carcinoma and grading of tumor. Ethical research committee of Medical university of Shiraz has approved the project under the resisted number 15230503971998.

After taking biopsy, immunohistochemically-staining assay performed as routine procedures for ER/PR/HER2 testing in the patient's specimens. The positive HER2 samples were definite as plus three, and other scoring (plus one and plus two) marked as negative.

RNA Extraction and cDNA Synthesis

RNA was extracted using Trizol ® (Invitrogen Company, Germany). Subsequently, DNase treatment and cDNA synthesis were done by Ampliqon ® (Denmark) as described by Company instructions.

Quantitative Expression Analysis of *EAF1* and *EAF2* Genes

We used here the *RPL27* housekeeper gene as internal normalizer for SYBR-Green based real-time-PCR. We designed three Primer pairs by primer3® for *EAF1*, *EAF2*, and *RPL27*, respectively. Primer sequences and corresponding product size are shown in the table 1. All q-PCR reactions were triplicated for more accuracy.

Statistical analysis: Graph Pad Prism8®was used to analyze Mann–Whitney *t*- test and Anova one way, and correlation coefficient of target genes.
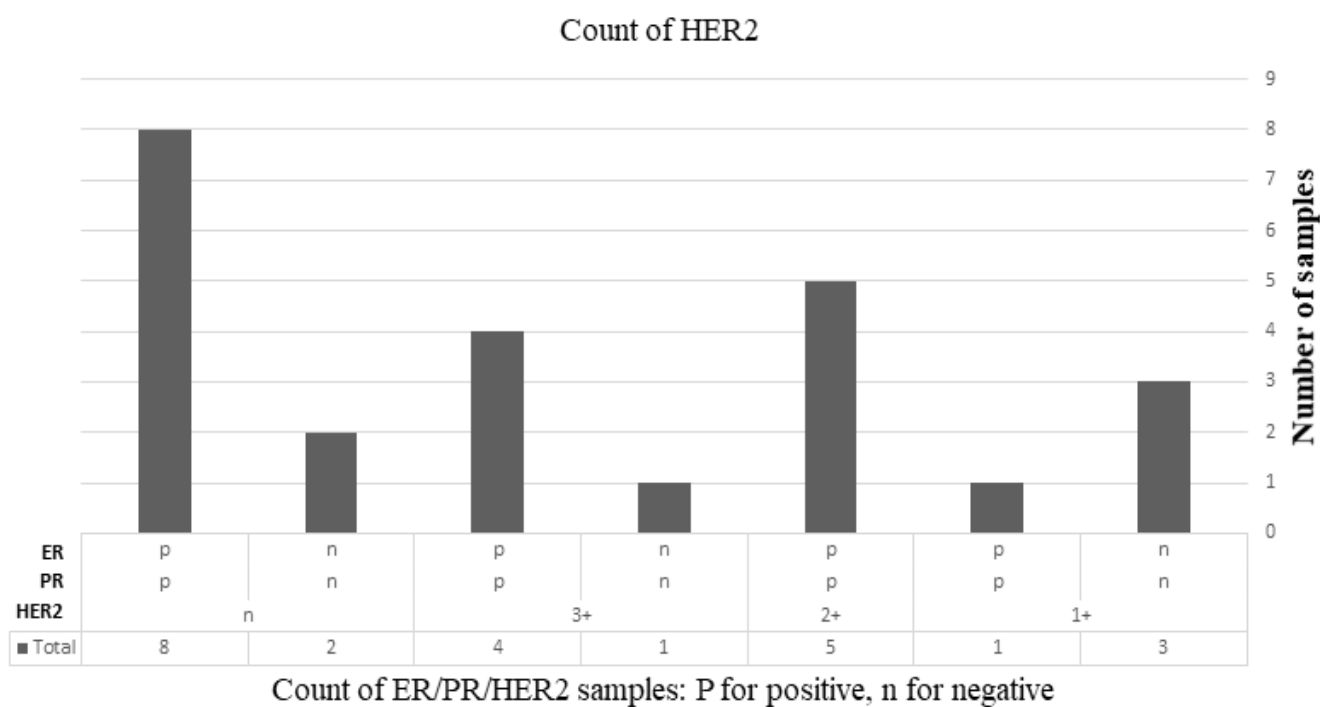
Table 1- We used Primer3® Software for Designing of Primers. The Size of each Product Ranged 105-160 base Pairs

Primer	Sequence	Accession number	Size
EAF1-F	5-CAAGTTGGCAAAGGAGATGAAG-3	NM_033083.7	105bp
EAF1-R	5-GTCTTTCTGGTAAGGCCGTTTG-3		
EAF2-F	5-GAAGGCAGAAGCTAGTCTAATGG-3	NM_018456.6	160bp
EAF2-R	5-ATGTCCTGAGACACAATTCCC-3		
RPL27-F	5-ATCGCCAAGAGATCAAAGATAA-3	NM_000988.5	123bp
RPL27-R	5-TCTGAAGACATCCTTATTGACG-3		

3. Results

We had to exclude 16 from 40 samples because of inappropriate RNA concentration, or failure of clinical data of patients. From these samples, six patients were negative for ER and PR, and 14 samples positive for HER2/neu. In Total, 10 samples were positive for triple marker. Four samples under 40 years old were positive for HER2/neu, but negative for PR/ER. The mentioned results were summarized in the figure 2.

Figure 1- Comparison of Triple Markers to each other (p: Positive; n: Negative)

Count of HER2

ER	p	n	p	n	p	p	n
PR	p	n	p	n	p	p	n
HER2		n		3+	2+		1+
■ Total	8	2	4	1	5	1	3

Count of ER/PR/HER2 samples: P for positive, n for negative

The fold change calculation ($2^{-\Delta\Delta Ct}$) revealed that the expression of both the *EAF1* and the *EAF2* genes in the tumor tissue compared to the normal tissue is significantly decreased (*p*-value for the *EAF1* gene: 0.028, and for the *EAF2* gene: 0.0134), respectively (figure 3).

Statically, there is no significant correlation between the *EAF1* and the *EAF2* genes in tumor tissue, regardless of demographic and clinical parameters (Tables 2 and 3).

Figure 2- Statically Analysis has shown significant down regulation of the *EAF1* and the *EAF2* genes (*p*-value for the *EAF1* gene: 0.028, and for the *EAF2* gene: 0.0134) in tumor tissue compared to normal tissue of BC patients.
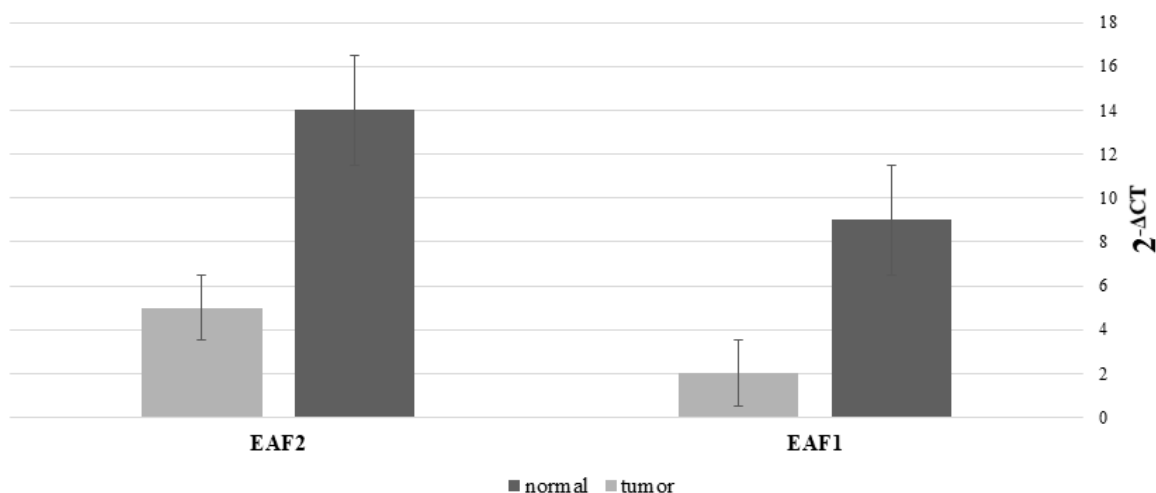

Table 2- Correlation of the *EAF1* Gene Expression and Clinic-pathological Characteristics

	Patient (T) vs. ER	Patient (T) vs. PR	Patient (T) vs. HER2	Patient (T) vs. Grade	Patient (T) vs. age
Spearman r	0/1447	0/1447	-0/1641	-0/02323	-0/1503
95% confidence interval	-0/3441 to 0/5718	-0/3441 to 0/5718	-0/5850 to 0/3265	-0/4836 to 0/4472	-0/5756 to 0/3391
P value					
P (two-tailed)	0/5545	0/5545	0/5020	0/9248	0/5392
P value summary	ns	ns	ns	ns	ns
Exact or approximate P value?	Approximate	Approximate	Approximate	Approximate	Approximate
Significant? (alpha = 0.05)	No	No	No	No	No
Number of XY Pairs	19	19	19	19	19

Table 3- Correlation of the *EAF2* gene expression and clinic-pathological characteristics

	patient(T) vs. ER	patient(T) vs. PR	patient(T) vs. HER2	patient(T) vs. Grade	patient(T) vs. age
Spearman r					
r	-0/1315	-0/1315	0/02332	0/1767	-0/1128
95% confidence interval	-0/5739 to 0/3703	-0/5739 to 0/3703	-0/4603 to 0/4963	-0/3297 to 0/6041	-0/5610 to 0/3865
P value (two-tailed)	0/6030	0/6030	0/9268	0/4831	0/6558
P value summary	ns	ns	ns	ns	ns
Exact or approximate P value?	Approximate	Approximate	Approximate	Approximate	Approximate
Significant? (alpha = 0.05)	No	No	No	No	No
Number of XY Pairs	18	18	18	18	18

4. Discussion

Knowingly, about eighty percent of BC patients are positive for ER/PR, and other twenty percent are positive for HER2/neu oncogene, which makes the tumor more aggressive (13). Usually, the age of onset in HER2/neu positive patients is lower (14). Frequently, HER2/neu negative younger women with BC have pathogenic mutations in the *BRCA1* gene. Here, we calculated the average age of ten BC patients negative for HER1/neu as 40.6 years old. It is to mention that the average age of onset for BC in Iran is about 46 to 49 years (15), which is reflecting in our total samples, but the average age of our patients negative for HER2/neu oncogene is much lower as general BC population in Iran. However, we recommend the *BRCA1* gene mutation screening for at least these ten cases. On the other hand, the mutation frequency in the *BRCA1* gene in BC affected women in Iran is much lower as reported for general BC patients, worldwide (16).

In the present study, we assessed for the first time the relative expression changes of the two tumor suppressor genes in Iranian women with the ductal type of breast cancer. Data showed that regardless of the age, triple marker condition, and tumor grading, the expression of the *EAF1* and the *EAF2* is significantly decreased. It could be possible that the significant down-regulation of both tumor suppressor genes promote tumorigenesis, at least in Iranian BC patients.

Recently, researchers have reported the significant down-regulation of both mentioned genes in prostate cancer in the mice model (17). They suggested that the cooperative working of the EAF1 and the EAF2 factors might be important for prostate epithelial hemostasis, and the failure of them would lead to malignancy in prostate tissue (17,18).

First evidence that the EAF1 and EAF2 factors might be a potent inhibitor of the canonical Wnt/β-catenin signaling reported by Liu and colleagues (18). They hypothesized that this ability might be a consequent of the TSG activity of both factors. Furthermore, the EAF family have conserved biological activities across diverse species (19). Previously, Nandini et al. suggested that the dysregulation of the Wnt-signaling pathway would be associated with metastasis in Triple negative BC (20), in despite of our study contain only few patients with triple negative BC. In addition, none of our patients was in metastasis stage. In this context, we can assume a higher risk of metastasis for such individuals, who eventually need more intensive care and therapy.

Recent report presumed a link between the Wnt/β-catenin signaling and the TNBC tumorigenesis by regulating the key tumor-associated characteristics, including migration, stemness, proliferation, and chemo resistance in TNBC cells and Cancer stem cells (CSCs) (21). Although, most of the participants in this study were triple positive, according to the gene expression results, it

can be assumed that the Wnt/β-catenin signaling might also be involved of yet unknown reasons in triple positive BC (TPBC) patients.

Our quantitative PCR data of these factors showed for the first time their significant downregulation in concurrent TNBC and TPBC, at least in Iranian women. Unfortunately, we could not access to a larger sample size to strengthen our data. Because of the importance of the possible novel involvement of the two TSG factors EAF1 and EAF2 in the breast tumorigenesis, and to increase the accuracy, we recommend to replicate the study with more BC samples.

Conflict of Interest

Acknowledgments

Author Contributions

- **Conceptualization:** Hamid Galehdari
- **Funding acquisition:** Hamid Galehdari, Sogand Heydaran
- **Supervision:** Hamid Galehdari, Sirus Naeimi
- **Writing - original draft:** Sogand Heydaran
- **Writing - review & editing:** Sogand Heydaran, Hamid Galehdari

References

Biswas, D., Milne, T.A., Basrur, V., Kim, J., Elenitoba-Johnson, K.S., Allis, C.D., & Roeder, R.G. (2011). Function of leukemogenic mixed lineage leukemia 1 (MLL) fusion proteins through distinct partner protein complexes. *Proceedings of the National Academy of Sciences, 108*(38), 15751-15756.

Simone, F., Luo, R.T., Polak, P.E., Kaberlein, J.J., & Thirman, M.J. (2003). ELL-associated factor 2 (EAF2), a functional homolog of EAF1 with alternative ELL binding properties. *Blood, 101*(6), 2355-2362.

Li, M., Wu, X., Zhuang, F., Jiang, S., Jiang, M., & Liu, Y.H. (2003). Expression of murine ELL- associated factor 2 (Eaf2) is developmentally regulated. *Developmental dynamics: an official publication of the American Association of Anatomists*, 228(2), 273-280.

Mathews, A., Holland, L., & Yankulov, K. (2009). The interaction between EAP30 and ELL is modulated by MCM2. *FEBS letters*, *583*(21), 3431-3436.

Kong, S.E., Banks, C.A., Shilatifard, A., Conaway, J.W., & Conaway, R. C. (2005). ELL-associated factors 1 and 2 are positive regulators of RNA polymerase II elongation factor ELL. *Proceedings of the National Academy of Sciences*, *102*(29), 10094-10098.

Ma, X., & Liu, J. X. (2013). Eafs control erythroid cell fate by regulating c-myb expression through Wnt signaling. *PloS one*, *8*(5), e64576.

Ai, J., Pascal, L.E., Wei, L., Zang, Y., Zhou, Y., Yu, X., & Wang, Z. (2017). EAF2 regulates DNA repair through Ku70/Ku80 in the prostate. *Oncogene*, *36*(15), 2054-2065.

Liu, J.X., Zhang, D., Xie, X., Ouyang, G., Liu, X., Sun, Y., & Xiao, W. (2013). Eaf1 and Eaf2 negatively regulate canonical Wnt/β-catenin signaling. *Development*, *140*(5), 1067-1078.

Wan, X., Ji, W., Mei, X., Zhou, J., Liu, J.X., Fang, C., & Xiao, W. (2010). Negative feedback regulation of Wnt4 signaling by EAF1 and EAF2/U19. *PloS one*, *5*(2), e9118.

Zangouri, V., Akrami, M., Tahmasebi, S., Talei, A., & Hesarooeih, A.G. (2018). Medullary breast carcinoma and invasive ductal carcinoma: a review study. *Iranian journal of medical sciences*, *43*(4), 365.

Jafarian, A.H., Kooshkiforooshani, M., Rasoliostadi, A., & Roshan, N.M. (2019). Vascular mimicry expression in invasive ductal carcinoma; a new technique for prospect of aggressiveness. *Iranian journal of pathology*, *14*(3), 232.

Iranshahi, N., Zafari, P., Yari, K., & Alizadeh, E. (2016). The most common genes involved in epigenetics modifications among Iranian patients with breast cancer: A systematic review. *Cellular and Molecular Biology*, *62*(12), 116-122.

Azami, A., Madani, S. H., Khazaei, S., Izadi, B., Saleh, E., & Sajadimajd, S. (2020). Survey of low ER-positive expression and its correlation with other clinical and pathological factors in breast cancer. *Indian Journal of Pathology and Microbiology*, *63*(5), 129.

Anwar, S.L., Dwianingsih, E.K., Avanti, W.S., Choridah, L., & Aryandono, T. (2020). Aggressive behavior of Her-2 positive colloid breast carcinoma: a case report in a metastatic breast cancer. *Annals of Medicine and Surgery*, *52*, 48-52.

Forat-Yazdi, M., Neamatzadeh, H., Sheikhha, M. H., Zare-Shehneh, M., & Fattahi, M. (2015). BRCA1 and BRCA2 common mutations in Iranian breast cancer patients: A meta analysis. *Asian Pacific Journal of Cancer Prevention*, *16*(3), 1219-1224.

Korolczuk, A., Amarowicz, M., Bąk, K., Korobowicz, E., & Koncewicz, T. (2016). Adenomyoepithelioma of the breast with late pulmonary metastases–case report and review of the literature. *Journal of cardiothoracic surgery*, *11*(1), 1-6.

Neamatzadeh, H., Shiryazdi, S.M., & Kalantar, S.M. (2015). BRCA1 and BRCA2 mutations in Iranian breast cancer patients: A systematic review. *Journal of research in medical sciences: the official journal of Isfahan University of Medical Sciences*, *20*(3), 284.

Pascal, L. E., Su, F., Wang, D., Ai, J., Song, Q., Wang, Y., & Wang, Z. (2019). Conditional deletion of Eaf1 induces murine prostatic intraepithelial neoplasia in mice. *Neoplasia*, *21*(8), 752-764.

Liu, R., Chen, C., Li, Y., Huang, Q., & Xue, Y. (2020). ELL-associated factors EAF1/2 negatively regulate HIV-1 transcription through inhibition of Super Elongation Complex formation. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, *1863*(5), 194508.

Dey, N., Barwick, B.G., Moreno, C.S., Ordanic-Kodani, M., Chen, Z., Oprea-Ilies, G., & Leyland-Jones, B.R. (2013). Wnt signaling in triple negative breast cancer is associated with metastasis. *BMC cancer*, *13*(1), 1-15.

Xu, J., Prosperi, J.R., Choudhury, N., Olopade, O.I., & Goss, K.H. (2015). β-Catenin is required for the tumorigenic behavior of triple-negative breast cancer cells. *PloS one*, *10*(2), e0117097.