RESEARCH ARTICLE

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E74-like Factor 5 Promoter Methylation in Circulating Tumor DNA as a Potential Prognostic Marker in Breast Cancer Patients

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Abstract

Background: Epigenetic alternations, such as DNA methylation, play a crucial role in breast tumor initiation and progression. The identification of noninvasive prognostic biomarkers has great importance in cancer management. Methylated cell-free DNA (cfDNA), circulating in the blood as a convenient tumor-associated DNA marker, can be used as a minimally invasive cancer biomarker. This study aimed to evaluate the promoter methylation status of E74-like factor 5 (*ELF5*) tumor suppressor gene in both tumors and plasma cell-free DNA of 80 breast cancer patients, compared with normal controls. **Methods:** Plasma cfDNA concentrations were measured using quantitative real-time PCR, and methylation pattern in the *ELF5* gene promoter region was performed using methylation-specific polymerase chain reaction (MS-PCR) technique. **Results:** The data revealed a statistically significant increase in cfDNA concentrations in breast cancer patients, particularly in those with higher stages of the disease, triple-negative status, and metastasis ($p<0.001$). *ELF5* promoter region hypermethylation was observed in 70% of breast cancer patients in both plasma cfDNA and tumor tissues. Notably, all patients with lymph node involvement and distant metastatic exhibited promoter hypermethylation in the *ELF5* gene. **Conclusion:** Our findings suggest that *ELF5* promoter methylation in circulating DNA could serve as a potential non-invasive prognostic molecular marker in breast cancer patients. However, further studies are warranted to evaluate its diagnostic value.

Keywords: Breast neoplasms- Cell-free nucleic acids- DNA methylation- Prognosis

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Introduction

Attempts have been made to develop noninvasive strategies for early cancer detection and the identification of diagnostic and prognostic biomarkers based on the analysis of extracellular nucleic acids in biological fluids. The liquid biopsy has emerged as a potential alternative to tissue biopsy, DNA can be release from both normal and apoptotic cells, including cancer cells, into the circulating blood system. The use of circulating DNA as a non-invasive method to obtain a personalized genomic snapshot of a patients' tumor holds tremendous potential. However increased plasma circulating DNA concentration alone cannot be considered as a specific indicator of tumor development, given that comparable increases in DNA concentration have also been observed in patients with other disorders such as rheumatoid arthritis, systemic lupus erythematosus, pancreatitis, glomerulonephritis, and hepatitis (Han et al., 2017). Therefore, the identification of informative biomarkers in circulating blood expressing tumors is of great importance in cancer management. Epigenetic alternations, such as gene promoter methylation, have emerged as novel cancer biomarkers with prognostic, diagnostic, or predictive value in different stages of the variety of cancers (Chen et al., 2017). Tumor-specific alterations, such as aberrant promoter methylation in circulating DNA recovered from plasma or serum of patients, have been reported in the variety of malignancies. Changes in the status of DNA methylation represent one of the frequent molecular alterations in human neoplasia (Cho et al., 2010), including breast cancer (Rauscher et al. 2015). These epigenetic alterations may contribute to the neoplastic process by transcriptionally silencing tumor suppressor genes or activating oncogenes, potentially playing a role in the initiation of tumor cell proliferation (Cho et al., 2010). Therefore, the analysis of gene methylation patterns holds profound significance for early detection of cancer. Over the past few years, research in this field has gained substantial momentum. A number of studies in metastatic breast cancer have shown the potential of circulating DNA to predict prognosis and treatment response. Exosomal miRNA and hypermethylated DNA in plasma have shown promise in terms of early breast cancer detection

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specificity and may serve as treatment response indicators (Beddowes et al., 2017).The complex network of genetic and epigenetic factors plays a vital role in regulating the development and hemostasis of breast tissue. Dysfunctions in different components of this network can initiate tumor progression. One such component is the transcription factor known as E74-like factor 5, also referred to as ESE-2, which belongs to the E twenty-six (Ets)-domain transcription factor family. Ets family proteins are involved in a wide spectrum of biological processes. They contribute not only to physiological development and differentiation, but also possess oncogenic or tumor suppressive activities play a role in regulating metastasis and the epithelial-mesenchymal transition (EMT) process. *ELF5* has been found to have an inhibitory effect on the *SNAIL2* gene, an important gene in the EMT process, thereby activating the reversed pathway of EMT called mesenchymal-epithelial transition (MET) (Wu et al., 2015). Several Ets factors have been associated with cancer initiation, progression, and metastasis. Similarly, studies have shown that loss of *ELF5* expression is frequently observed in human breast cancer tissues and cell lines, suggesting a potential tumor suppressive role for this transcription factor (Piggin et al., 2016).

However, due to the complexity and multifunctional nature of *ELF5*, questions remain regarding its precise role in metastasis. It is believed that the cooperation between genetic and epigenetic factors plays a prominent role in tumorigenesis and the acquisition of various features such as metastasis and drug resistance. The most prevalent type of cancer among Iranian women is invasive ductal carcinoma breast cancer (Jazayeri et al., 2015). Therefore, in this study, we sought to recognize whether there are significant differences in the promoter methylation patterns of the *ELF5* gene in breast cancer tumors compared to normal breast tissue. Additionally, we sought to determine whether these differences can be detectable in plasma samples from patients, providing a noninvasive approach to cancer biomarker detection. Therefore, we assessed the concentration of circulating cell-free DNA (cfDNA), as well as the promoter region methylation of the *ELF5* gene in cfDNA derived from plasma and breast tumors, among breast cancer patients with different stages and clinicopathological characteristics. Our goal was to identify potential prognostic biomarkers in breast cancer that are readily accessible and can be easily detected.

Materials and Methods

Sample Collection

The test samples were divided into two categories: breast tissues and plasma. For the breast tissues, tumor and normal adjacent breast tissues were collected from 80 patients with operable breast cancer before the initiation of any therapy. Additionally, 20 normal breast tissues were obtained from individuals who underwent surgery due to cosmetic purposes, primarily breast reduction. To serve as blood control group samples, peripheral blood (10 mL) was collected from 75 unaffected female blood donors who did not have any breast lesion either in themselves or among their first-degree relatives. The inclusion criteria for patient samples were a histopathological diagnosis of ductal carcinoma and availability of immunohistochemistry (IHC) results for human epidermal growth factor 2 (HER-2), estrogen receptors (ER), progesterone receptor (PR) status, and other relevant diagnostic information. Patients who had received chemotherapy or radiotherapy before recruitment and had a history of familial breast disease or malignancy were excluded from the study. The demographic and histoclinical data of the patients and controls are summarized in Table 1.

Approval for this study was obtained from the Ethical Committee of on the National Institute of Genetic Engineering and Biotechnology, in accordance with the principles outlined in the Declaration of Helsinki. All individuals provided informed consent to participate in the study. Tumor staging was performed according to the tumor, node, and metastasis (TNM) classification system. Blood samples were collected before surgery.

Plasma Cell-Free DNA Extraction

Peripheral blood (10 mL in Ethylenediaminetetraacetic acid (EDTA)) was collected by venipuncture ,discarding the first 2 mL of blood. The remaining freshly collected blood was processed within 1hour by centrifugation at 1000 g for 10 minutes at 4°C. The resulting supernatant was carefully transferred to a Falcon tube, taking care not to disturb the cellular layer, and subjected to a second centrifugation step for 10 minutes to ensure complete removal of any residual cells. The cell-free plasma was then divided into aliquots and stored at -80°C. DNA was performed using a 0.5 ml plasma aliquot and the QIAmp DNA Blood Midi Kit (Qiagen, Hiden, Germany) following the manufacturer's instructions. The extracted DNA was then stored at -20°C for further analysis.

DNA Extraction from Tissues

Tissue samples, including both tumor and normal tissues, were immediately snap-frozen and stored at -70°C until use. DNA extraction from the tissues was performed using the DNeasy Blood and Tissue Kit (Qiagen, Hiden, Germany) according to the manufacturer's protocol.

Plasma DNA Quantification by Real-Time PCR

Plasma DNA concentration was determined by quantitative real-time PCR, with the human *β-actin* gene used as a reference gene. The protocol described by Skrypkina et al., (2016) was followed, with a few modifications. The primer sequences uused were as follows: forward primer: 5' CCACACTGTGCCCATCTACG 3' and reverse primer: 5' AGGATCTTCATGAGGTAGTCAGTCAG 3', generating a 99 base pair amplicon. A standard curve was constructed sing serial 10-fold dilutions of genomic DNA from pooled peripheral blood lymphocytes of ten healthy donors, with the concentration determined by ultraviolet (UV) absorbance measurements using a NanoVue Plus Spectrophotometer (Buckinghamshire, UK). The dynamic range of the calibration curve was set between 0.01 and100 ng of DNA. The QPCR was performed using the ABI 7500/7500 fast real-time system (CA, USA). Each PCR reaction mixture consisted of 10 µl of Applied Biosystems™ SYBR™ Green PCR Master Mix (ABI,

CA, USA), 1.0 µl each primer (0.4 mM), 2 µl water, and 6 µl of extracted DNA. For blank samples, the DNA was replaced with an equal volume of water. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60 °C for 60 seconds, and extension at 72 °C for 60 seconds. Each data point was performed in duplicates, and melting curves were obtained from 56 to 92°C with reads every 0.2°C for each amplicon. The sample DNA concentration was extrapolated from the standard curve.

Methylation-Specific PCR (MS-PCR)

The isolated DNA underwent Bisulfite treatment using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The methylation status of the *ELF5* gene was determined qualitatively by the methylation-specific polymerase chain reaction (MS-PCR). The primer sequences used for MS-PCR analysis, along with the PCR product size and primer annealing temperature, are as follows: *ELF5* methylated-specific forward: 5'TAAAAATGTATTTGTAGGTTATGTGCG3' and reverse: 5'ATTCTTACTTATTACCCAAACCGTC3' (49.5°C, 258 base pair), and *ELF5* un-methylated forward: 5'TAAAAATGTATTTGTAGGTTATGTGTGT3' and reverse: 5'ATTCTTACTTATTACCCAAACCATC3' (49.5°C, 258 base pair). Four µL of bisulfite-modified DNA was used as a template for PCR amplification in a final reaction volume of 25 µL, including 12.5µl of 2x EpiTect MSP Kit (Qiagen, Hilden, Germany) and 0.5 µM of each primer.

PCR was performed with an initial 10-minute incubation at 95°C, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 49.5°C for 30 seconds, extension at 72°C for 60 seconds, and a final 10-minute hold at 72°C. Each sample was assessed in duplicate, and each run included a no template control (NTC) and the EpiTek PCR control DNA set (methylated and unmethylated DNA) as an external universal control (Qiagen, Hilden, Germany). The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

Statistical analysis

The data were analyzed using SPSS 16.0 (SPSS Inc. Chicago, USA). The Mann-Whitney U test and Kruskal-Wallis test were performed for numerical data, while the chi-square test was used to analyze the relationship between categorical parameters. Correlation and consistency were analyzed using Pearson correlation analysis. Numerical data are presented as mean ± standard deviation (SD). Statistical significance was considered at a p-value <0.05.

Results

The results of the present study are categorized into two main parts, as follow: Part I, Concentration Quantification of cfDNA and Part II- Determination of *ELF5* promoter methylation status in breast tissues and plasma.

Part I: cfDNA Concentration Quantification

The concentration of plasma cfDNA in Breast Cancer Patients Compared to Unaffected Donors

The concentration of plasma cell-free DNA (cfDNA) was assessed in 80 breast cancer patients compared to 75 unaffected donors (control group). Quantitative real-time PCR was performed to amplify the plasma *β-actin* gene in all samples. The efficiently of amplified plasma DNA were tested in all samples. The concentration values ranged from 0.6 to100 ng ml^{-1}. The Real-Time PCR assay performance demonstrated high linearity of product amplification, as indicated by the mean slope (-3.20) and mean correlation coefficient (R2=0.99) of all constructed

Table 1. Demographic and Histoclinical Characteristics of Patients and Normal Controls

	Patient N (%)	Control N (%)
Number	80	75
Age (years)		
Mean	47.2±12.6	48.5±16.4
Range	27-84	25-80
Stage at diagnosis		
Stage II	42 (52.5)	
Stage III	26 (32.5)	
Stage IV	12 (15)	
Lymph node status		
N0	34 (42.5)	
N+	46 (57.5)	
Distance metastasis		
Yes	12 [2 bone, 10 lung] (15)	
No	68 (85)	
Hormone receptor status (IHC)		
ER-positive	48 (60)	
ER-negative	32 (40)	
PR-positive	42 (52.5)	
PR-negative	38 (47.5)	
HER-2 status (IHC)		
+ + +	22 (27.5)	
Negative	50 (62.5)	
Triple-negative breast cancer	8 (10)	
Menopause status		
Yes	42 (52.5)	36 (48)
No	38(47.5)	39 (52)
Smoking		
Yes	20 (25)	24 (32)
No	60 (75)	51 (68)
Pregnancy at term		
Yes	66 (82.5)	60(80)
No	14 (17.5)	15 (20)
HRT		
Yes	18 (22.5)	15 (20)
No	62 (77.5)	60 (80)

HRT, hormone replacement therapy; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; N, number

Table 2. Cell-free DNA Concentration in Plasma Samples of Breast Cancer and Normal Control Groups

Sample	Mean (ng/ml)±SD	Range (ng/ml)	Median (ng/ml)
Normal control	11.6±17.2	(0.6-68)	3
Breast cancer	25.9±22.8	(5.1-100)	21.5

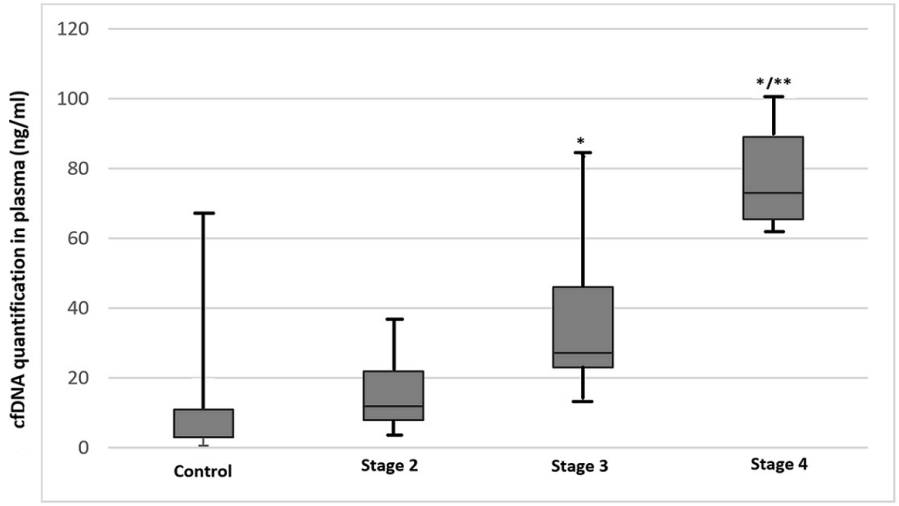

Figure 1. Plasma Cell-free DNA Quantification in Different Stages of Breast Cancer Compared with Normal Control. **, compared with other breast cancer stages, Kruskal-Wallis test, p<0.001. *, compared with normal control, Mann-Whitney U test, p<0.001

standard curves.

The specificity of real-time PCR amplification products was verified by melting curve analysis. All plasma DNA samples demonstrated a single peak corresponding to 84 ^{0}C in their melting curves, confirming the high specificity of the selected primers and the absence of nonspecific amplification products.

As shown in Table 2, quantitative real-time PCR revealed a statistically significant increase in plasma cfDNA concentration in breast cancer patients compared to the normal control group (p<0.001).

Plasma cfDNA Concentration Comparison in Patients with Different Stages of Breast Cancer

The breast cancer patient test group comprised of stages II, III and IV. As shown in Figure 1, the plasma cfDNA concentration was dramatically elevated in the highest stage, stage IV (p<0.001). The mean plasma cfDNA concentration was 77.25±14.2 ng/ml in stage IV, while it was 22.5±22.1, 15±10.2 and 11.25±16.4 ng/ml in stages III, II, and the normal control group, respectively.

Plasma cfDNA Concentration Comparison in Breast Cancer Patients Based on Tumor Hormone Receptors Status

The patients were divided into groups based on their tumor hormone receptor status, including the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2), as well as the triple negative (ER-, PR-, HER2-) or non-triple-negative groups. As shown in Figure 2, the plasma cfDNA concentration was significantly higher in the triple-negative group, with the mean of 72.6±19.1 ng/ml, compared to the other breast cancer groups (p<0.001).

Comparison of Plasma cfDNA Concentration in Breast Cancer Patients Based on Nodal Involvement

The patients were categorized into two groups based on lymph node involvement: LN+ (lymph node positive) and LN- (lymph node negative). As shown in Figure 3, the plasma cfDNA concentration was significantly elevated in LN+ group compared to the LN- (p<0.001). The mean cfDNA concentration in the LN- groups was 36.24±25.35 and 13.2±8 ng/ml, respectively.

Table 3. Categorization of *ELF5* Promoter Methylation Status

Sample type	Total number	Methylated ELF5 promoter (%)	Un-methylated ELF5 promoter (%)	Both methylated and un-methylated ELF5 promoter (%)	P value X2 test
BC/plasma	80	56 (70)	22 (27.5)	2 (2.5)	*
N/plasma	75	0 (0)	75 (100)	0 (0)	*
BC/tumor	80	56 (70)	20 (25)	4 (5)	*
Nadj/tissue	80	6 (7.5)	24 (30)	50 (62.5)	*
NC/ tissue	20	0 (0)	20 (100)	0 (0)	*

BC, Breast cancer; N, Normal; Nadj, Normal adjacent; NC, Normal control; *, X^2 test; p ≤ 0.0001

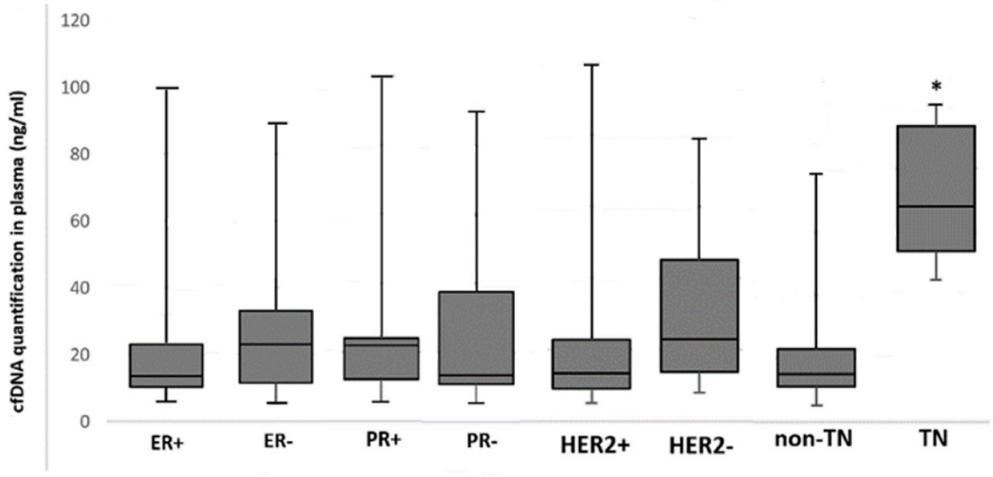

Figure 2. Characterization of Quantified Plasma Cell-free DNA based on Tumor Hormone Receptors. A) ER, estrogen receptor possession; B) PR, progesterone receptor possession; C) HER2, human epidermal growth factor receptor 2 possession; D) TN, triple negative (ER-, PR-, HER2-). *, compared with other breast cancer hormone receptor situations, Kruskal-Wallis test p<0.001.

Part II:
Plasma and Breast Tissues ELF5 Promoter Methylation Study

The *ELF5* promoter methylation status in the plasma and breast tissues of breast cancer patients was compared with that of normal control group, as summarized in Table 3. The data showed that 70% of breast tumors exhibited methylation in the *ELF5* gene promoter region. Interestingly the same methylation results were observed in the plasma samples of these patients. In other words, the methylation status detected in breast tumors was also traceable in the plasma. In the normal adjacent control group, most of the samples (approximately 62.5%) showed both methylated and un-methylated *ELF5* promoter regions. However in the normal control group, which comprised of healthy individuals with no cancer history and who underwent cosmetic surgery, all the samples (100%) showed an unmethylated *ELF5* promoter.

ELF5 Promoter Methylation Status in Breast Cancer Patients with Various Histopathology Situations

The frequency of methylated the *ELF5* promoter regions in different subtypes of breast cancer patients based on nodal involvement, hormone (estrogen and progesterone) receptors, and HER2 situations, as well as TNM staging is shown in Figure 4.

The data indicated that the stage IV and lymph node positive groups with 100% *ELF5* promoter methylation had significantly higher methylated promoter frequency compared to the other studied breast cancer subtypes (p<0.0001). There was no statically significant difference in *ELF5* promoter methylation frequency in other mentioned groups in Figure 4.

Discussion

The phenomenon of plasma cfDNA in cancer patients

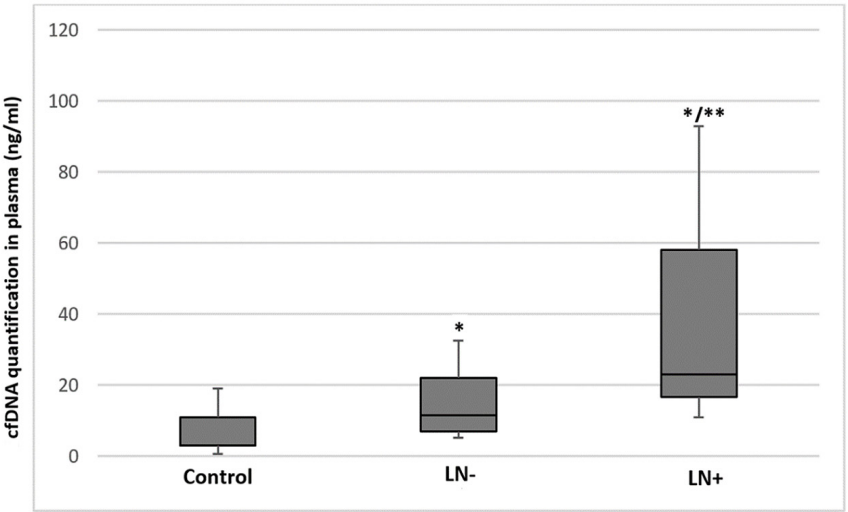

Figure 3. Plasma Cell-free DNA Quantification in Different Breast Cancer Patients Based on Nodal Involvement Situations Compared with Normal Control. LN, lymph node; **, compared with LN-, Mann-Whitney U test, p<0.001; *, compared with normal control, Mann-Whitney U test, p<0.001

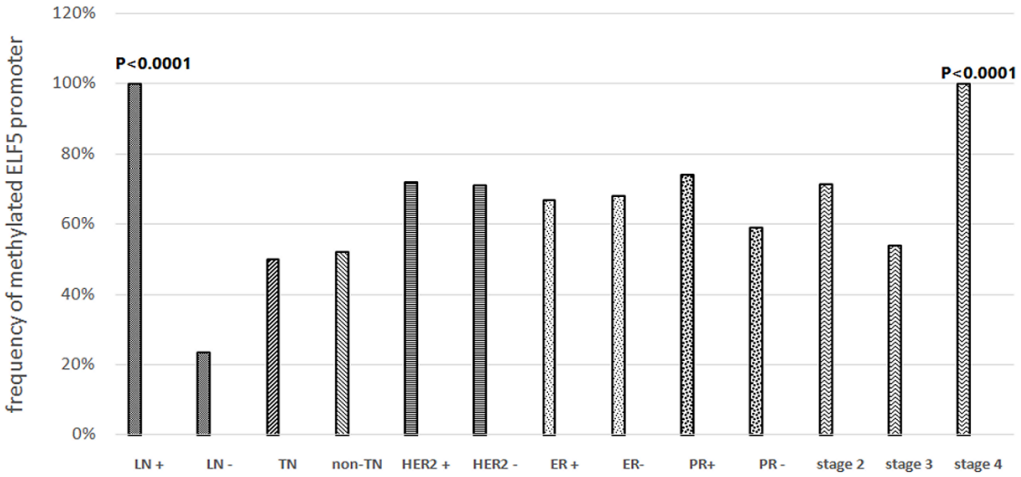

Figure 4. Comparison of Methylated ELF-5 Promoter Frequency in Different Breast Cancer Groups Based on Histopathology Situations. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TN, triple negative; LN, lymph node involvement

has been extensively studied in recent years. The level of plasma cfDNA have been considered as a universal marker indicating malignancy (Han et al., 2017). Various studies have shown that cfDNA concentration can be correlated with prognosis, development, and survival of patients suffering from cancer. Increased cfDNA concentration has been observed in patients with different kinds of cancers (Rave-Fränk et al.,2017; Li et al. 2017).

It has been suggested that elevated cfDNA concentration in cancer patients is associated with cancer cells, necrosis, and apoptosis in the tumor microenvironment (Chen et al. 2005). Numerous cancer-specific alterations, such as methylation, allelic imbalances, and mutations have been identified in blood cfDNA (Kirkizlar et al., 2015; Schwarzenbach et al., 2012). Monitoring cfDNA levels monitoring in peripheral blood has also been reported as a potential biomarker for indicating therapy response in different cancer types (Oellerich et al., 2017). These findings have attracted much attention to the potential use of elevated levels of circulating DNA as a tumor marker.

Although the concentration of circulating DNA in blood is limited, and its lower invasiveness and cost-effectiveness make it a prominent focus of research. In this study, we utilized real-time PCR with SYBR Green detection, a highly precise and reproducible method for quantifying total DNA (Park et al., 2012), to high-quality DNA quantification. Our results showed that plasma cfDNA concentration, quantified by measuring *β-actin* gene amplification, was significantly higher in breast cancer patients compared to the normal control group. Moreover, this higher cfDNA concentration was associated with higher cancer stages and lymph node involvement.

The data revealed the highest levels of plasma cfDNA concentration in samples from stage IV patients and those with LN+. Nodal involvement and higher stages are considered poor prognostic and invasive characteristic of tumors. It can be concluded that higher plasma cfDNA concentration may be associated with cancer invasion. These findings are consistent with a study in Egyptian breast cancer patients, which reported higher cfDNA levels as well as long cfDNA fragments in breast cancer patients compared to controls. They also found a correlation between higher cfDNA concentration, cfDNA integrity, Her-2 positivity, metastasis, and poor treatment response (Ibrahim et al., 2016).

The higher levels of cfDNA concentration in cancer patients compared with normal individuals may be attributed to the inefficient removal of cells, apoptotic DNA, and necrotic DNA released by macrophages (Schwarzenbach et al., 2011).

In addition to quantitative changes, cfDNA in tumor cells may also undergo qualitative changes such as mutations, microsatellite instabilities, and methylations (Kasi et al., 2017; Barault et al., 2017). Gene promoter methylation is a well-known mechanism for gene expression regulation. Aberrant gene promoter methylation in cfDNA has been reported as a noninvasive biomarker for detection, differential diagnosis, prognosis, and therapy response in various cancers (Warton et al., 2015; Leygo et al., 2017).

In this study, we investigated the methylation status of the *ELF5* gene promoter in tumor and normal breast tissues, as well as the corresponding plasma sample. The observed correlation between the methylation status of tumor and the corresponding plasma samples confirmed that the plasma *ELF5* methylation pattern may represent the tumor methylation status. Additionally, we analyzed the association of *ELF5* methylation status with various clinicopathological characteristics of breast cancer. The results revealed a significant association between the *ELF5* promoter methylation status in both tumor and plasma samples and malignant indicators such as lymph node involvement, metastasis, and higher cancer stages. It could be concluded that the *ELF5* promoter methylation in breast cancer patients can be considered as a poor prognosis and an invasiveness indicator. The data unveiled a bimodal methylated pattern in the *ELF5* promoter regions within normal margin tissues. This phenomenon

may be stem from the influence of neighboring cancer cells on the adjacent normal cells, potentially leading to an adoption of their methylation pattern. Consequently, it raises the possibility that, under certain conditions, normal adjacent tissues may not serve as an ideal control group.

ELF5 exerts an inhibitory effect on *SNAIL2*, a pivotal mediator of epithelial- to-mesenchymal transition (EMT). This inhibition of *SNAIL2* by *ELF5* reduces metastasis in cancerous cells. Additionally, *ELF5* plays a lesser-known role in inducing mesenchymal-to-epithelial transition (MET), the reverse process of EMT, thereby stabilizing tumor cells in their original location (Mathsyaraja et al., 2012). These multifaceted functions of *ELF5* emphasize its capacity to inhibit metastasis.

Our data indicated that all the stage IV breast cancer patients with distant metastasis as well as LN positives showed *ELF5* promotor methylation. It could be concluded that *ELF5* promoter methylation may have the significant association with invasion and aggressive behavior in cancerous cells.

Despite the limited sample size, our data somehow confirms the possible utilization of cfDNA assessment both qualitative and quantitative as an informative marker in breast cancer management. In conclusion, the present study provided shreds of evidence that *ELF5* methylation in circulating DNA may be an effective noninvasive possible poor prognosis molecular marker. However, further studies need to evaluate its diagnostic value.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

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Conflict of Interest

The authors report no conflicts of interest.

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