RESEARCH ARTICLE

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Association of Elevated Peripheral Blood Micronucleus Frequency and *Bmi-1* mRNA Expression with Metastasis in Iranian Breast Cancer Patients

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Abstract

Background: In order to find cytogenetic and molecular metastasis biomarkers detectable in peripheral blood the spontaneous genomic instability expressed as micronuclei and *Bmi-1* expression in peripheral blood of breast cancer (BC) patients were studied in different stages of the disease compared with unaffected first-degree relatives (FDRs) and normal control. **Methods:** The Cytokinesis Block Micronuclei Cytome (CBMN cyt) and nested real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assays, were respectively used to measure genomic instability and *Bmi-1* gene expression in 160 Iranian individuals comprised of BC patients in different stages of the disease, unaffected FDRs and normal control groups. **Result:** The frequency of micronuclei and *Bmi-1* expression were dramatically higher in distant metastasis compared with non-metastatic BC. In spite of micronucleus frequency with no association with lymph node (LN) involvement and hormone receptor status, the *Bmi-1* expression level was higher in LN positive and triple negative patients. **Conclusion:** Our results indicate that increased genomic instability expressed as micronuclei and higher *Bmi-1* expression in peripheral blood are associated with metastasis in breast cancer. Therefore implementation of micronucleus assay and *Bmi-1* expression analysis in blood as possible cytogenetic and molecular biomarkers in clinical level may potentially enhance the quality of management of patients with breast cancer.

Keywords: Breast cancer- gene expression- micronucleus- metastasis biomarkers- oxidative stress

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Introduction

Sporadic breast cancer is the common type of female's malignancies in women worldwide. Its etiology is multifactorial. Predisposition to breast cancer may be consequent of mutation in genes involved in the processing of DNA damage and repair known as low penetrance genes (Hemalatha et al., 2014).

The majority of solid tumors are of epithelial origin. Breast cancer cells that have to undergo epithelial-to-mesenchymal transition (EMT) obtain malignant characteristic, however, the molecular mechanism and/or cytogenetic characteristic underlying this transition are poorly understood. It was shown that genomic instability expressed as aneuploidy and chromosomal rearrangements is closely related to tumor development and tumor progression. Highly aneuploid breast tumors generally progressed faster and were clinically more aggressive than their counterparts without aneuploidy (Li et al., 2008). These data clearly indicate that genomic instability may be considered as an important factor for tumor development and progression including distant metastases.

DNA damages expressed as micronuclei are scored specifically in once-divided binucleated cells arrested at cytokinesis in Cytokinesis Block Micronucleus Cytome (CBMN cyt) assay technique. The micronucleus (MN) is an established biomarker for genomic instability indicating chromosome breakage and/or whole chromosome loss. MN arises from acentric chromosome fragments or chromosomes which are not incorporated into daughter nuclei during mitosis. MN scoring in interphase cells has been proposed and used as the quick and easy substitute for the more difficult and time-consuming metaphase aberration analysis. The nuclear bud (NBUD) is considered as a biomarker of elimination of amplified DNA and/or DNA repair complexes and the nucleoplasmic bridge (NPB) is a biomarker of DNA misrepair and/or telomere end-fusions. These three biomarkers, i.e., MN, NPB and, NBUD, are the endpoints evaluated with this technique. The Cytostatic effect is measured via nuclear division index (NDI) considering the proportion of mono-, bi-, and multinucleated cells. The cytotoxicity effect which is detectable via this technique determines apoptotic and/or necrotic cell ratios (Fenech, 2007; Salimi et al., 2016).

The processes of metastasis are distinctive features

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of breast cancer progression. Although markers such as large tumor size, poorly-differentiated histopathological grade, and lymph-node metastasis are common established prognostic markers related to metastasis, distant metastasis still occurs in 20-30% of the patients with negative lymph-node involvement (Loda et al., 2010).

The exact molecular mechanism of breast cancer metastasis remains unclear due to the cancer heterogeneity and represents a new prerequisite for developing better treatment strategies.

The polycomb (PcG) proteins constitute a global system with important roles in cancer, multi-cellular development and, stem cell biology. B-lymphoma Moloney murine leukemia virus insertion region-1 (*Bmi-1*) is the first functional mammalian Polycomb group (PcG) proto-oncogene to be recognized. The PcG consists of several proteins that form multiprotein complexes that regulate gene activity at the chromatin level. They were initially identified as part of the memory system that ensures the faithful transmission of cell identities throughout cell division. Although PcG protein expression is tightly regulated in normal cell proliferation and differentiation, it is often deregulated in several types of human cancer (Li et al., 2014). *Bmi-1* is known to play an important role in carcinogenesis as it was originally identified as an oncogenic partner of c-Myc in murine lymphomagenesis (Joensuu et al., 2011). Previous studies revealed that *Bmi-1* is involved in the regulation of stem-cell-associated genes to control cell self-renewing and differentiation. Moreover *Bmi-1* may be involved in the carcinogenesis and metastasis of breast cancer due to have role in leading mammary epithelial cells (HMECs) to bypass senescence and immortalize by activation of human telomerase reverse transcriptase (hTERT), which extended the replicative life span lifespan (Silva et al., 2007; Guo et al., 2011) also, a significant correlation has been observed between *Bmi-1* expression and axillary lymph node metastasis in invasive ductal breast cancer (Silva et al., 2007). Although some shreds of evidences have shown that *Bmi-1* expression is associated with unfavorable prognosis, other studies have not confirmed these findings (Choi et al., 2009; Nalwoga et al., 2010; Shao et al., 2014). Shao (2014) in a meta-analysis study reported that high *Bmi-1* expression was significantly associated with poor survival in Asian patients with esophageal carcinoma, gastric cancer, lung cancer, colorectal cancer and cervical carcinoma, whereas the high level of *Bmi-1* can predict better prognosis in Caucasian patients with breast cancer. In spite of the aforementioned link between *Bmi-1* and cancer, very few studies have focused on the molecular mechanism and clinical outcome of *Bmi-1* in breast cancer metastasis. Majority of *Bmi-1* expression studies in breast cancer have been focused on its expression in breast cancer tissues and there is only one report analyzing *Bmi-1* gene expression at mRNA level in plasma (Silva et al., 2007). In the present study for the first time, we have traced *Bmi-1* expression at RNA level in total peripheral blood using nested Real-time RT-PCR. Many tumors shed stray cells, vesicles, and traces of DNA and RNA into the blood and other body fluids, such debris can serve as markers to monitor disease progression and even help to diagnose cancers before symptoms appear. Considering advantages to find cancer biomarkers detectable in blood, in the present study the relative influence of peripheral blood genomic instability expressed as MN and *Bmi-1* expression at RNA level was investigated in Iranian breast cancer patients using CBMN and nested real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assays.

Materials and Methods

Study population

The study was carried out as a case-control study in a group of 160 Iranian females (70 ductal carcinoma breast cancer patients, 40 unaffected first-degree relatives of the studied patients and 50 unaffected matched controls). The protocol was approved by the ethical committee of the National Institute of Genetic Engineering and Biotechnology (NIGEB), based on Helsinki declaration. Patients and controls signed a written informed consent letter before enrolment. Table 1 shows clinical and analytical data for test and control groups. About 5 ml of blood was collected from each donor (breast cancer, unaffected first-degree relatives of patients and normal control, all were female) by venipuncture into heparinized and EDTA tubes. Breast cancer patients were collected from patients referred to ''Imam Khomeini hospital, Tehran, Iran''. All donors completed a written questionnaire to obtain information related to their lifestyle, such as dietary habits, medical history and exposure to chemical and physical agents.

The inclusion criteria for the patient samples were the histopathological diagnosis of ductal carcinoma and availability of immunohistochemistry (IHC) results for human epidermal growth factor 2 (HER-2), estrogen receptors (ER) and progesterone receptor (PR) status and other pathologic diagnostic information. Receiving chemotherapy or radiotherapy before recruitment and any history of familial breast disease or malignancy considered as exclusion criteria in our study.

The patients were distributed into three groups according to tumor stage (stage II to IV), which was determined by a pathologist in compliance with common standards. Details of the patient clinicopathological parameters are presented in Table 1.

CBMN assay

Cell culturing

Blood samples were drawn by venipuncture into sodium-heparin vacutainers and processed within 3 hours after retrieved from the hospital. For each individual, four lymphocyte cultures were set up by adding 0.5 mL of whole blood into 4.5 mL of RPMI 1,640 medium supplemented with 15% Fetal Bovine Serum (FBS), 1% antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) and 0.15 mL phytohaemagglutinin were also added to the cultures (all provided by Gibco Life Technologies, Paisley, UK). Lymphocytes were cultured at 37°C for 72 hr. After 44 h, 6 µg/mL cytochalasin B [Gibco, Northumberland, UK] was added to the culture to arrest cells at cytokinesis. At 72 hours of incubation, cultures

were harvested by centrifugation at 120g for 8 min followed a brief hypotonic treatment (2–3 min in 0.075 M KCl at 48°C). The cells were centrifuged, then fixed and washed in methanol/ acetic acid (3:1 v/v) solution three times. The resulting cells were resuspended and dropped onto clean slides. Slides were coded and stained with 10% of Giemsa (Merck, Darmstadt, Germany) in phosphate buffer (pH 6.8) for 5 min.

Scoring and data evaluation

The scoring criteria established by Fenech (2007) were used for CBMN Cyt assay analysis. To determine the frequency of CBMN assay endpoints (micronuclei, nucleoplasmic bridge, and nuclear buds) as well as apoptosis and necrosis a total of 1,000 binucleated cells with well-preserved cytoplasm were blindly scored on coded slides. In addition, a total of 500 lymphocytes were scored to determine the percentage of cells with one, two, or more nuclei in order to calculate the nuclear division index (NDI).

Bmi-1 mRNA expression analysis

RNA extraction and cDNA synthesis

Total RNA was purified from EDTA saturated fresh blood using TRI reagent BD (sigma, Darmstadt, Germany), 2 µg of total RNA was digested by 2 µg DNase 1 (Fermentas, Manchester, UK) to remove genomic DNA contamination and then 1 µg of RNA was used for cDNA synthesis, with Precision qScriptt Reverse Transcription Kit (Primerdesign, Chandlers's Ford, UK). All the steps were done following the manufacturer's instructions.

Standard curve construction

Amplification efficiency for each primer pairs was determined by the amplification of a linear standard curve (from 0.24 to 1,000 ng) of total cDNA assessed by ultraviolet spectrophotometer. Standard curves showed good linearity and amplification efficiency (100%) for the primer set of experimental (*Bmi-1*) and reference (beta-actin) genes.

Nested Real-time RT-PCR analysis

Gene-specific primers were designed manually. Because the *Bmi-1* gene was expressed at low levels in Peripheral blood RNA, nested real-time PCR was used to quantitate gene expression. The first round of PCR was carried out in 50 µl reaction containing 5 µl of cDNA, 10 µl 10X buffer, 1 µl 10 mM dNTP, 3 µl gene-specific primer mix (20 µM), 5 units Taq Polymerase and amplified on a thermal cycler at 95°C for 2 minutes followed by 40 cycles of 95°C for 30 seconds, 51°C for 35 seconds and 72°C for 35 seconds, then 1 cycle of 72°C for 10 min, ending at 4°C. The PCR primers for the 1st round amplification of *Bmi-1* are F1: 5′TAATGCCATCTGATTCTTAC3′ and R1: 5′CATGTCACTGTGAATAACG3′. Real-time RT-PCR reactions were performed to detect the expression of each gene in duplicate, in 25 µl reaction volume using 5 µl of 1st round PCR product, 12.5 µl SYBR Select Master Mix (Applied Biosystems), 0.25 µl primer mix (2 µM final) and 7.25 µl water. Beta-actin gene is a housekeeping gene and was used for normalization. The real-time RT-PCR primers for *Bmi-1* and beta actin are F2: 5′CCGCTTTTAGGCATACAGATTG3′, R2: 5′GATTTATACTTCTCTGTTGCTAC3′ and F: 5′CAGCAGATGTGGATCAGCAAG3′, R: 5′GCATTTGCGGTGGACGAT3′, respectively. All reactions were carried out on the ABI 7500/7500 fast real-time system (Applied Biosystem, CA, USA). Using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) the data were presented as the fold change in gene expression normalized to an endogenous reference gene (beta-actin) and relative to the normal controls.

Statistical Analyses

Statistical computations were performed using the SPSS version 16.0 (SPSS, Chicago, IL). The comparison of the data between patient and control groups was carried out using an analysis of variance (ANOVA) test. A Student's t-test was performed for comparisons between two groups. For all analyses, differences were accepted as statistically significant at $p < 0.05$. Numerical data are presented as the mean ± standard deviation (SD).

Results

Characteristics of the study populations

Table 1 summarizes the demographic and clinical data for the different groups of patients and control. There were no significant differences in the distribution of body mass index, the age of menarche, number of children (data not shown) and smoking habits and use of hormone replacement therapy.

CBMN assay in the studied populations

The background MN frequency, as well as nuclear buds and nucleoplasmic bridges in binucleated peripheral blood lymphocyte and micro nucleated cell frequency in breast cancer, first degree relatives of breast cancer patients (FDR), and control groups, are summarized in Table 2. The background frequency of micronuclei was significantly higher in breast cancer (BC) group compared with both unaffected FDR and control groups ($p<0.001$).

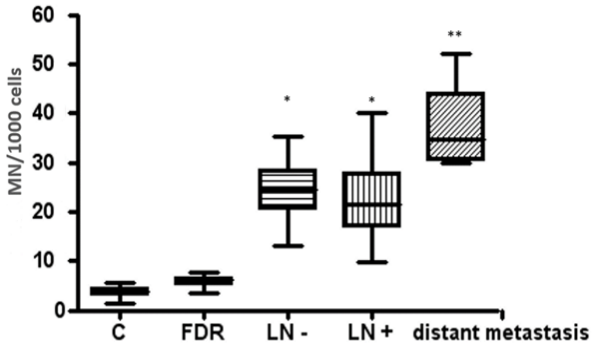

Figure 1. MN Frequency in Different Breast Cancer Groups based on Metastasis Situation Compared with FDR and Control Groups. C, control; FDR, first degree relatives; LN -/+, lymph node negative/positive; MN, mean of micronuclei frequency. * Statically significant compared with C and FDR groups ($P\leq 0.001$). **Statically significant compared with C, FDR, LN+ and, LN – groups ($P\leq 0.001$).

Table 1. Characteristics of Breast Cancer Patients and Controls

	Patient N (%)	Control N (%)	First degree relatives
Number	70	50	40
Age (years)			
Mean	46.8±12.8	48.5± 16.4	45.6± 10.8
Range	27-84	25-82	20-75
Stage at diagnosis			
Stage II	36 (51.4%)		
Stage III	24 (34.3%)		
Stage IV	10 (14.3%)		
Lymph node status			
N0	30 (42.9%)		
N+	40(57.1%)		
Distance metastasis			
yes	10 [2 bone, 8 lung] (14.3%)		
No	60 (85.7%)		
Hormone receptor status (IHC)			
ER and/or PR positive	56 (80%)		
ER and PR negative	14 (20%)		
HER-2 status (IHC)			
+ + +	22 (31.4%)		
Negative	48 (68.6%)		
Triple-negative breast cancer	9 (12.5%)		
Smoking			
Yes	18 (25.7%)	12 (24%)	15 (37.5%)
No	52 (74.3%)	38 (76%)	25 (62.5%)
Menopause status			
Yes	36 (51.4%)	19 (38%)	13 (32.5%)
No	34 (48.6%)	31 (62%)	27 (67.5%)
HRT			
Yes	15 (21.4%)	10 (20%)	7 (17.5%)
No	55 (78.6%)	40 (80%)	33 (82.5%)
Pregnancy at term			
Yes	60 (85.7%)	41 (82%)	25 (62.5%)
No	10 (14.3%)	9 (18%)	15 (37.5%)

HRT, hormone replacement therapy

The mean MN frequency was also higher in unaffected FDR group compared with normal control ($p<0.01$). The MN frequencies are 28.36±8.34, 6.21±1.64, 4.2 ±1.24, for breast cancer patients, FDR and control groups respectively ($p<0.001$).

As shown in figure 1 when the breast cancer patients stratified according to the metastasis situation (metastasis to lymph node (LN+ / LN-) and distant metastasis) no significant differences were observed between LN+ and LN- based on micronucleus frequency whereas this frequency was significantly higher in breast cancer patients with distance metastasis ($p\leq0.001$).

Mean of micronucleus frequency in breast cancer patients stratified according to immunohistochemistry (IHC) results for hormone receptors which commonly used in clinical practice was not different between ER+/ ER- or PR+/PR- or HER2+/ HER2- breast cancer patients

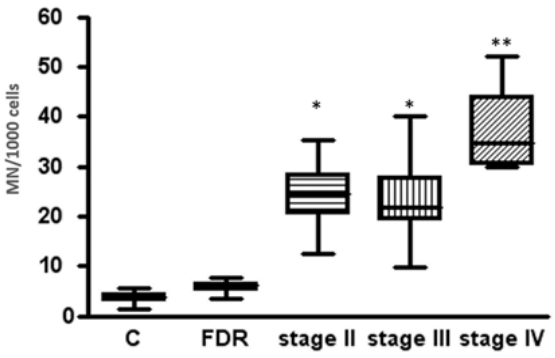

Figure 2. Mean Micronucleus Frequency in Breast Cancer Group Compared with FDR and Control Groups. Patients were grouped considering breast tumor TNM staging. C, control; FDR, First degree relatives; MN, micronucleus. * Statically significant compared with C and FDR groups ($p \leq 0.001$). **Statically significant compared with C, FDR and other groups (stages II & III) ($p \leq 0.01$).

(p>0.05).

When the breast cancer patients categorized based on clinical stages, the mean MN frequency was significantly higher in stage IV ($p\leq0.01$) whereas no significant differences were observed between other stages (Figure 2). The data showed that the background frequency of both nucleoplasmic buds and nucleoplasmic bridges were significantly higher than control in breast cancer patients group ($p<0.001$) (table 2).The rate of apoptosis in breast cancer group was significantly higher than other groups also this frequency was higher in FDR group compared with normal control ones ($p<0.001$) whereas no statistically significant difference was between necrosis rate among all three test and control groups ($p>0.05$) (Table 2).

Bmi-1 expression results

Compared to FDR and normal control groups, the average amount of peripheral blood *Bmi-1* RNA expression was significantly higher in breast cancer patients (Figure 3). There were no significant differences between levels of *Bmi-1* expression in FDR and the

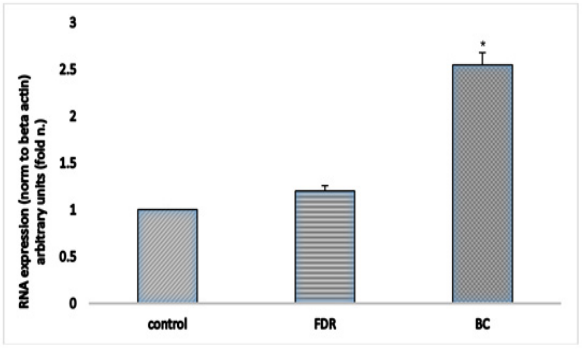

Figure 3. Nested Real-Time RT-PCR Analysis of Bmi-1 Expression in BC Patients, FDR, and Control. BC, Breast cancer; FDR, unaffected first-degree relatives. * Statically significant compared with C and FDR groups ($p < 0.001$).

Table 2. Background Cytogenetic and Cytotoxicity Parameters in the Studied Populations Breast Cancer, First Degree Relatives and Controls (C)

	Breast cancer (mean ±SD)	First degree relatives (mean ±SD)	Control (mean ±SD)
Cytogenetic			
Background MN	28.36±8.34*a/*c	6.21±1.64**a	4.2 ±1.24 *b
Background Micro nucleated cell	26.05±7.54*a/*c	6.02±1.01**a	4.12±1.02*b
Background NBUD frequency	4.56±1.83*a/*c	1.56±1.4	1± 0.82
Background NPB frequency	5.4±2.18*a/*c	2.06±1.89	1.84±1.04
Cytostatic			
Background NDI	2.1±0.3	1.9±0.31	1.9± 0.31
Cytotoxicity			
Background apoptosis	8.06±2.02**a	7.78±1.76**a	5±1.13
Background necrosis frequency	4.34±1.35	4.06±1.34	3±1.48

MN, Micronuclei; PBL, peripheral blood lymphocyte; SD, standard deviation; NBUD, nucleoplasmic bud; NPD, nucleoplasmic bridge; NDI, nuclear dividing index: (mononucleated cells + 2X binucleated cells + 3X trinucleated cells + 4 X tetranucleated cells)/total number of cells.a, t-test with the control group as the reference; b, ANOVA test; C versus first degree relatives and breast cancer groups; c, ANOVA test : BC versus first degree relatives and C groups.*P≤ 0.001 ; **P≤0.01

normal control group (p>0.05).

When *Bmi-1* expression data stratified in BC patients based on estrogen/ progesterone and human epidermal growth factor receptors status, our results showed that mean of RNA expression in triple negative breast cancer tumors (ER-, PR-, HER2-) was significantly higher than non-triple negative ones (P<0.001) (Figure 4).

In figure 5, nested real-time RT- PCR analysis of *Bmi-1* expression in different breast cancer groups based on metastasis situation compared with first degree relatives and control groups is shown. As this figure indicates the mean of *Bmi-1* mRNA expression was dramatically higher in metastatic groups both distant metastasis and lymph node metastasis compared with lymph node negative (LN-) breast cancer patients, FDR and normal control groups (p<0.001). The peripheral blood mRNA expression was 4.81±1.29, 4 ± 2, 0.75 ± 0.68 and 1. 2±0.7 in breast cancer with distant metastasis, LN +, LN- and FDR respectively.

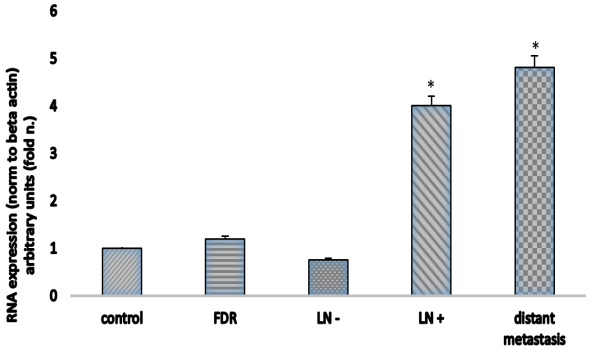

Figure 5. Real-Time RT- PCR Analysis of Bmi-1 Expression in Different Breast Cancer Groups Based on Metastasis Situation Compared with Unaffected First Degree Relatives and Control Groups. C, control; FDR, unaffected first degree unaffected first-degree relatives; LN, lymph node metastasis.*Statically significant compared with C, FDR, and LN – groups (p ≤ 0.001).

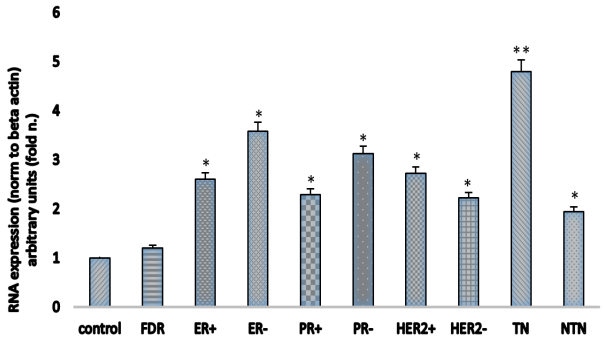

Figure 4. Nested Real-Time RT-PCR Analysis of Bmi-1 Expression in Different Breast Cancer Groups Based on Estrogen, Progesterone, and Human Epidermal Growth Factor 2 Receptor Status Compared with Unaffected First Degree Relatives and Control Groups. ER, estrogen receptors; PR, progesterone receptor; HER-2, human epidermal growth factor 2; TN, triple negative; NTN, non-triple negative; FDR, unaffected first-degree relatives. * Statically significant compared with C, FDR (p < 0.001). **Statically significant compared with C, FDR, NTN, and other groups (p ≤ 0.001).

It could be concluded from our data that breast cancer patients with the highest levels of both MN frequency and *Bmi-1* expression detectable in blood would have major possibility to undergo distant metastasis (figure 1 and 5).

Discussion

In the present study, we supposed MN frequency and *Bmi-1* expression as two cytogenetic and molecular possible metastasis biomarkers detectable in peripheral blood. In the case of MN frequency, CBMN assay was done in peripheral blood of breast cancer patients, FDR, and normal control groups. CBMN assay has been applied to examine the effect of the variety of factors such as genetics, lifestyle, dietary and environmental on chromosomal stability and mitotic function (Salimi et al., 2014; Bitgen et al., 2015; Salimi et al., 2015; Salimi et al., 2016).

Our data demonstrated that the frequency of DNA damage expressed as nuclear aberrations were significantly

higher in the breast cancer patient group compared with FDR and normal controls. We studied different CBMN assay endpoints (Table 2) and the frequency of micronuclei was chosen as a biomarker of effects. This biomarker has great biological relevance since MN represent fixed genetic damage resulting from both clastogenic and aneugenic mechanisms (Fenetch, 2007) and it is considered as a well surrogate marker of cancer risk (Giovannini et al., 2014).

Our results showed that the frequency of micronuclei in BC patients and FDR groups were higher than the normal control group (Table 2). Our result was somehow in line with some studies have reported the higher frequency of micronuclei in cancer patients compared with normal unaffected individuals (Paz et al., 2018; Santos et al., 2010; Milosević-Djordjević et al., 2010; Bonassi et al., 2011). The micronuclei scoring as a biomarker on fine needle aspiration cytology smears of breast carcinoma was done and confirmed the association of high MN frequency and breast cancer (Hemalatha et al., 2014). Micronucleus assay in buccal smears of breast carcinoma patients showed that micronucleated cells are significantly increased in buccal cells of the breast carcinoma cases (Flores-Garcia et al., 2014). We may conclude from our data and most works of literatures that the increased number of MN in different sample types of BC raises the possibility that the genetic damage in breast cancer patients is generalized and predicted MN scoring could be used in biomonitoring of DNA damage and early detection of high-risk cases of carcinoma of the breast in future. In contrast, Bolognesi et al. reported no significant role of micronucleus frequency as a biomarker of breast cancer risk/susceptibility (Bolognesi et al., 2014).

The higher frequency of micronuclei in FDR group compared with control that was shown in Figure 1 was somehow in line with a study reported that the FDRs of patients having head and neck cancer (HNC) showed significantly higher chromosomal damage in terms of MN frequencies in lymphocytes when compared with those of controls, thus reflecting an increased susceptibility to HNC in FDRs (Burgaz et al., 2011).

This higher MN frequency in FDR compared with normal control group, clearly demonstrates that MN frequency is determined by genetic factors to a major part. The strong reflection of the genetic background supports the idea that MN frequency represents an intermediate phenotype between molecular DNA repair mechanisms and the cancer phenotype and affirms the approaches that are made to utilize them as predictors' cancer risk (Surowy et al., 2011). Our results expressed both higher MN frequency and *Bmi-1* expression in breast cancer patients compared with control.

We examined levels of peripheral blood micronuclei and *Bmi-1* mRNA expression in LN-positive and negative and metastatic breast cancer cases. Our data showed that the MN frequency was not associated with lymph node involvement but was significantly higher in peripheral blood of patients harboring distant metastasis. Whereas *Bmi-1* expression was significantly correlated with both nodal involvement and distant metastasis. Other studies reported the higher *Bmi-1* expression at the mRNA level in breast tissue of early-stage patients with no lymph node metastasis (Surowy et al., 2011). Also, it was reported that *Bmi-1* expression may be associated with favorable overall survival in breast cancer patients, especially in patients with ER-positive breast cancer (Choi et al., 2009). Controversially, up-regulation of *Bmi-1* was shown to be associated with the invasion and poor survival prediction in nasopharyngeal (Song et al., 2009) and with nodal involvement, distant metastasis and clinical stage in uterine cervical and gastric cancers (Zhang et al., 2010).

In a study a xenograft mice model was used to elucidate that *BMI-1* was necessary in tumor development by assessing tumor volume and Ki67 expression. They found that Hedgehog (Hhg) signaling exerted synergized functions together with *Bmi-1*, implicating the importance of *BMI-1* in Hhg signaling. They concluded that downregulation of *BMI-1* could be an effective strategy to suppress tumor growth, which supports the potential clinical use of targeting *Bmi-1* in breast cancer treatment (Yan et al., 2017)

Previously the *Bmi-1* expression in breast cancer tumor and cells were investigated and showed the positive association between *Bmi-1* overexpression and clinical features, such as tumor size, lymph node involvement, distant metastasis and clinical stage (Wang et al., 2015; Gavrilescu et al., 2012). In contract in a study done on pulmonary squamous cell carcinoma, *BMI-1* expression was reported to be associated with a favorable prognosis and considered as a possible prognostic factor of pulmonary squamous cell carcinoma (Abe et al., 2017). Our data for the first time showed that *Bmi-1* expression in blood was significantly higher in BC compared with FDR and normal control groups (Figure 3, $p<0.001$) also the positive association was observed between *Bmi-1* expression levels and Lymph node involvement and distant metastasis (Figure 5, $p<0.001$).

Our results indicate that high *Bmi-1* peripheral blood expression predicts an unfavorable patient prognosis and serves as a high-risk indicator in breast cancer. Furthermore, we also shed light on the biological impact of *Bmi-1* on the invasive and metastatic properties of breast cancer. The main line of evidence involving *Bmi-1* in tumorigenesis is the repression of INK4a/ARF suppressor proteins, deregulating both pRb and p53 cell cycle control pathways, facilitating cell proliferation, and desensitizing cells to apoptosis. However, the effect of *Bmi-1* overexpression on the inactivation of the INK4a/ARF transcripts in human breast cancer is unclear. As mentioned earlier the overexpression of *Bmi-1* enhances the motility and invasiveness, facilitates concurrent EMT-like molecular changes, and promotes the stabilization of Snail and the activation of the Akt/GSK3b pathway. In addition, repression of *Bmi-1* reverses the expression of EMT markers and inhibits the Akt/GSK3b pathway (Guo et al., 2011).

Since distant metastasis still occurs in 20-30% of the patients with negative lymph-node involvement (Loda et al., 2010), finding other biomarkers represents metastasis has great value. Our results provide the evidence that *Bmi-1* overexpression and high micronucleus frequency measured in lymphocytes may be considered as two

unfavorable molecular and cytogenetic possible patient prognosis biomarkers detectable in blood and serve as high-risk metastasis indicators in breast cancer. Therefore implementation of micronucleus assay and *Bmi-1* expression analysis in blood as possible cytogenetic and molecular biomarkers in clinical level may potentially enhance the quality of breast cancer management.

Statement conflict of Interest

No potential conflicts of interests were disclosed by the authors.

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