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# Evaluation of Aberrant Methylation of *RASSF1A*, *BCL-XL*, *ITGA6*, *TCF3* and *SNAIL2* Genes in Peripheral Blood Leukocyte DNA in Breast Cancer Patients

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## Abstract

**Purpose:** Nowadays, breast cancer is the most common cancer in women that caused by defects in the signaling mechanisms that control cell proliferation and apoptosis. Recent findings suggest that epigenetic alterations are the key factors in the development of breast cancer. Methylation changes occur within CpG islands of promoters and induce gene silencing. Abnormal methylation can be used as a potential biomarker for diagnosis of various diseases including cancer. In this study, methylation changes of *RASSF1A*, *TCF3*, *BCL-XL*, *SNAIL2* and *ITGA6* genes were assessment as epigenetic biomarkers of breast cancer.

**Methods:** 70 breast cancer samples and 70 normal samples were selected and identified with different Clinical and pathological data, which might be related with methylation changes. Breast cancer patients and normal blood samples were collected, and DNA was extracted from white blood cells. DNA samples were digested using methylation-sensitive restriction enzymes to identify methylated sites. Unlike hypomethylated positions, hypermethylated sites were not digested using these enzymes, thus replication occurs by PCR reaction.

**Results:** *RASSF1A* and *TCF3* (in some cases) were significantly hypermethylated in breast cancer cases ($P<0.05$) compared to normal samples. *ITGA6* was significantly hypomethylated in breast cancer cases ($P<0.05$) compared to normal samples.

According to statistical analysis, no significant correlation was observed between methylation changes and clinical factors (stage of disease, age of patients, Estrogen Receptor (ER), Progesterone Receptor (PR), and human epidermal growth factor 2 (HER2) status) in patients with breast cancer ($P>0.05$) except *RASSF1A* gene ethylation changes that shown reverse correlation with age of patients ($P<0.05$).

**Conclusion:** This study demonstrated that *RASSF1A*, *ITGA6* and *TCF3* genes methylation status were changed during breast cancer and they can be used as molecular biomarkers for breast cancer diagnosis.

**Keywords:** Breast cancer; Methylation; *RASSF1A*; *BCL-XL*; *TCF3*; *SNAIL2*; *ITGA6*

## Introduction

Cancer is a devastating life-threatening disease arises from both genetic and environmental factors and caused by defects in the signaling mechanisms that control cell proliferation and apoptosis. Molecular defects that make disturbance in cellular growth and death, allow tumor cells to have uncontrolled division and metastasis. Most cancers are named for the organ or type of cell in which they start [1].

Breast cancer is one of the most numerous cancers in women and occurrence of it is increased globally. Despite of conventional therapies and novel progressed techniques in diagnosis and therapy, breast cancer still a devastating disease worldwide [1,2]. Breast cancer is a heterogeneous disease in clinical and morphological parameters such as tumor size, histological grade, age; or molecular biomarkers like estrogen receptor (ER), progesterone receptor (PGR) and human epidermal growth factor receptor 2 (HER2) [3].

Breast cancer patients classified in four different subgroups of breast tumors including: normal-like phenotype, luminal phenotype (estrogen receptor (ER)-positive tumors, expression of E-cadherin and cytokeratins CK8, 18, and 19), ER-negative tumors (overexpression HER2) and basal-like phenotypes [4].

Epigenetic alterations (such as DNA methylation) are mitotically heritable changes in gene expression without changes in DNA sequence [2].

DNA methylation is an enzymatic change that frequently occurs at cytosine residues in CpG dinucleotides by DNA methyltransferase enzymes such as DNMT1, DNMT3a and DNMT3b in mammalian cells. Methylation occurs within promoters and enhancers and controls gene regulation and generally induces gene silencing by the blocking of transcription factor binding or formation of heterochromatin state [5].

DNA hypomethylation can be associated with proto-oncogenes over expression and hypermethylation is associated with tumor suppressor genes suppression in cancer cells [6].

Abnormal methylation can be used as a potential biomarker for diagnosis of disease including cancer, psychiatric and neurodegenerative disorders and prediction of drug sensitivity and treatment [7]. Biomarkers classify to different groups including risk biomarkers, diagnostic biomarkers, prognostic biomarkers and predictive biomarker [8-10].

Cancer detection and diagnosis tests are commonly blood-based DNA methylation analysis and less invasive tests. These methods

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based on differential detection between methylated and unmethylated fragments by changing their sequence. For example, bisulfite modification can convert unmethylated cytosines to uracils and methylation sensitive restriction enzymes cannot destroy methylated DNA and these fragments detect by PCR amplification [7].

DNA methylation-based biomarker genes including B-cell lymphoma 2 like 1 (*BCL-X$_L$*), Ras Association Domain Family Member 1 (*RASSF1A*), Transcription Factor 3 (*TCF3*), Snail Family Transcriptional Repressor 2 (*SNAIL2*) and Integrin Subunit Alpha 6 (*ITGA6*) are good examples of diagnostic, prognostic and predictive biomarkers in breast cancer.

In human *BCL-XL* is critical antiapoptotic factor that is encoded by *BCL2L1* gene [11]. Cancer cells prevent apoptosis by overexpression of anti-apoptotic proteins such as *BCL-XL* protein and suppression of pro-apoptotic proteins such as BAX and BAK proteins [12]. *BCL-XL* be discovered to regulate necrosis by interacting with the mitochondrial phosphatase PGAM5 [13]. *BCL-XL* interacts with Beclin 1 and evades the autophagy [14,15]. *BCL-XL* induced epithelial–mesenchymal transition (EMT), cell migration and metastasis [16].

*RASSF1A* is a member of the RASSF family of tumor suppressors that expressed in all epithelial cells and inactivated in breast cancer tumors by epigenetic silencing including promoter hypermethylation [17,18]. *RASSF1A* promote apoptosis and restrict the cell cycle. *RASSF1A* modulate the cell cycle by binding, polymerizing and stabilizing the microtubules [19]. *RASSF1A* interacts with a, b and g tubulins and microtubule associated proteins (MAPS) [20,21]. *RASSF1A* induces two apoptotic pathways by activating Hippo and Bax [22,23].

In mammals, *TCF3* is a member of the TCF family with various isoforms [24,25]. Wnt signaling pathway contributes to the regulation of *TCF3* and Overexpression of this gene has been detected in different cancers such as breast cancer. *TCF3* implicated in epithelial to mesenchymal transition, tumor aggressiveness, E-cadherin repression, pluripotency and self-renewal [26-28].

*Snail2* (also known as Slug) is EMT-inducing transcription factor by repression of E-cadherin and a regulator of cancer stem cells (CSCs) [29]. Different factors such as TGFB, Notch, TNFα, EGF, FGF, hypoxia, and estrogens induce *Snail2*. *Snail2* overexpression induces Bcl2 (anti apoptotic factor) and protects cancer cells against apoptosis for their survival. *Snail2* is associated with multidrug resistances [30].

*ITGA6* overexpression has been shown in cancer stem cells which have mesenchymal features (cell adhesion, migration, and invasion) and breast cancer tissue that is associated with a poor prognosis and reduced survival rates [31,32]. *ITGA6* cleavage and its interaction with HER2 promote cell invasion and migration [33,34]. In addition, overexpression of *ITGA6* induced resistance to radiotherapy and suppressed apoptosis and cell cycle arrest in cancer cells [35].

In this paper, the methylation profile of *BCL-X$_L$*, *RASSF1A*, *TCF3*, *SNAIL2* and *ITGA6* genes were studied in patient and normal cases as the epigenetic biomarkers of cancer.

## Materials and Methods

### Sample collection

Control and cancer blood samples were obtained from thirty healthy and seventy cancer patient donors in EDTA coated tubes after informed consent in accordance with local ethics guidelines and stored at -20°C. Patient donors were hospitalized at Imam Khomeini Hospital in 2016. Different clinical factors of all patients including age, stage of disease and bio-markers such as ER, PR and HER2 were collected to assess their correlation to the methylation profile of candidate genes.

### DNA isolation

White blood cells were separated from archived whole blood samples. DNA was extracted from cell population of each sample by Roche DNA extraction Kit (Roche Diagnostics, Germany). DNA concentrations are measured by Nano Drop™ spectrophotometer at a wavelength of 280/260 nm.

### Select the appropriate methylated region in promoter and primer design

Gene promoter sequences obtained from a transcriptional regulatory element database (TRED). Methylated regions in the promoter sequences were determined from EMBOSS Cpgplot database. The region that the percentage of methylation was close to 100% was selected from all methylated regions in promoter. Restriction endonuclease recognition sites were studied in selected methylated position using NEBcutter analysis tool and a methylated sensitive restriction enzyme that its recognition site was located in this area was selected. Then primer sequences were designed on both sides of selected restriction enzyme recognition site (Figure 1).

### Restriction endonuclease quantitative PCR (RE-PCR)

Detection of hyper or hypo methylated CpG islands in the promoter region of the candidate gene including *BCL-XL*, *RASSF1A*, *TCF3*, *SNAIL2* and *ITGA6* were carried out by methylation-specific PCR (MS-PCR). Restriction endonuclease quantitative PCR method was used in this study [36]. Isolated DNA samples were digested using methylation-sensitive restriction endonuclease enzymes (RE-enzymes) (TaKaRa, Japan) such as SacII, SmaI and NaeI. For each enzyme, methyl groups block the cleavage site and digestion was suppressed. The ratio between unmethylated and methylated promoters in different samples was analyzed using MS-PCR in the LightCycler system (Rotor-GeneQ, Qiagen). Unmethylated (hypomethylated) DNA samples were digested using RE-enzymes and PCR products were not detected. Digested methylated (hypermethylated) DNA samples were amplified by PCR and products were detected.

### Digestion of DNA samples

40 ng of each DNA sample was digested using specific RE-enzyme at 37°C for overnight.

### RE-PCR

PCR amplification was done on treated and untreated DNA samples that performed in a lightcyclerTM system (Rotor-GeneQ, Qiagen). PCR (35 cycles of denaturation for 60 s at 95°C, annealing for 40 s at 60°C, and extension for 45 s at 72°C) was performed using specific primers (Table 1).

### Real time PCR

All real time PCR reactions were performed in a lightcyclerTM system (Corbett Real-Time Thermal) using specific primers and SYBR Green Master mix (Bioneer, Daejeon, Korea) following these conditions: 95°C for 15 minutes followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 60°C for 30 seconds.

Methylation index= {Ct value of treated DNA} - {Ct value of untreated DNA}.

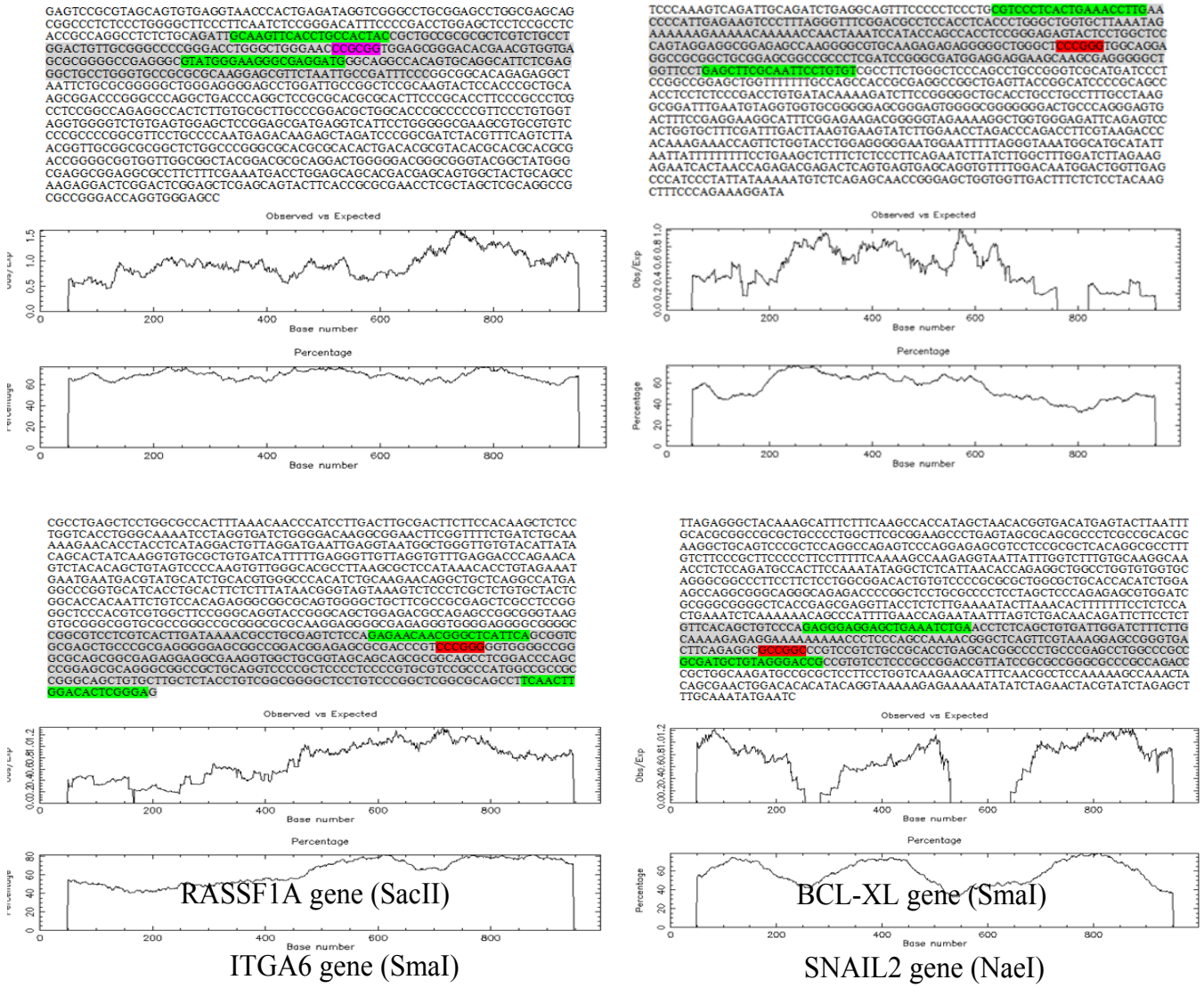


**Figure 1:** Select the appropriate methylated region in promoter and primer design.

| Gene | Size (bp) | Strand | Sequence (5'→3') | Annealing temperature |
|---|---|---|---|---|
| *BCL-XL* | 308 | F | CGTCCCTCACTGAAACCTTG | 60 |
| | | R | ACACAGGAATTGCGAAGCTC | 60 |
| *ITGA6* | 297 | F | GAGAACAACGGGCTCATTCA | 60 |
| | | R | TCCCGAGTGTCCAAGTTGA | 60 |
| *TCF3* | 488 | F | GCCTGAGTTCTGTCCAAAGTC | 60 |
| | | R | CTGTGCGCTTAGTCCATGAC | 60 |
| *RASSF1A* | 144 | F | GCAAGTTCACCTGCCACTAC | 60 |
| | | R | CATCCTCGCCCTTCCCATAC | 60 |
| *SNAIL2* | 198 | F | GAGGGAGGAGCTGAAATCTGA | 60 |
| | | R | CGGTCCCTACAGCATCGC | 60 |

**Table 1:** Primer sequences for RE-PCR and real-time PCR.

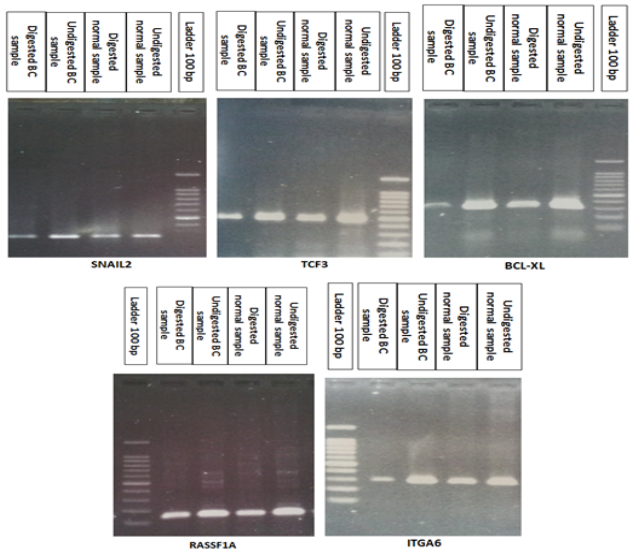


**Figure 2:** RE-PCR analysis of five genes (*SNAIL2, TCF3, BCL-XL, RASSF1A* and *IRGA6*) methylation in normal and breast cancer samples. Electrophoresis was used for digested and undigested mentioned genes PCR products for cancer and normal samples. PCR products were shown in both digested and undigested DNA in normal and cancer samples. For *RASSF1A* gene, digested DNA in cancer samples has more PCR products than digested DNA in normal samples. Thus, the RASSF1A promoter is hypermethylated in breast cancer. For *ITGA6, BCL-XL, TCF3* and *SNAIL2* genes, digested DNA in cancer samples has fewer PCR products than digested DNA in normal samples. Thus, the *ITGA6, BCL-XL, TCF3* and *SNAIL2* promoter is hypomethylated in breast cancer.

## Statistical analysis

Percent of promoter methylation change in cancer and normal samples were analyzed using gel analyzer software (GelAnalyzer 2010a). The Real time RT-PCR data analyzed with LinReg software which estimates the efficiency and Crossing Threshold (CT) for each reaction. The SPSS version 21.0 software (Chicago, SPSS Inc) was used for statistical analysis. Differences in promoter methylation of candidate genes between patient and normal samples were analyzed by T-test. Information about stages of disease, age, PR, ER and Her 2 patients were collected, and their association to the candidate genes methylation was analyzed by One-Way Analysis of Variance (ANOVA). p value<0.05 was accepted as a statistically significant.

# Results

## Methylation changes between normal and cancerous samples using RE-PCR

Digested and undigested DNA used as a template for PCR using specific primers. PCR products were assessed by Gel-doc system on 1.5% agarose gel that stained using ethidium bromide (Figure 2). Results were demonstrated that *RASSF1A* gene was hypermethylated and *BCL-X$_L$*, *ITGA6*, *TCF3* and *SNAIL2* genes were hypomethylated in breast cancer patients compared to normal samples. Promoter methylation percent of five genes in normal and cancer samples were shown in Figure 3.

## Real-time PCR

Real-Time PCR was performed to differences assessment in methylation status between normal and cancerous samples quantitatively. Results of gene amplification are demonstrated in Figure 4. Mean value comparison of ΔCT between normal and patient samples were statistically analyzed using the SPSS software. A significant difference (p-value<0.05) in all of the genes methylation was observed between patient and normal samples.

ΔCT=CT amount of digested DNA sample- CT amount of undigested DNA sample

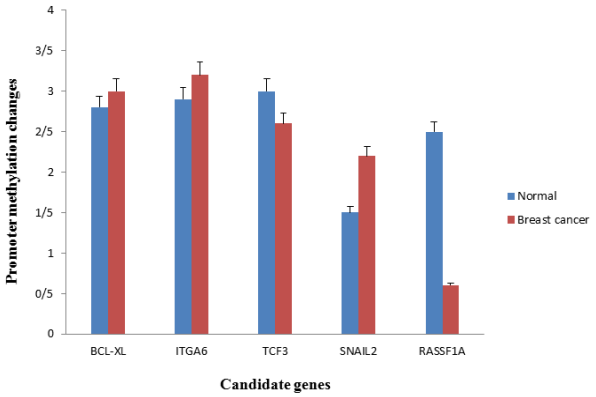


**Figure 3:** Promoter methylation percent of five genes in normal and cancer samples. Digested and undigested DNA used as a template for PCR using specific primers. PCR products were assessed electrophoresis and analyzed using gel analyzer software. Results demonstrated that *RASSF1A* and *TCF3* genes were hypermethylated and *BCL-X$_L$*, *ITGA6* and *SNAIL2* genes were hypomethylated in breast cancer patients compared to normal samples. Only methylation changes in *RASSF1A*, *TCF3* and *ITGA6* genes were significant (P-value<0.05) but methylation changes of *BCL-XL* and *SNAIL2* genes were not significant (P-value>0.05).

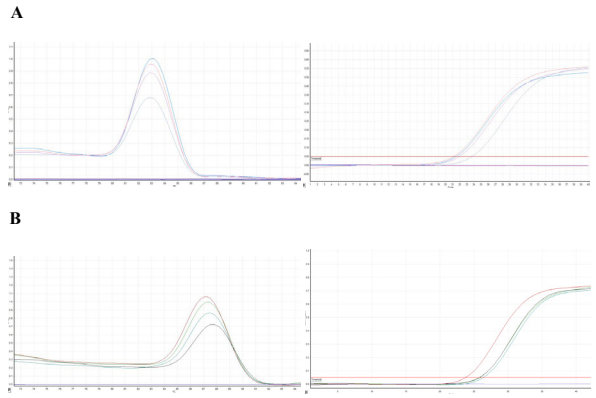


**Figure 4:** A) The melting and amplification curves of oncogenes in digested and undigested patient sample comparison to normal sample and negative control. B) The melting and amplification curves of tumor suppressor gene in digested and undigested patient sample comparison to normal sample and negative control.

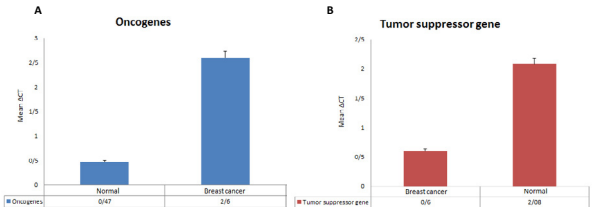


**Figure 5:** A) Mean ΔCT of oncogenes in breast cancer samples comparison to normal samples. B) Mean ΔCT of tumor suppressor gene in breast cancer samples comparison to normal samples. There is significant difference between the two groups (P-value<0.05) in oncogenes and tumor suppressor gene.

| Clinic pathological factors | Breast cancer patients (%) |
|---|---|
| **Age** | |
| <45 years | 23 (33.3) |
| ≥ 50 years | 47 (66.7) |
| **Stage** | |
| II | 5 (6.6) |
| III | 18 (26.6) |
| II, III | 36 (51.5) |
| High | 11 (15.3) |
| **Estrogen receptor** | |
| Positive | 31 (44.4) |
| Negative | 39 (55.6) |
| **Progesterone receptor** | |
| Positive | 30 (45) |
| Negative | 40 (55) |
| **Her 2** | |
| Positive | 16 (22.2) |
| Negative | 54 (77.8) |

**Table 2:** Clinic pathological factors in a population of 70 women diagnosed with breast cancer.

In *RASSF1A* gene that hypermethylated in cancerous samples compared to normal samples, the ΔCT's mean value of the normal group showed a greater amount than the patient group. Unlikely in *BCL-X*$_L$, *ITGA6*, *TCF3* and *SNAIL2* genes that hypomethylated in cancerous samples compared to normal samples, the ΔCT's mean value of the normal group showed a fewer amount than the patient group (Figure 5).

## Clinical and pathological data

The age range of normal samples was 20-35 years old and the age range of breast cancer samples was 30-70 years old. Patients with stage II (6.6%), III (26.6%), II, III (40%) and high (13.3%) were diagnosed by pathology examination. Patients were classified in three groups including 22.2% HER2$^+$, 44.4% ER$^+$, and 45% PR$^+$ (Table 2).

### Association between clinic pathological factors and candidate genes methylation changes

The relation between candidate genes methylation changes and clinic pathological factors including age, stage of cancer, HER2, PR and ER status were investigated in this study. According to statistical analysis, no significant correlation was observed between methylation changes and clinical factors in patients with breast cancer (P>0.05) except *RASSF1A* gene methylation changes that shown reverse correlation with age of patients (P<0.05).

## Discussion

Breast cancer is the most commonly detected cancer and the main reason of mortality from cancer among females, which is approximately 23% of the total cancers and 14% of the cancer deaths [37]. Changes in DNA methylation or chromatin structure has been frequently observed in cancer cells. Tumor suppressor genes are repressed in cancer cells by Hypermethylation and oncogenes are over expressed by hypomethylation. Predictive and prognostic biomarkers of breast cancer were discovered for increasing of survival rate. Discovery and use of blood-based epigenetic biomarkers are being developed [38].

In this study, the genomic DNA of white blood cells was isolated from normal and breast cancer samples. The methylation changes in five gene (*ITGA6*, *BCL-XL*, *TCF3*, *RASSF1A* and *snail2*) were assessed by RE-PCR technique. Genomic DNA was digested using methylation sensitive restriction endonucleases and PCR was performed [39]. Our study is the first to demonstrate the DNA methylation status of five gene including *ITGA6*, *BCL-XL*, *TCF3*, *RASSF1A* and *snail2* simultaneously in breast cancer cases comparison to normal cases. In the present study, we evaluated methylation in five genes in the normal and breast cancer cases. Our results showed that promoter hypermethylation of *RASSF1A* (tumor suppressor genes) and hypomethylation of four oncogenes (*ITGA6*, *BCL-XL*, *TCF3* and *snail2*) were associated with breast cancer cases in comparison to normal cases. Relationship of methylation changes and age of patients, disease stage, and the status of clinical/pathological factors such as estrogen receptor (ER), progesterone receptor (PR) and HER2 were checked in 70 patient blood samples [40]. Epigenetic molecular markers have important applications in cancer progression, diagnosis and personalization of treatment [41,42]. The cancer incidence is more common in older ages. In 50 years old women and older, 79% of new cases of breast cancer and 88% of death were happened [43]. Breast cancer specific biomarkers such as ER, PR, and HER2 can be used in prognosis and prediction [44]. Assessment of tumor aggression and select the best treatment for patients determine using ER, PR, and HER2 tests. Estrogen and progesterone receptors are necessary for tumor growth and disease progression. Hormone positive types of breast cancers respond better to the treatment [45].

### *BCL-XL*

Apoptosis were regulated by two pathways including extrinsic (FAS receptor and FAS ligand) and intrinsic (BCL2 family such as BAX and *BCL-XL*). Intrinsic pathway including activation of pro apoptotic factors such as BAX and inhibition of anti-apoptotic factors such as *BCL-XL* will happen during normal and tumor associated angiogenesis for blood vessel growth [46]. Our study showed that hypomethylation of *BCL-XL* gene is not significant in breast cancer cases comparison to normal cases (P>0.05). No correlation was seen between methylation of *BCL-XL* gene and stage of cancer (P>0.05). Data analysis show that no correlation between methylation of *BCL-XL* gene and age of patients (P>0.05). Relation between ER, PR, and HER2 status and the methylation of *BCL-XL* gene in patient samples is not significant (P>0.05). We found no correlations of *BCL-XL* methylation status with clinical/pathological factors.

Our observations suggested that *BCL-XL* hypomethylation were happened in normal and tumor associated angiogenesis in patient and normal samples and it cannot be used as molecular biomarker for breast cancer diagnosis. According to several studies, overexpression of *BCL-XL* can induce chemo resistance in cancer patients [47]. As a result, methylation status of *BCL-XL* gene in patient samples reflects the drug sensitivity or resistance in patients.

### *ITGA6*

Most solid tumors overexpress Hypoxia-Inducible Factor transcription factors (HIFs) in response to oxygen depleted situation.

HIF transcription factors induce *ITGA6* expression in the following of hypoxia condition in solid tumors. Integrin interactions with the extra cellular matrix can induce migration and invasion in cancer cells. Integrin subunit alpha (*ITGA6*) is over expressed in cancer stem cells with mesenchymal features [32]. Our study showed that hypomethylation of *ITGA6* gene is significant in breast cancer cases comparison to normal cases (P<0.05). No correlation was seen between methylation of *ITGA6* gene and stage of cancer (P>0.05). Data analysis show that no correlation between methylation of *ITGA6* gene and age of patients (P>0.05). Relation between ER, PR, and HER2 status and the methylation of *ITGA6* gene in patient samples is not significant (P>0.05). We found no correlations of *ITGA6* methylation status with clinical/pathological factors. These results show that *ITGA6* hypomethylation were happened in cancer cases in comparison to normal cases. It suggested that this gene can be used as molecular biomarker for breast cancer diagnosis. According to several studies, overexpression of *ITGA6* can induce chemo resistance in cancer patients [48]. As a result, hypomethylation of *ITGA6* gene reflects the drug resistance in patient samples.

### *TCF3*

Over expression of *Tcf3* can promote self-renewal and differentiation in stem cells of the normal or cancerous breast cells. *Tcf3* affects the tumor growth initiation ability and colony formation in breast cancer cells in the early stages of cancer [49]. Our study showed that hypermethylation of *TCF3* gene is significant in breast cancer cases comparison to normal cases (P<0.05). This result was due to the high stage of breast cancer in patients. No correlation was seen between methylation of *TCF3*gene and stage of cancer (P>0.05). Data analysis show that no correlation between methylation of *TCF3*gene and age of patients (P>0.05). Relation between ER, PR, and HER2 status and the methylation of *TCF3* gene in patient samples is not significant (P>0.05). We found no correlations of *TCF3* methylation status with clinical/pathological factors. These results show that *TCF3* hypermethylation were happened in final stages of cancer in comparison to normal cases. It suggested that this gene can be used as molecular biomarker for breast cancer diagnosis in early stages of cancer. According to several studies, overexpression of *TCF3* can induce chemo resistance in cancer patients [50]. As a result, hypomethylation of *TCF3* gene reflects the drug resistance in patient samples.

### *RASSF1A*

*RASSF1A* is a tumor suppressor gene that reduces tumor growth through connection to microtubules and protects cells from microtubule destabilizing agents. *RASSF1A* is involved in cell cycle regulation and mitotic progression. The promoter of *RASSF1A* is often hypermethylated in cancer cells [51]. Our study showed that hypermethylation of *RASSF1A* gene is significant in breast cancer cases comparison to normal cases (P<0.05). No correlation was seen between methylation of *RASSF1A* gene and stage of cancer (P>0.05). Data analysis show that reverse correlation between methylation of *RASSF1A* gene and age of patients (P<0.05). Relation between ER, PR, and HER2 status and the methylation of *RASSF1A* gene in patient samples is not significant (P>0.05). These results show that *RASSF1A* hypermethylation were happened in cancer cases in comparison to normal cases. It suggested that this gene can be used as molecular biomarker for breast cancer diagnosis. According to several studies, inhibition of *RASSF1A* expression can induce chemo resistance in cancer patients [52]. As a result, hypermethylation of *RASSF1A* gene reflects the drug resistance in patient samples.

### *Snail2*

*Snail2* (Slug) are transcription factors that regulate cell movements and induce the Epithelial-to-Mesenchymal Transition (EMT). EMT is an essential process during tumor invasion and metastasis [53]. Our study showed that hypomethylation of *snail2* gene is not significant in breast cancer cases comparison to normal cases (P>0.05). No correlation was seen between methylation of *snail2* gene and stage of cancer (P>0.05). Data analysis show that no correlation between methylation of *snail2* gene and age of patients (P>0.05). Relation between ER, PR, and HER2 status and the methylation of *snail2* gene in patient samples is not significant (P>0.05). We found no correlations of *snail2* methylation status with clinical/pathological factors. Our observations suggested that *snail2* hypomethylation cannot be used as molecular biomarker for breast cancer diagnosis. According to several studies, overexpression of *snail2* can induce chemo resistance in cancer patients [54]. As a result, methylation status of *snail2* gene in patient samples reflects the drug sensitivity or resistance in patients.

## Conclusion

In the cancer cells, DNA hypomethylation is associated to over expression of proto-oncogenes and hypermethylation is associated to inhibition of tumor suppressor genes. Based on these results, we can conclude that hypomethylation of INTGA6 and *TCF3* oncogenes and hypermethylation of *RASSF1A* tumor suppressor gene and *TCF3* (in some cases) were significant (P<0.05) in cancer samples comparison to normal samples. So, these genes can be useful as epigenetic markers in breast cancer diagnosis and treatment. According to statistical analysis, no significant correlation was observed between methylation changes and clinical factors in patients with breast cancer (P>0.05) except *RASSF1A* gene methylation changes that shown reverse correlation with age of patients (P<0.05).

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#### References

1. Vo AT, Millis RM (2012) Epigenetics and breast cancers. Obstet Gyn Int 602720.
2. Slatkin M (2009) Epigenetic inheritance and the missing heritability problem. Genet 182: 845-850.
3. Eroles P, Bosch A, Fidalgo PAJ, Lluch A (2012) Molecular biology in breast cancer: Intrinsic subtypes and signaling pathways. Cancer Treat Rev 38: 698-707.
4. Sarri D, Pinilla RMS, Hardisson D, Cano A, Bueno MG, et al. (2008) Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res 68: 989-997.
5. Clark SJ, Melki J (2002) DNA methylation and gene silencing in cancer: which is the guilty party? Oncogene 21: 5380-5387.
6. Jovanovic J, Ronneberg JA, Tost J, Kristensen V (2010) The epigenetics of breast cancer. Mol Oncol 4: 242-254.
7. Levenson VV (2010) DNA methylation as a universal biomarker. Expert Rev Mol Diagn 10: 481-488.
8. Strimbu K, Tavel JA (2010) What are biomarkers? Current Opinion in HIV and AIDS 5: 463-466.
9. Ogino S, Lochhead P, Chan TA, Nishihara R, Cho E, et al. (2013) Molecular pathological epidemiology of epigenetics: Emerging integrative science to analyze environment, host, and disease. Modern Pathol 26: 465-484.

10. Mikeska T, Craig JM (2014) DNA methylation biomarkers: cancer and beyond. Genes 5: 821-864.

11. Boise LH, García MG, Postema EC, Ding L, Lindsten T, et al. (1993) *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74: 597-608.

12. Galluzzi L, Vitale I, Abrams MJ, Alnemri SE, Baehrecke HE, et al. (2012) Molecular definitions of cell death subroutines: Recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 19: 107-120.

13. Niture SK, Jaiswal AK (2014) Inhibitor of Nrf2 (INrf2 or Keap1) protein degrades Bcl-xL via phosphoglycerate mutase 5 and controls cellular apoptosis. J biolog Chem 289: 22019.

14. Maiuri MC, Toumelin LG, Criollo A, Rain CJ, Gautier F, et al. (2007) Functional and physical interaction between Bcl-x*L* and a BH3 like domain in Beclin-1. EMBO J 26: 2527-2539.

15. Michels J, Kepp O, Senovilla L, Lissa D, Castedo M, et al. (2013) Functions of BCL-XL at the interface between cell death and metabolism. Int J Cell Biolog.

16. Choi S, Chen Z, Tang HL, Fang Y, Shin JS, et al. (2016) Bcl-xL promotes metastasis independent of its anti-apoptotic activity. Nat Commun 7: 10384.

17. Ye M, Xia B, Guo Q, Zhou F, Zhang X (2007) Association of diminished expression of RASSF1A with promoter methylation in primary gastric cancer from patients of central China. BMC cancer 7: 120.

18. Alvarez C, Garrido G, Gajardo P, Quiroz A, Cornejo V, et al. (2013) Silencing of tumor suppressor genes *RASSF1A, SLIT2,* and *WIF1* by promoter hypermethylation in hereditary breast cancer. Mol carcinogen 52: 475-487.

19. van Der Weyden L, Adams DJ (2007) The Ras-association domain family (RASSF) members and their role in human tumourigenesis. Biochim Biophys Acta 1776: 58-85.

20. El-Kalla M, Onyskiw C, Baksh S (2010) Functional importance of RASSF1A microtubule localization and polymorphisms. Oncogene 29: 5729-5740.

21. Donninger H, Barnoud T, Nelson N, Kassler S, Clark J, et al. (2011) RASSF1A and the rs2073498 cancer associated SNP. Front Oncol 1: 54.

22. Donninger H, Allen N, Henson A, Pogue J, Williams A, Gordon L, et al. (2011) Salvador protein is a tumor suppressor effector of RASSF1A with hippo pathway-independent functions. J Biol Chem 286: 18483-18491.

23. Donninger H, Clark JA, Monaghan MK, Schmidt ML, Vos M, et al. (2014) Cell cycle restriction is more important than apoptosis induction for RASSF1A tumor suppression. J Biol Chem.

24. Cadigan KM, Waterman ML (2012) TCF/LEFs and Wnt signaling in the nucleus. Cold Spring Harbor Perspectives in Biology 4: a007906.

25. Kuwahara A, Sakai H, Xu Y, Itoh Y, Hirabayashi Y, et al. (2014) Tcf3 represses Wnt–β-Catenin signaling and maintains neural stem cell population during neocortical development. PloS one 9: e94408.

26. Cole MF, Johnstone SE, Newman JJ, Kagey MH, Young RA (2008) Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. Genes Dev 22: 746-755.

27. Li C, Cai S, Wang X, Jiang Z (2014) Hypomethylation-associated up-regulation of TCF3 expression and recurrence in stage II and III colorectal cancer. PloS one 9: e112005.

28. Cubillo E, Diaz-Lopez A, Cuevas EP, Moreno-Bueno G, Peinado H, et al. (2013) E47 and Id1 interplay in epithelial-mesenchymal transition. PloS one 8: e59948.

29. Chakrabarti R, Hwang J, Andres Blanco M, Wei Y, Lukačišin M, et al. (2012) Elf5 inhibits the epithelialâ€“mesenchymal transition in mammary gland development and breast cancer metastasis by transcriptionally repressing Snail2. Nat Cell Biol 14: 1212-1222.

30. Sánchez-Tilló E, Liu Y, de Barrios O, Siles L, Fanlo L, et al. (2012) EMT-activating transcription factors in cancer: beyond EMT and tumor invasiveness. Cell Mol Life Sci 69: 3429-3456.

31. Takebe N, Warren RQ, Ivy SP (2011) Breast cancer growth and metastasis: interplay between cancer stem cells, embryonic signaling pathways and epithelial-to-mesenchymal transition. Breast Cancer Res 13: 211.

32. Brooks DL, Schwab LP, Krutilina R, Parke DN, Sethuraman A, et al. (2016) ITGA6 is directly regulated by hypoxia-inducible factors and enriches for cancer stem cell activity and invasion in metastatic breast cancer models. Mol Cancer 15: 26.

33. Kacsinta AD, Rubenstein CS, Sroka IC, Pawar S, Gard JM, et al. (2014) Intracellular modifiers of integrin alpha 6p production in aggressive prostate and breast cancer cell lines. Biochem Biophys Res Commun 454: 335-340.

34. Gupta P, Srivastava SK (2015) Inhibition of HER2-integrin signaling by Cucurbitacin B leads to *in vitro* and *in vivo* breast tumor growth suppression. Oncotarget 7: 1812-1828.

35. Hu T, Zhou R, Zhao Y, Wu G (2016) Integrin α6/Akt/Erk signaling is essential for human breast cancer resistance to radiotherapy. Sci Rep.

36. Dehbid M, Vaziri H (2016) Evaluation of DNA methylation *FGFR2* gene in Breast Cancer.

37. Heyn H, Carmona FJ, Gomez A, Ferreira HJ, Bell JT, et al. (2013) DNA methylation profiling in breast cancer discordant identical twins identifies DOK7 as novel epigenetic biomarker. Carcinogenesis 34: 102-108.

38. Jemal A, Bray F, Center MM, Ferlay J, Ward E, et al. (2011) Global cancer statistics. CA Cancer J Clin 61: 69-90.

39. Siegel RL, Miller KD, Jemal A (2015) Cancer statistics, 2015. CA Cancer J Clin 65: 5-29.

40. Bock C (2009) Epigenetic biomarker development. Epigenomics.

41. Nakayama M, Gonzalgo ML, Yegnasubramanian S, Lin X, De Marzo AM, et al. (2004) GSTP1 CpG island hypermethylation as a molecular biomarker for prostate cancer. J Cell Biochem 91: 540-552.

42. Bastian PJ, Palapattu GS, Lin X, Yegnasubramanian S, Mangold LA, et al. (2005) Preoperative serum DNA GSTP1 CpG island hypermethylation and the risk of early prostate-specific antigen recurrence following radical prostatectomy. Clin Cancer Res 11: 4037-4043.

43. Joensuu K, Leidenius M, Kero M, Andersson LC, Horwitz KB, et al. (2013) ER, PR, HER2, Ki-67 and CK5 in early and late relapsing breast cancer--reduced CK5 expression in metastases. Breast Cancer 7: 23.

44. Ludwig JA, Weinstein JN (2005) Biomarkers in cancer staging, prognosis and treatment selection. Nat Rev Cancer 5: 845-856.

45. Alteri RC, Barnes, Burke A (2013) American cancer society. Breast cancer facts and figures.

46. Watson EC, Koenig MN, Grant ZL, Whitehead L, Trounson E, et al. (2016) Apoptosis regulates endothelial cell number and capillary vessel diameter but not vessel regression during retinal angiogenesis. Development 143: 2973-2982.

47. Abdullah LN, Chow EKH (2013) Mechanisms of chemoresistance in cancer stem cells. Clin Transl Med.

48. Yamakawa N, Kaneda K, Saito Y, Ichihara E, Morishita K, et al. (2012) The increased expression of integrin α6 (ITGA6) enhances drug resistance in EVI1 high leukemia. PloS one 7: p. e30706.

49. Slyper M, Shahar A, Bar-Ziv A, Granit RZ, Hamburger T, et al. (2012) Control of breast cancer growth and initiation by the stem cells associated transcription factor TCF3. Cancer Res 72: 5613-5624.

50. Patel D, Chaudhary J (2012) Increased expression of bHLH transcription factor E2A (TCF3) in prostate cancer promotes proliferation and confers resistance to doxorubicin induced apoptosis. Biochem Biophys Res Commun 422: 146-151.

51. Dittfeld C, Richter AM, Steinmann K, Klagge-Ulonska A, Dammann RH, et al. (2012) The SARAH domain of RASSF1A and its tumor suppressor function. Mol Biol Int.

52. Lai D, Visser-Grieve S, Yang X (2012) Tumour suppressor genes in chemotherapeutic drug response. Biosci Rep 32: 361-374.

53. Villarejo A, Cortés-Cabrera A, Molina-Ortíz P, Portillo F, Cano A (2014) Differential role of *Snail1* and *Snail2* zinc fingers in E-cadherin repression and epithelial to mesenchymal transition. J Biol Chem 289: 930-941.

54. Sarrió D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, et al. (2008) Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res 68: 989-997.