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 subtype (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 are done via DNA methylation analysis of peripheral blood leukocyte DNA (PBLD) [11-15].

DNA methylation analysis of peripheral blood leukocyte DNA (PBLD) has been recently used as a biomarker for diagnosis of cancer and other diseases because of less invasive and more economic tests. DNA methylation analysis of peripheral blood leukocyte DNA (PBLD) has been recently used as a biomarker for diagnosis of cancer and other diseases because of less invasive and more economic tests.

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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-X L is critical antiapoptotic factor that is encoded by BCL2L1 gene [11]. Cancer cells prevent apoptosis by overexpression of anti-apoptotic proteins such as BCL-X L protein and suppression of pro-apoptotic proteins such as BAX and BAK proteins [12]. BCL-X L is discovered to regulate necrosis by interacting with the mitochondrial phosphatase PGAM5 [13]. BCL-X L interacts with Beclin 1 and evades the autophagy [14,15]. BCL-X L 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 TGFβ, Notch, TNFα, EGFR, FGF, hypoxia, and estrogens induce Snail2. Snail2 overexpression induces Bcl2 (antia 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 multidrug resistances [30,32]. 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 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 Nael. 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.
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-XL, 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.

\[ \Delta CT = CT \text{ amount of digested DNA sample} - CT \text{ amount of undigested DNA sample} \]

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size (bp)</th>
<th>Strand</th>
<th>Sequence (5'→3')</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL-XL</td>
<td>308</td>
<td>F</td>
<td>CGTCCTCTACTGAAACCTTG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>ACACGGAACTGGGAAAGCTC</td>
<td>60</td>
</tr>
<tr>
<td>ITGA6</td>
<td>297</td>
<td>F</td>
<td>GAGCGACAGGGGTCTATTCA</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>TCCCGAGTGTCCAAGTTGA</td>
<td>60</td>
</tr>
<tr>
<td>TCF3</td>
<td>488</td>
<td>F</td>
<td>GCCTGAGTTCTGTCAAGTC</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CTGTCGCTTGTATCCATGAC</td>
<td>60</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>144</td>
<td>F</td>
<td>GCAAGTTCTGACTGCTCAG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CATCCCTACACTGACATGC</td>
<td>60</td>
</tr>
<tr>
<td>SNAIL2</td>
<td>198</td>
<td>F</td>
<td>GAGGGAGGAGCTGAAATCTGA</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CGGTCCCTAGACATGCAC</td>
<td>60</td>
</tr>
</tbody>
</table>

Figure 2: RE-PCR analysis of five genes (SNAIL2, TCF3, BCL-XL, RASSF1A and ITGA6) 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 digest 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 digest 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
Clinic pathological factors | Breast cancer patients (%) | 
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;45 years</td>
<td>23 (33.3)</td>
</tr>
<tr>
<td>≥ 50 years</td>
<td>47 (66.7)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>5 (6.6)</td>
</tr>
<tr>
<td>III</td>
<td>18 (26.6)</td>
</tr>
<tr>
<td>II, III</td>
<td>30 (45.1)</td>
</tr>
<tr>
<td>High</td>
<td>11 (15.3)</td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>31 (44.4)</td>
</tr>
<tr>
<td>Negative</td>
<td>39 (55.6)</td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>30 (45)</td>
</tr>
<tr>
<td>Negative</td>
<td>40 (55)</td>
</tr>
<tr>
<td>Her 2</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>16 (22.2)</td>
</tr>
<tr>
<td>Negative</td>
<td>54 (77.8)</td>
</tr>
</tbody>
</table>

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. Unlike in BCL-XL, 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 INTG4A6 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).

Acknowledgements

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