**Original Article**
Middle East Journal of Cancer; October 2014; 5(4): 207-213

# Methylation Status of Tumor-Related Genes in Mcf7, Mda-Mb-468 and Bt-474 Breast Cancer Cell Lines with Different Estrogen Receptor Status

**Mozhgan Rasti*♦, Rita Arabsolghar*,**, Sajad Azmand*****

*Department of Biochemistry, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran
** Laboratory Sciences Department, Paramedical School, Shiraz University of Medical Sciences, Shiraz, Iran
***Pharmaceutical Science Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

## Abstract

**Background:** Many breast cancer tumor suppressor genes have been reported to undergo hypermethylation, including *ER, RASSF1A, HIC, CDH1, GSPT1, APC, TWIST* and *CCND2*. We first determined the promoter methylation of the above eight tumor suppressor genes as epigenetic markers of breast cancer in three cell lines with different ER status.

**Methods:** This study was an experimental study performed on three cell lines: ER-positive MCF-7, ER-negative MDA-MB-468 and the ER- and HER2-positive breast cancer cell line (BT-474). For this purpose DNA was extracted from these cell lines and gene promoter methylation was analyzed by methylation-specific PCR.

**Results:** Our results showed that all studied genes were hypermethylated in ER-negative MDA-MB-468 cells. We detected hypermethylation of *GSPT1, RASSF1A* and *CCND2* genes in these three cell lines. In addition, the ER-positive MCF-7 cells showed methylation of *TWIST* and *APC* genes.

**Conclusion:** The numbers of methylated molecular biomarkers is increased with tumor progression.This finding may be important in breast cancer therapy for different ER status.

***Keywords:*** Hypermethylation, Breast cancer, ER status

♦Corresponding Author:
Mozhgan Rasti, PhD
Department of Biochemistry, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran
Tel/Fax: +98-711-2303029
Email: rasti31@yahoo.com

## Introduction

Epigenetic alteration is one of the causes of breast cancer which induces a neoplastic process by aberrant transcriptional regulation of genes implicated in cellular proliferation, survival and differentiation.[1] DNA methylation of CpG islands which is located in the upstream promoter and Exon1 region of most tumor

Received: April 8, 2014; Accepted: June 7, 2014

suppressor genes results in transcriptional silencing in tumors[2] and is known to be an early molecular event in carcinogenesis.[3] Many tumor suppressor genes have been identified from breast neoplasms as predictive and prognostic biomarkers.[1,4] Hypermethylated genes such as *estrogen receptor α (ER), glutathion-S-transferase P1 (GSTP1), adenomatous polyposis coli (APC), ras-associated domain family 1 (RASSF1A), hypermethylated in cancer (HIC1), human basic helix-loop-helix DNA binding protein (TWIST), cyclin D2 (CCND2)* and *E-cadherin (CDH1)* are used as hallmarks of breast cancer prognosis.[1]

On the other hand, the ER-positive breast tumors response to hormone therapy and the presence of ER acts as a predictive marker. It has been reported that up to one-third of breast cancers do not express ER at the time of diagnosis and even some ER-positive breast cancers lose ER during tumor progression.[5] There are findings which suggest that ER status of breast cancer affects methylation of some breast tumor-related genes.[6,7] Methylation-specific PCR(MSP)-based studies of primary breast tumors have demonstrated that ER promoter methylation has been observed in approximately 18% of Iranian female patients.[8] To investigate the epigenetic changes of breast tumor-related genes in association with ER status, we assessed the methylation of *GSTP1, APC, RASSF1A, HIC1, TWIST, CCND2* and *CDH1* in ER-positive and ER-negative breast cancer cell lines.

The *GSTP1* gene encodes an enzyme which detoxifies hydrophobic electrophils and protects the body from environmental and dietary carcinogens.[9] Increased *GSTP1* methylation has been shown with tumor size and metastasis.[10] The *CDH1* and *APC* genes encode proteins involved in cell adhesion and their loss allows tumor cell metastasis.[11,12] Loss of *CDH1* expression is correlated with poor tumoral differentiation, increased invasion and decreased patient survival.[13,14] Our group has previously found that *CDH1* methylation was present in 41% of primary breast cancers and a correlation existed with increased tumor size and lymph node metastasis in Iranian patients.[8] *APC* plays a role in the Wnt signal transduction pathway which is involved in cell proliferation and epithelial-mesenchymal transition in epithelial breast tumors.[15] *RASSF1A* gene encodes a cellular growth-inhibitory signaling protein[16] which is involved in cell cycle control[17] and apoptosis signaling.[18,19] *RASSF1A* hypermethylation has been observed in advanced

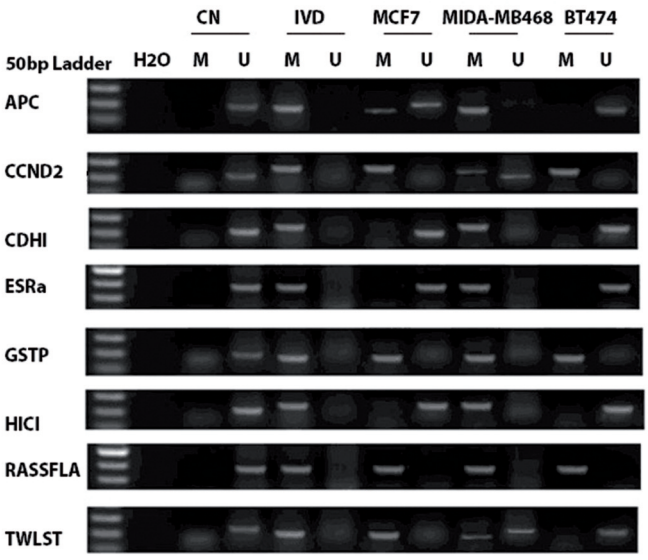


**Figure 1.** DNA methylation analysis of the indicated genes by MSP. U and M show amplified unmethylated and methylated bands, respectively. In vitro-methylated DNA (IVD) was used as a positive methylated control. CN represents DNAs from normal tissue. H2O is a negative control reaction without DNA.

**Table 1.** Methylated and unmethylated specific primer sequences.

| Name | Primer pair sequences (5' 3') | Annealing temperature (˚C) |
|---|---|---|
| CDH1-M | TTAGGTTAGAGGGTTATCGCGT | 57 |
| | TAACTAAAAATTCACCTACCGAC | |
| CDH1-U | TAATTTTAGGTTAGAGGGTTATTGT | 53 |
| | CACAACCAATCAACAACACA | |
| ESRα-M | TTTGGGATTGTATTTGTTTTCGTC | 59 |
| | ACAAAATACAAACCGTATCCCCG | |
| ESRα-U | TTTGGGATTGTATTTGTTTTTGTTG | 59 |
| | AAACAAAATACAAACCATATCCCCA | |
| HIC1-M | TCGGTTTTCGCGTTTTGTTCGT | 60 |
| | AACCGAAAACTATCAACCCTCG | |
| HIC1-U | TTGGGTTTGGTTTTTGTGTTTTG | 60 |
| | CACCCTAACACCACCCTAAC | |
| RASSF1A-M | GGGTTTTGCGAGAGCGCG | 64 |
| | GCTAACAAACGCGAACCG | |
| RASSF1A-U | GGTTTTGTGAGAGTGTGTTTAG | 64 |
| | CACTAACAAACACAAACCAAAC | |
| CCND2-M | TACGTGTTAGGGTCGATCG | 54 |
| | CGAAAACATAAAACCTCCACG | |
| CCND2-U | GTTATGTTATGTTTGTTGTATG | 54 |
| | TAAAATCCACCAACACAATCA | |
| GSTP1-M | TTCGGGGTGTAGCGGTCGTC | 61 |
| | GCCCCAATACTAAATCACGACG | |
| GSTP1-U | GATGTTTGGGGTGTAGTGGTTGTT | 61 |
| | CCACCCCAATACTAAATCACAACA | |
| TWIST-M | TTTCGGATGGGGTTGTTATCG | 61 |
| | GACGAACGCGAAACGATTTC | |
| TWIST-U | TTGGATGGGGTTGTTATTGT | 56 |
| | ACCTTCCTCCAACAAACACA | |
| APC-M | TATTGCGGAGTGCGGGTC | 60 |
| | TCGACGAACTCCCGACGA | |
| APC-U | GTGTTTTATTGTGGAGTGTGGGTT | 60 |
| | CCAATCAACAAACTCCCAACAA | |

M:Methylated-specific primers; U:Unmethylated-specific primers.

tumor stages and is associated with poor prognosis.[16,20,21] *HIC1* is aberrantly hypermethylated in breast cancers.[22] Promoter methylation of *RASSF1A* and HIC1 have been shown to occur more frequently (51% and 79%, respectively) in invasive metastatic breast tumors in Iranian patients.[23] *TWIST*1 is a transcription factor which plays an important role in cell differentiation and survival.[24,25] Increased *TWIST*1 methylation has been observed in malignant breast cancers.[26] *CCND2* is involved in cell cycle regulation and its methylation has been correlated with poor prognosis.[27]

In the present study we have investigated the methylation status of eight tumor suppressor gene promoters in three cell lines, one of which (MDA-MB-468 cell line) is methylated and the others (MCF-7 and BT-474 cell lines) are unmethylated for the ER promoter. We subsequently evaluated whether ER status in these cell lines was associated with epigenetic changes in these breast cancer markers.

## Materials and Methods

### *Cell culture and DNA extraction*

This was an experimental study. We obtained ER-negative MDA-MB-468, ER-positive MCF-7 and BT-474 (also HER2 positive) cells from the National Cell Bank of Iran (Pasture Institute, Iran). The human breast cancer cell lines MDA-MB-468, MCF-7 and BT-474 were grown in

RPMI 1640 (Biosera, UK) supplemented with L-glutamine to 2mM and 10% fetal calf serum (Cinnagen, Iran), after which they were sub-cultured 2-3 times in a week. There were 20 human normal breast tissue samples obtained from the Department of Pathology at Shiraz University of Medical Sciences, Shiraz, Iran. DNA was isolated from the cells by a standard phenol/chloroform procedure and frozen until analysis at -20˚C.

### *Methylation-specific PCR (MSP)*

We used the MSP method to study eight epigenetic breast cancer markers in three breast cancer cell lines. This method is the most widely used technique for studying the methylation of CpG islands, however it is non-quantitative.[4] Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which are then converted to thymidine during the subsequent PCR step, giving sequence differences between methylated and unmethylated DNA.[28] Genomic DNA was treated with sodium bisulfate as reported by Hermanet al.[28] Briefly, 2 µg of genomic DNA in a volume of 50 µl was denatured by NaOH (final concentration: 0.2 M) for 10-15 min at 50°C. Freshly prepared 30µl of 10 mM hydroquinone (Fluka, UK) and 520 µl of 3 M sodium bisulfite (Fluka, UK) at pH 5 were added and mixed, after which samples were incubated at 50°C for 16 h. Modified DNA was purified using a DNA purification kit (Fermentas, UK) and eluted into 50 µl of water. Extracted DNA was treated with NaOH (final concentration, 0.3 M) for 5-10 min at room temperature. DNA was ethanol precipitated and resuspended in water. Modified DNA samples were used immediately or stored at -20°C until use. Table 1 summarizes the sequence of PCR primers used to distinguish methylated and unmethylated tumor related genes and annealing temperatures. The PCR reaction mixture contained lx PCR buffer, dNTPs (each at 0.3 mM), and MgCl2 (3mM) which were all provided by Fermentas, primers (0.5 mM each per reaction), and bisulfite-modified DNA (100-150 ng) or unmodified DNA (100-150 ng) in a final volume of 25 µl. Reactions were hot-started at 94°C for 5 min before the addition of 1.25 units of *Taq* DNA polymerase (Fermentas, UK). Amplification of two genes were performed under the following conditions: 35 cycles at 95°C for 30 s, the specific annealing temperature listed in Table 1 for 30 s, and at 72°C for 30 s; and a final extension for 4 min at 72°C. Human normal DNA samples were used as the controls for unmethylated alleles. A reaction without DNA was used as the negative control for each set of PCR reactions. In each case in vitro-methylated DNA (IVD) was used as the positive control. A total of 8 µl of each PCR reaction was loaded onto a 2% agarose gel and visualized under UV illumination. The PCR for samples was repeated three times to confirm the reproducibility of the results. All analyses were performed using the t-test by SPSS version 11.5 statistical software (Chicago, IL).

## Results

### *Analysis of methylation distribution in MCF7, MDA-MB-468 and BT-474 cell lines*

We searched for aberrant promoter methylation at ERα, *GSTP1*, *APC*, *RASSF1A*, *HIC1*, *TWIST*, *CCND2* and *CDH1* gene loci in the MCF7, MDA-MB-468 and BT-474 cell lines. These data are summarized in Table 2. Our results (Figure1) showed that all studied genes were hypermethylated in ER-negative MDA-MB-468 cells. However, all of these sites were unmethylated in normal samples. We used 20 human normal DNA samples as controls. The controls were all unmethylated for the studied alleles. CpG islands are usually unmethylated in DNA from normal tissues. The results for control samples are presented in Figure 1. We detected hypermethylation of GSPT1, RASSF1A and *CCND2* genes in the three cell lines. In addition, the ER-positive MCF-7 cells showed methylation of *TWIST* and *APC* genes. We observed *CDH1* and HIC methylation in ER-negative MDA-MB-468 cells and unmethylated in ER-positive MCF7 and BT-474 cell lines.

**Table 2.** Promoter methylation status of breast tumor-related genes in breast cancer cell lines.

| Cell lines | ERα | GSTP | APC | RASSF1A | HIC1 | TWIST | CCND2 | CDH1 |
|---|---|---|---|---|---|---|---|---|
| MCF-7 | U | M | U+M | M | U | M | M | U |
| BT-474 | U | M | U | M | U | U | M | U |
| MDA-MB-468 | M | M | M | M | M | U+M | U+M | M |

U:Unmethylated; M:Methylated; U+M:Heterogenous, both methylated and unmethylated PCR products.

## Discussion

Estrogen receptors play an important role in the initiation, progression and prognosis of breast cancers. It has been reported that two-thirds of breast cancers express ER protein at levels higher than normal breast tissues. They are more differentiated and responsive to hormonal therapies. However one-third of all breast cancers are ER-negative, which are associated with poorer differentiation, higher growth fraction and worse clinical outcome compared with ER-positive tumors.[29]

Breast cancer is a disease driven by epigenetic alterations. These alterations change the expression patterns of genes implicated in cellular proliferation, survival and differentiation. Epigenetic changes occur by DNA methylation and histone modification. Tumor suppressor gene hypermethylation can lead to transcriptional silencing of these genes. Many tumor suppressor genes have been identified as epigenetic markers of breast cancer.[1] Promoter methylation as a cancer marker occurs more than mutations and is shown to be an early event in tumor genesis.[3] These molecular biomarkers have been reported as important targets for breast cancer diagnosis in early stage and therapy.[1]

We chose eight molecular biomarkers - ERα, *GSTP1*, *APC, RASSF1A, HIC1, TWIST, CCND2* and *CDH1* in the present study. We compared our results for these three cell lines with data found for methylation of these genes in ER-positive and ER-negative breast cancer tumors and cell lines. *GSTP1* gene was shown to express in ER-negative rather than ER- positive breast cancer cell lines.[30] This relationship was postulated to be the result of methylation-associated inactivation of the *GSTP1* gene by estrogen metabolites.[31] Despite this, our results showed *GSTP1* to be methylated in both ER-negative and ER-positive breast cancer cell lines. We also detected *RASSF1A* and *CCND2* methylation in ER-positive and ER-negative breast cell lines. The association of *RASSF1A* methylation and ER-positive breast cancer tumors was reported in previous studies.[6,7,32,33] However, methylation status of *CCND2*, *GSTP1*, *APC* and *TWIST*, whose promoter methylations have been reported to be associated with ER positivity of breast cancer samples,[6,7,34] failed to show correlations with ER status in other studies[33] and the current study. We found differences in the methylation of *CDH1* and HIC between ER-positive and ER-negative cell lines. In contrast, other studies showed no differences in methylation of CDH1[7,35] between ER-positive and ER-negative groups. Our previous studies in Iranian patients also showed that methylation of *RASSF1A, HIC1* and *CDH1* did not correlate with ER status.[8,23] This discrepancy might be due to the limited number of cell lines studied. In some reports HER2 status was also shown to be correlated with epigenetic alteration.[7,34] In the present study we observed differences in methylation status of *TWIST* and *APC* genes between the HER2-positive cell line (BT-474) and ER-positive cell line (MCF7). These findings showed lesser gene methylation in the HER2-positive cell line compared to the ER-positive cell line. Different studies have shown, however, that methylation of many tumor suppressor genes is not restricted to ER status. In contrast, tumor suppressor methylation occurs at high frequencies in both ER-positive and negative tumors. CpG island methylation of tumor-related genes accumulates with breast cancer progression,[33] consistent with the idea that increased numbers of methylated molecular biomarkers which started from an early stage of

breast cancer in either ER-positive or ER-negative tumors may not be restricted to the special tumor suppressor genes. It was also suggested that DNA hypermethylation makes step-wise transformation from normal to invasive breast cancer.[36]

The strong point of our study is that the MDA-MB-468 cell line which is a more progressive form of breast cancer than the other two studied cancer cell lines has shown hypermetylation of all studied epigenetic markers; however this should be researched more through the study of a large number of breast cancer cell lines.

In conclusion we demonstrated hypermethylation of all studied epigenetic markers of breast cancer in the ER-negative breast cell line, which should be additionally researched. In comparison with the other studies which reported accumulation of DNA hypermethylation in breast tumors, it could not be concluded that there was an association between many epigenetic markers of breast cancer and ER status of breast tumors. This was consistent with the idea that the number of methylated molecular biomarkers has been shown to increase with tumor progression. It seems HER2 status does not associate with promoter methylation of these epigenetic markers. This finding may be important in breast cancer therapy for different ER and HER2 status.

## Acknowledgment

This work was supported by grant no: 89-01-36-2199 from the Pharmaceutical Science Research Center, Shiraz University of Medical Sciences, Iran as Mr. Sajad Azmand's thesis.

## Conflict of interest

No conflict of interest is declared.

## References

1. Lo PK, Sukumar S. Epigenomics and breast cancer. *Pharmacogenomics.* 2008;9(12):1879-902.
2. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci USA.* 2002;99(6):3740-5.
3. Suijkerbuijk KP, van Diest PJ, van der Wall E. Improving early breast cancer detection: focus on methylation. *Ann Oncol.* 2011;22(1):24-9.
4. Suijkerbuijk KP, Pan X, van der Wall E, van Diest PJ, Vooijs M. Comparison of different promoter methylation assays in breast cancer. *Anal Cell Pathol (Amst).* 2010;33(3):133-41.
5. Giacinti L, Claudio PP, Lopez M, Giordano A. Epigenetic information and estrogen receptor alpha expression in breast cancer. *Oncologist.* 2006;11(1):1-8.
6. Feng W, Shen L, Wen S, Rosen DG, Jelinek J, Hu X, et al. Correlation between CpG methylation profiles and hormone receptor status in breast cancers. *Breast Cancer Res.* 2007;9(4):R57.
7. Sunami E, Shinozaki M, Sim MS, Nguyen SL, Vu AT, Giuliano AE, et al. Estrogen receptor and HER2/neu status affect epigenetic differences of tumor-related genes in primary breast tumors. *Breast Cancer Res.* 2008;10(3):R46.
8. Rasti M, Entezam M, Monabati A. Hypermethylation of E-Cadherin and estrogen receptor-α gene promoter and its association with clinicopathological features of breast cancer in Iranian patients. *IJMS.* 2009;34(3):186–92.
9. Key TJ, Allen NE, Spencer EA, Travis RC. Nutrition and breast cancer. *Breast.* 2003;12(6):412-6.
10. Shinozaki M, Hoon DS, Giuliano AE, Hansen NM, Wang HJ, Turner R, et al. Distinct hypermethylation profile of primary breast cancer is associated with sentinel lymph node metastasis. *Clin Cancer Res.* 2005;11(6):2156-62.
11. Caldeira JR, Prando EC, Quevedo FC, Neto FA, Rainho CA, Rogatto SR. *CDH1* promoter hypermethylation and E-cadherin protein expression in infiltrating breast cancer. *BMC Cancer.* 2006;6:48.
12. Liu Z, Yang L, Cui DX, Liu BL, Zhang XB, Ma WF, et al. Methylation status and protein expression of adenomatous polyposis coli (*APC*) gene in breast cancer [Article in Chinese]. *Ai Zheng.* 2007;26(6):586-90.
13. Debies MT, Welch DR. Genetic basis of human breast cancer metastasis. *J Mammary Gland Biol Neoplasia.* 2001;6(4):441-51.
14. Hazan RB, Qiao R, Keren R, Badano I, Suyama K. Cadherin switch in tumor progression. *Ann N Y Acad Sci.* 2004;1014:155-63.
15. Klarmann GJ, Decker A, Farrar WL. Epigenetic gene silencing in the Wnt pathway in breast cancer. *Epigenetics.* 2008;3(2):59-63.
16. Dammann R, Schagdarsurengin U, Seidel C, Strunnikova M, Rastetter M, Baier K, et al. The tumor suppressor *RASSF1A* in human carcinogenesis: an update. *Histol Histopathol.* 2005;20(2):645-63.
17. Mathe E. *RASSF1A*, the new guardian of mitosis. *Nat Genet.* 2004;36(2):117-8.
18. Oh HJ, Lee KK, Song SJ, Jin MS, Song MS, Lee JH, et al. Role of the tumor suppressor *RASSF1A* in Mst1-mediated apoptosis. *Cancer Res.* 2006;66(5):2562-9.
19. Reu FJ, Leaman DW, Maitra RR, Bae SI, Cherkassky

L, Fox MW, et al. Expression of *RASSF1A*, an epigenetically silenced tumor suppressor, overcomes resistance to apoptosis induction by interferons. *Cancer Res.* 2006;66(5):2785-93.
20. Honorio S, Agathanggelou A, Schuermann M, Pankow W, Viacava P, Maher ER, et al. Detection of *RASSF1A* aberrant promoter hypermethylation in sputum from chronic smokers and ductal carcinoma in situ from breast cancer patients. *Oncogene.* 2003;22(1):147-50.
21. Martins AT, Monteiro P, Ramalho-Carvalho J, Costa VL, Dinis-Ribeiro M, Leal C, et al. High *RASSF1A* promoter methylation levels are predictive of poor prognosis in fine-needle aspirate washings of breast cancer lesions. *Breast Cancer Res Treat.* 2011;129(1):1-9.
22. Parrella P, Scintu M, Prencipe M, Poeta ML, Gallo AP, Rabitti C, et al. HIC1 promoter methylation and 17p13.3 allelic loss in invasive ductal carcinoma of the breast. *Cancer Lett.* 2005;222(1):75-81.
23. Rasti M, Tavasoli P, Monabati A, Entezam M. Association between HIC1 and *RASSF1A* promoter hypermethylation with MTHFD1 G1958A polymorphism and clinicopathological features of breast cancer in Iranian patients. *Iran Biomed J.* 2009;13(4):199-206.
24. Lee MS, Lowe GN, Strong DD, Wergedal JE, Glackin CA. *TWIST*, a basic helix-loop-helix transcription factor, can regulate the human osteogenic lineage. *J Cell Biochem.* 1999;75(4):566-77.
25. Maestro R, Dei Tos AP, Hamamori Y, Krasnokutsky S, Sartorelli V, Kedes L, et al. *Twist* is a potential oncogene that inhibits apoptosis. *Genes Dev.* 1999; 13(17):2207-17.
26. Gort EH, Suijkerbuijk KP, Roothaan SM, Raman V, Vooijs M, van der Wall E, et al. Methylation of the *TWIST*1 promoter, *TWIST*1 mRNA levels, and immunohistochemical expression of *TWIST*1 in breast cancer. *Cancer Epidemiol Biomarkers Prev.* 2008; 17(12):3325-30.
27. Padar A, Sathyanarayana UG, Suzuki M, Maruyama R, Hsieh JT, Frenkel EP, et al. Inactivation of cyclin D2 gene in prostate cancers by aberrant promoter methylation. *Clin Cancer Res.* 2003;9(13):4730-4.
28. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA.* 1996;93(18):9821-6.
29. Yan L, Yang X, Davidson NE. Role of DNA methylation and histone acetylation in steroid receptor expression in breast cancer. *J Mammary Gland Biol Neoplasia.* 2001;6(2):183-92.
30. Jhaveri MS, Morrow CS. Methylation-mediated regulation of the glutathione S-transferase P1 gene in human breast cancer cells. *Gene.* 1998;210(1):1-7.
31. Cavalieri EL, Stack DE, Devanesan PD, Todorovic R, Dwivedy I, Higginbotham S, et al. Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proc Natl Acad Sci USA.* 1997; 94(20):10937-42.
32. Muggerud AA, Rønneberg JA, Wärnberg F, Botling J, Busato F, Jovanovic J, et al. Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and PTEN in ductal carcinoma in situ and early invasive breast cancer. *Breast Cancer Res.* 2010;12(1):R3.
33. Park SY, Kwon HJ, Lee HE, Ryu HS, Kim SW, Kim JH, et al. Promoter CpG island hypermethylation during breast cancer progression. *Virchows Arch.* 2011;458(1):73-84.
34. Swift-Scanlan T, Vang R, Blackford A, Fackler MJ, Sukumar S. Methylated genes in breast cancer: associations with clinical and histopathological features in a familial breast cancer cohort. *Cancer Biol Ther.* 2011;11(10):853-65.
35. Li S, Rong M, Iacopetta B. DNA hypermethylation in breast cancer and its association with clinicopathological features. *Cancer Lett.* 2006;237(2):272-80.
36. Chen KM, Stephen JK, Raju U, Worsham MJ. Delineating an Epigenetic Continuum for Initiation, Transformation and Progression to Breast Cancer. *Cancers (Basel).* 2011;3(2):1580-92.