EPIGENETIC MODIFICATIONS OF p16, E-CADHERIN, RARβ AND DAPK GENE PROMOTERS IN BREAST CANCER

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The breast cancer is the result of multiple associations of genetic and epigenetic alterations, which lead to overexpression of the proto-oncogenes and loss of expression of tumor suppressor genes. Such new expression patterns determine the cell genetic reprogramming results in the oncogenesis process and therefore may represent possible important markers for diagnostic and prognostic in cancer therapy. The epigenetic process is frequently associated with the aberrant silencing of tumor suppressor genes through the CpG islands located in their promoter region. These regions are normally unmethylated while in tumor cells are abnormally hypermethylated and associated with transcriptional silencing. Such local hypermethylation processes represent frequently an alternative mechanism for mutations of tumor suppressor genes, being an early and ordinary process for many tumor types, including breast cancer.
The aim of this study is to analyze the promoter methylation pattern of some tumor suppressor genes (p16, E-cadherin, RARβ and DAPK) in invasive ductal breast cancers, in order to identify new molecular markers for diagnosis and prognosis.

Key words: Breast cancer; DNA methylation; Tumor suppressor gene.

INTRODUCTION

Tumor suppressor genes protect cells from undergoing malignant transformation and function by one of the following mechanisms: defend the genome from mutagenic events, impede deregulated progression through the cell cycle, induce apoptosis in cells that escape normal cell cycle controls, and inhibit cellular migration and metastasis. Tumor suppressor genes have been described to acquire loss of function mutations or deletions leading to their inability to impede malignant transformation. Alternatively, epigenetic events, such as methylation, represent a distinct mechanism of tumor suppressor gene inactivation. Aberrant gene promoter methylation is associated with gene silencing and is functionally equivalent to a mutated gene.

The aim of this study is to analyze the promoter methylation pattern of some tumor suppressor genes (p16, E-cadherin, RARβ and DAPK) which proved to be critical in breast cancer.

The p16 gene encodes a cyclin-dependent kinase inhibitor, p16INK4A, which regulates the transition from G1- to S-phase *via* its effect on Rb phosphorylation.

In many cancers, including breast cancer the loss of p16INK4A function was related to genetic modifications (deletions and point mutation in the promoter region) and epigenetic modification (methylation of the gene promoter or the first exon)[1].

The E-cadherin gene encodes a cell-surface adhesion protein that is important in maintaining homophilic cell–cell adhesion in epithelial tissues

Evidence shows that loss of expression and function of E-cadherin protein contributes to increased proliferation, invasion and metastasis in breast cancer.[2] Genetic alterations like point mutations and deletions clearly play a role in loss of the E-cadherin expression and function[3,4] which was correlated with the increase of cell division rate, tumor development, invasion and metastasis.[5] Several studies demonstrated that epigenetic silencing of the E-cadherin gene by 5′CpG methylation occurs in some human breast cancer cell lines too as well as about 50% of unselected primary breast cancers.[5]

The RARβ gene is a member of the super-family of nuclear receptors and its role is in growth limiting of different tumor types: breast, pulmonary, etc.

Recently, it was found that RARβ was not expressed in a number of malignant tumors, including lung carcinoma, squamous cell carcinoma of head and neck, and breast cancer[6, 7].

DAPK – Death-associated protein kinase 1 is a positive mediator of gamma-interferon induced programmed cell death.

The first hint for involvement of DAP- kinase in the tumorigenesis process emerged from the finding that DAP-kinase expression was lost in various tumor cell lines.[8] In this respect it was found that DAP-kinase mRNA and protein expression levels were below detection limits in 80% of B-cell lymphoma and leukemia cell lines, and in 30–40% of cell lines derived from bladder, breast, and renal cell carcinomas.

MATERIALS AND METHODS

Biological samples consisted in 25 breast tumor specimens surgically obtained from patients (age between 42–62 years). All patients were diagnosed with invasive ductal carcinoma in different stages, according the American Joint Committee on Cancer (AJCC) TNM system, after the clinical and histological exam. The percentage of tumor stages is summarized below:

DNA extraction was performed using High Pure PCR Template Preparation Kit (Roche). Cells were lysed during a short incubation with Proteinase K in the presence of a chaotropic salt, which immediately inactivate all nucleases. The lysate was then applied to a High Pure Spin Filter Tube. Under the buffer conditions used in the procedure, nucleic acids bound to the filter in the High Pure tube, while contaminating substances (salts, proteins and other cellular contaminants) did not. Brief wash-and-spin steps readily removed those contaminants. Once purified, DNAs were eluted in 50 µl volume of low salt buffer and stored at -20^0C.

Unmethylated C residues conversion was performed with bisulphite treatment using EpiTect Bisulfite kit (Qiagen). Incubation of the target DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged.

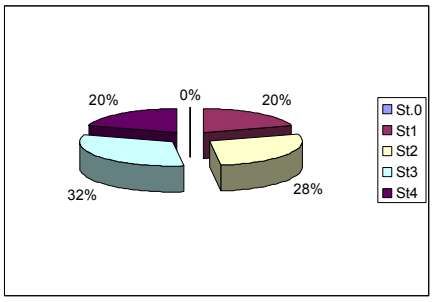

Fig. 1 Tumor stages distribution:

(Stage 0: Tis, N0, M0; Stage I: T1, N0, M0; Stage–II: IIA - T0, N1, M0 / T1, N1, M0 / T2, N0, M0; IIB - T2, N1, M0 / T3, N0, M0; Stage–III: IIIA - T0-2, N2, M0 / T3, N1-2, M0; IIIB - T4, N0-2, M0; IIIC - T0-4, N3, M0; Stage IV: T0-4, N0-3, M1).

MS-PCR. The design of selected primers for methylated and unmethylated sequences discriminates between the methylated and unmethylated status of the gene promoter and does not allow primers to align with other DNA sequences. The primers were designed using the online bioinformatics tool MethPrimer. The program converts the DNA sequence in a DNA sequence bisulphite treated and shows the density of targeted CpG islands. A typical MS-PCR consists of two different types of PCR, using the same DNA sample treated with bisulphite, and the primer pairs for methylated and unmethylated sequences. A DNA isolated from blood belonging to a healthy person was treated with bisulphite and used as negative control.

The sequences of the primers used:

p16

M
Forward(5’-3’): TTATTAGAGGGTGGGGCGGATCGC
Reverse(5’-3”): GACCCCGAACCGCGACCGTAA

U
Forward(5’-3’): TTATTAGAGGGTCGGTGGATTTGT
Reverse(5’-3”): CAACCCCAAACCACAACCATAA
Product size M: 150pb; U: 151pb

E - cadherin

M
Forward(5’-3’): GTGGGCGGGGTCGTTAGTTTC
Reverse:(5’3”):
CTCACAAATACTTTACAATTCCGACG
U
Forward(5’-3’): GGTGGGTGGGTTGTTAGTTTGT
Reverse(5’-3”): AACTCACAAATCTTTACAATTCCAAC
Product size M: 110pb; U: 100pb

RARβ2

M
Forward(5’-3’): GGTTAGTAGTTCGGGTAGGGTTTATC
Reverse(5’-3”): CCGAATCCTACCCCGACG
U
Forward(5’-3’): TTAGTAGTTGGGTAGGGTTTATT
Reverse(5’-3”): CCAAATCCTACCCCAACA
Product size M: 235pb; U: 233pb

DAPK

M
Forward(5'-3'): GGATAGTCGGATCGAGTTAACGTC
Reverse(5'-3"): CCCTCCCAAACGCCGA

U
Forward(5'-3'): GGAGGATAGTTGGATTGAGTTAATGT
Reverse(5'-3"): CAAATCCCTCCCAAACACCAA
Product size M: 98pb; U: 106pb

MS-PCR of the target sequences was performed using Platinum® Taq DNA Polymerase (Invitrogen) which presents a high fidelity for copying and could amplify traces of DNA.

The protocol for PCR is described in Table 1.

Table 1

The protocol used for PCR

PCR reaction components	Volume	Final concentration
10X PCR Buffer, Minus Mg	2,5µl	1X
10 mM dNTP mix (Invitrogen)	0,5 µl	0,2mM each
50 mM MgCl2	0,75 µl	1,5mM
Primer mix (10 µM each)	0,5 µl	0,2 µM each
Template DNA	1 µl	Depending on concentration
Platinum® *Taq* DNA Polymerase	0,1 µl	1 unit
Water nuclease free	19,65 µl	
Final volume	25 µl	

Table 2

The PCR program for each target gene

	P16	RARβ2	E-cadh	DAPK
Initial denaturation	94°C/12'	94°C/12'	94°C/5'	94°C/12'
Denaturation	94°C/1'	94°C/1'	94°C/45"	94°C/1'
Aligner	60°C/1'	58°C/1'	54°C/45"	58°C/1'
Elongation	72°C/1'	72°C/1'	72°C/45"	72°C/1'
Final elongation	72°C/7'	72°C/7'	72°C/7'	72°C/7'
Cycle number	35	35	35	35

RESULTS AND DISCUSSION

p16

According to literature methylation of the promoter and exon 1 regions was observed in both human breast cancer cell lines and 20–30% of primary breast cancers.[9, 10]

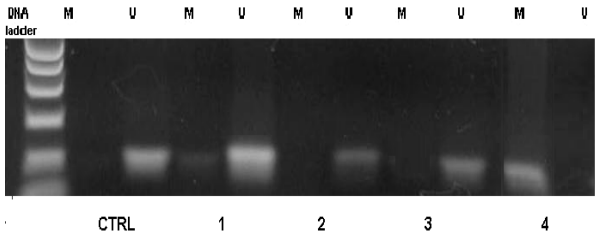

Fig. 2. The electrophoresis pattern for methylation status of p16 gene promoter.

DNA samples which were bisulphite treated shows both methylated and unmethylated status of gene p16 promoter. p16 promoter was found to be methylated in 48% of cases (12 of 25).

The p16 promoter methylation has been suggested as an early detection marker of breast cancer because it has been detected in early lesions.

E - cadherin

Previous studies demonstrated that hyper-methylation of the E-cadherin CpG islands was evident in about 30% of ductal carcinomas *in situ* and significantly increased to nearly 60% of metastatic lesions, suggesting a role of this process in tumor progression.[11]

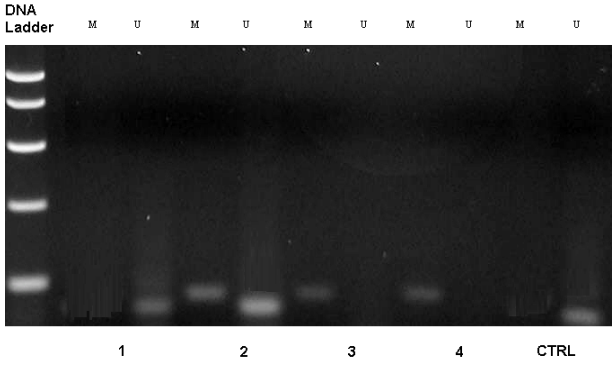

Fig. 3. The electrophoresis pattern for methylation status of E-cadherin gene promoter.

A number of studies have shown that E-cadherin is completely inactivated in invasive lobular carcinoma. According Mastracci *et al.* study, we can conclude that lobular lesions, whether hyperplasia or carcinoma in situ, lack E-cadherin expression.[12]

We found that in invasive ductal carcinomas the E-cadherin gene promoter is methylated in about 61.5% of advanced stages with lymph node

metastasis and 52% of all cases (13 of 25). E-cadherin gene is one of the first targets for epigenetic silencing in association with CpG island hypermethylation within the promoter region and is presumably involved in the observed high mitotic activity, poorer tumor differentiation, and increased tendency for regional lymph node metastases.

RAR β

High frequency of RAR β methylation was observed in breast cancer metastasis in bones, brains and lungs RAR β has been reported to be methylated in 20–42% breast cancers.[13]

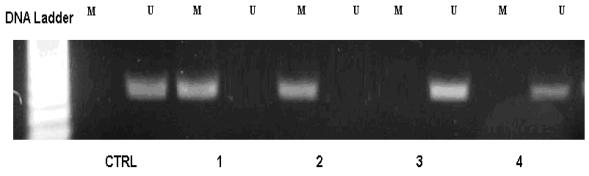

Fig. 4. The electrophoresis pattern for methylation status of RAR β gene promoter.

RARβ gene promoter shows a hypermethylated pattern in a percentage 56% of patients (14 of 25).

All of these findings support the concept that the specific loss of RAR β expression may be an important event in oncogenesis.

DAPK

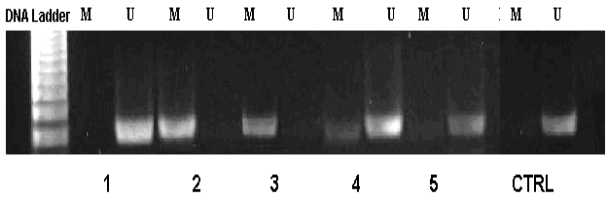

Fig. 5. The electrophoresis pattern for methylation status of DAPK gene promoter.

Our study shows that the promoter of DAPK gene was hypermethylated in about 64% of high grade stages of breast cancer. It has also been shown that loss of DAPK gene expression was associated with aggressive and metastatic phenotype in many tumor types, so the high percentage of gene promoter methylation in advanced stages of breast cancer provided a prognosis about the metastatic invasion.[14]

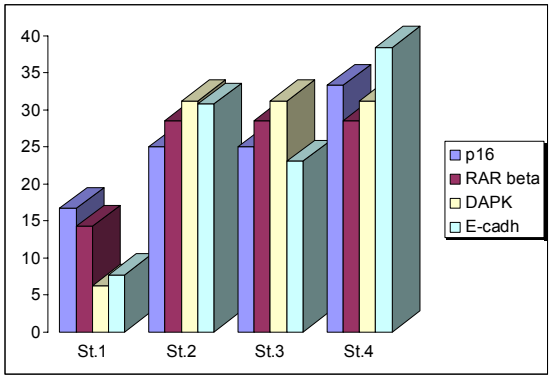

Fig.6. The distribution of methylated target genes in each stage of breast cancer.

We note that the percent of gene promoter methylation for RARβ and p16 is higher than for DAPK and E-cadherin in the stage I, but the promoter methylation percentage for all genes is increased in the next stages. For E-cadherin the highest percent is observed in stage IV.

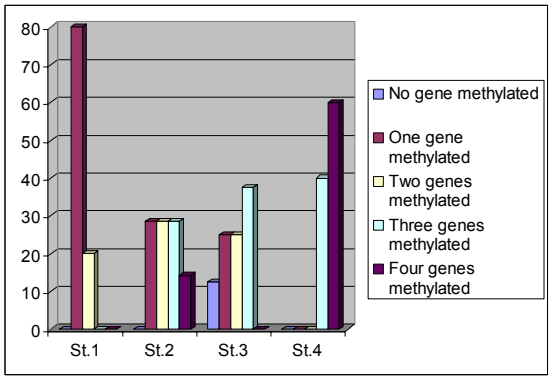

Fig.7. Number of genes with methylated promoters in different breast cancer stages.

The methylation pattern of gene promoters can be a marker for disease progression. In our study the number of methylated gene promoters is increasing with the advancement of disease.

CONCLUSIONS

The methylation of RARβ and p16 promoter is present in many cases starting with first stage of breast cancer, and might considered as early detection markers. DAPK and E-cadherin gene promoters are not methylated in stage I of breast cancer, but are highly methylated in advanced cancers. Methylation of E-cadherin gene promoter starting with stage II of breast cancer can be a marker for early metastasis.

The methylation status evaluation of the of tumor suppressor gene promoters during the breast oncogenesis processes may provide important data for the molecular tumor phenotype, aggressively level, proliferation, metastasis, angiogenesis, and therefore it can be used for the establishment the optimum way to treat this disease.

Methylation of tumor suppressor genes promoter is a frequent phenomenon in the development of a tumor cell. This phenomenon is added by genetic alterations, and other epigenetic changes like chromatin remodeling and RNA interference.

REFERENCES

1. Liu, T., Niu, Y., Feng, Y., Niu, R., Yu,Y., Lv,. A., Yang, Y., *Methylation of CpG islands of p16INK4a and cyclinD1 overexpression associated with progression of intraductal proliferative lesions of the breast, Human Pathology*, **2008**, Volume 39, Issue 11, Pages 1637 – 1646.
2. Ilyas, M., Tomlinson, I.P., *The interactions of APC, E-cadherin and beta-catenin in tumour development and progression. Journal of Pathology,* **1997**, 182, 128–137.
3. Bringuier, P.P., Umbas, R., Schaafsma, H.E., Karthaus, H.F., Debruyne, F.M., Schalken, J.A., *Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors*, *Cancer Research,* **1993**, 53**,** 3241–3245.
4. Graff, J.R., Herman, J.G., Lapidus, R.G., Chopra, H., Xu, R., Jarrard, D.F., Isaacs, W.B., Pitha, P.M., Davidson, N.E., Baylin, S.B., *E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Research,* **1995**, 55, 5195–5199.
5. Hiraguri, S., Godfrey, T., Nakamura, H., Graff, J., Collins, C., Shayesteh, L., Doggett, N., Johnson, K., Wheelock, M., Herman, J., Baylin, S., Pinkel, D., Gray, J., *Mechanisms of inactivation of E-cadherin in breast cancer cell lines, Cancer Research,* **1998,** 58**,** 1972–1977.
6. Houle, B., Rochette-Egly, C., Bradley, W.E.C., *Tumor-suppressive effect of the α retinoic acid receptor in human epidermoid lung cancer cells. Proc. Natl. Acad. Sci.USA***, 1993**, 90, 985–89.
7. Xu, X-C., Ro, J. Y., Lee, J. S., Shin, D.M., Hong, W.K., Lotan, R., *Differential expression of nuclear retinoid receptors in normal, premalignant and malignant headand neck tissues. Cancer Res*., **1994,** 54, 3580–3587.
8. Kissil, J., Feinstein, E., Cohen, O., Jones, P.A., Tsai, Y.C., Knowles, M.A., Eydmann, M.E., Kimchi, A., *DAP-kinase loss of expression in various carcinoma and B cell lymphoma cell lines: possible implications for role as tumor suppressor gene, Oncogene,* **1997**, 15, 403–407.
9. Sharpless, N.E., DePinho, R.A., 1 *The INK4A/ARF locus and its two gene products*,*Current Opinion in Genetics and Development,* ***1*999**, 9, 22–30.
10. Woodcock, D.M., Linsenmeyer, M.E., Doherty, J.P., Warren, W.D., *DNA methylation in the promoter region of the p16 (CDKN2/MTS–1/INK4A) gene in human breast tumors. British Journal of Cancer,* **1999**, 79, 251–256.
11. Nass, S.J., Herman, J.G., Gabrielson, E., Iversen, P.W., Parl, F.F., Davidson, N.E., Graff, J.R., *Aberrant methylation of the estrogen receptor and E-cadherin 5'CpG islands increases with malignant progression in human breast cancer*, *Cancer Research,* **2000**,60, 4346– 4348.
12. Mastracci, T. L., Tjan, S., Bane, A.L., O'Malley, F.P., Andrulis, I.L., *E-cadherin alterations in atypical lobular hyperplasia and lobular carcinoma in situ of the breas, Modern Pathology,* **2005**, 18, 741–751.
13. Shukla, S., Mirza, S., Sharma, G., Parshad, R., Gupta S.D., Ralhan, R.; *Detection of RASSF1A and RARbeta hypermethylation in serum DNA from breast cancer patients,* **2006,**1(2), 88–93.
14. Jeronimo, C., Monteiro, P., Henrique, R., Dinis-Ribeiro, M., Costa, I., Costa, V.L., Filipe, L., Carvalho,A.L., Hoque, M.O., Pais, I., Leal, C., Teixeira, M.R., Sidransky, D., *Quantitative hypermethylation of a small panel of genes augments the diagnostic accuracy in fine-needle aspirate washings of breast lesions, Breast Cancer Research and Treatment,* **2007**, 109(1), 27–34.