STUDY OF EPIGENETIC ALTERATION OF THE PROMOTER REGION OF P16, BRCA1, GSTP1 GENES AND SPORADIC BREAST CANCER

E. VARRIALE[1], A. SANGERMANO[2], L. POSTIGLIONE[3], M. DI PAOLO[4-5], A.P. TOMMASELLI[4], L. MORELLI[4], D. DELL'EDERA[6]

[1]UOS di Oncologia Ospedale Fatebenefratelli, Naples, Italy
[2]Dia-Chem srl Biologia Molecolare, Naples, Italy
[3]Dipartimento di Scienze Mediche Translazionali, Università degli Studi "Federico II", Naples, Italy
[4]CETAC Research Center, Caserta, Italy
[5]GORI, Gruppo Oncologi Ricercatori Italiani, Pordenone, Italy
[6]Presidio Ospedaliero "Madonna delle Grazie", Azienda Sanitaria di Matera, Matera, Italy

Absract – *Background:* Breast cancer is a heterogeneous disease, presents various pathological signs such as axillary lymph node metastasis which is associated with a high risk of recurrence and considered as an important prognosis factor in the early stages of the disease.
Invasion and metastasis are two important hallmarks of malignant tumors caused by complex genetic and epigenetic alterations. The present study investigated the contribution of aberrant methylation profiles of cancer related genes, BRCA1, GSTP1, and, p16 (CDKN2A), in the sporadic Breast Cancer biopsy.
Patients and Methods: A total of 35 subjects, (7 healthy women with a family hystory of breast cancer and 26 with monolateral breast cancer) have been studied in order to detect the hypermethylation status of the promoter region of the following genes: p16, GSTP1 and BRCA1.
Results: The DNA methylation analysis of the candidate genes showed higher methylation proportion in the primary tumor tissue than that of the matched normal tissue and the differences were significant for the, BRCA1, and p16, promoter regions ($p<0.05$). Among those candidate methylated genes, BRCA1 and p16 displayed higher methylation proportion in the matched lymph node metastasis than that found in the normal tissue ($p<0.05$).
Conclusions: The results of the present study showed methylation heterogeneity between primary tumors and metastatic lesion. The pathway analysis revealed that, BRCA1 and p16 have a role in prevention of neoplasm metastasis. The contribution of aberrant methylation alterations of, BRCA1and p16 genes in lymph node metastasis might provide a further sign to establish useful biomarkers for screening metastasis. In summary, our DNA methylated assay performed from blood cells from BRCA1 carriers is able to predict breast cancer risk years in advance of diagnosis.

KEY WORDS: *Methylation, Metastasis, Breast cancer, Biomarker.*

INTRODUCTION

Breast cancer is one of the most common malignancies with a high mortality rate among women Cancer is a multistep process resulting from an accumulation of genetic mutations leading to dysfunction of critical genes, including tumors T suppressor genes. Epigenetic changes are

recognized as an alternative mechanism of gene inactivation. The aberrant methylation of the promoter region of a gene can lead to silencing and contribute to the development of tumors. Hypermetylation of DNA is a post-synthetic modification which, by transferring methyl group from S-adenosylmetionine to 5methyl-Cytosine (5mC) as a new base on DNA. Promoter regions CpG islands are usually unmethylated in all normal tissues. Their aberrant methylation cause the transcriptional repression of the gene. The silencing of oncosuppressor genes may lead to the inactivation of the apoptotic pathway in different stages: at the beginning (p14-DAPkinase) – at the end (caspase) – in independent pathway (p15-p16), as previously described in breast cancer[1].

The phenomenon of regional DNA hypermethylation and silencing of tumor suppressor genes in cancer has been the focus of attention in the last decade. A significant amount of data has established a list of genes hypermethylated in cancer and recently whole genome approaches have identified methylation signatures of breast cancer cells[2]. These methylation signatures, which are the unique combination of methylated CpG islands in a cancer cell were correlated with breast cancer stage and have been proposed to be a diagnostic marker of breast cancer cells. In addition to their diagnostic value in breast cancer it is clear from the repertoire of methylated genes that silencing of these genes by DNA methylation plays a role in the transformation process. Amongst the methylated genes are tumor suppressor genes such as p16 whose methylation is proposed to silence this gene and override cell growth regulatory signals. Another group of methylated genes in breast cancer is composed of damage response genes such as BRCA1, which is also mutated in familial breast cancer and glutathione detoxification-system GSTP1[3].

DNA methylation is a naturally occurring event in both prokaryotic and eukaryotic organisms. In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA, and in higher eukaryotes DNA methylation functions in the regulation/control of gene expression. It has been demonstrated that aberrant DNA methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis. DNA methylation has also been shown to play a central role in gene imprinting, embryonic development, X-chromosome gene silencing, and cell cycle regulation. DNA methylation consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme. The majority of DNA methylation in mammals occurs in 5'-CpG-3' dinucleotides, but other methylation patterns do exist. In fact, about 80 percent of all 5'-CpG-3' dinucleotides in mammalian genomes are found to be methylated, whereas the majority of the twenty percent that remain unmethylated are within promoters or in the first exons of genes.

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, as well as many other important aspects of biology. To date, a number of methods have been developed to detect/quantify DNA methylation including: high-performance capillary electrophoresis and methylation-sensitive arbitrarily primed PCR[4]. However, the crucial step is the isolation of DNA[5]. To date, For 5'-CpG-3' detection, the most common technique used remains the bisulfite conversion method[5]. This technique involves treating methylated DNA with bisulfite, which converts unmethylated cytosines into uracil. Methylated cytosines remain unchanged during the treatment. Once converted, the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing.

The aim of this study is the evaluation of epigenetic alteration of p16, GSTP1 and BRCA1 as early biomarker of sporadic neoplastic process of breast.

MATERIALS AND METHODS

35 patients, among them 7 healthy women with a family hystory of breast cancer and 26 with monolateral breast cancer have been studied in order to detect the hypermethylation status of the promoter region of the following genes: p16, GSTP1 and BRCA1. We performed the MSP technique (Methylation Specific PCR), which allows, using specific primers for the methylated and unmethylated sequences, to detecte specifically the methylated regions using DNA extracted by ductal wash, fixed tissue, biopsy or serum. A commercial kit, manufactured by DIA-CHEM srl, (Naples, Italy), has been used. The assay has been performed in accordance with the instruction of the manufacturer. The procedure consists of 4 steps: 1) extraction of DNA; 2) Modification of extracted DNA with sodium bisulfite; 3) amplification; 4) detection on agarose gel.

Free DNA Isolation
Free DNA was isolated according to DIA-CHEM's protocol. Modification with sodium bisulfite:DNA was chemically modified with sodium bisulfite, which converts all unmethylated cytosines to uracil whyle all methylated cytosine remain unchanged.
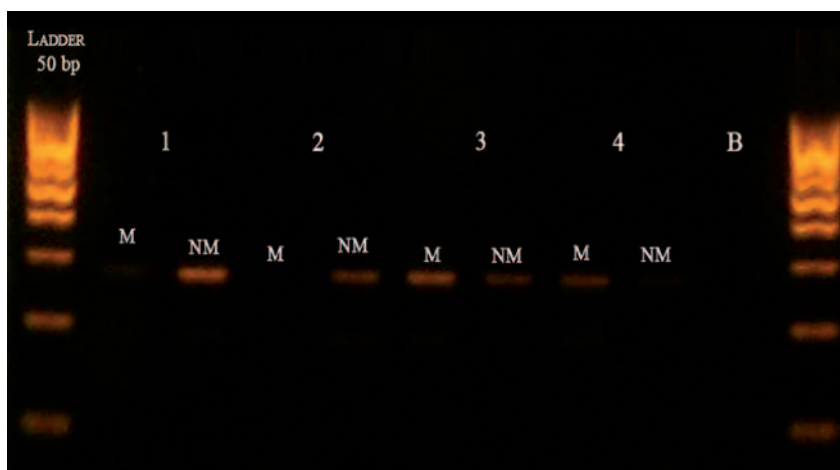
Fig. 1. Electrophoresis results of Me-
thylation Sensitivity-PCR.
M: Methylated signal
NM: No-Methylsted signal
B: Blank (no template control)
Samples for examples from 1 to 4

Methylation bisulfite conversion of DNA

Based on the three-step reaction that takes place between cytosine and sodium bisulfite where cytosine is converted into uracil. The product's innovative in-column desulphonation technology eliminates otherwise cumbersome precipitations. The process is designed to reduce template degradation, minimize DNA loss during treatment and clean-up, while ensuring complete conversion of the DNA

Amplification Step

The extracted DNA of every sample, after the modification step with sodium bisulfite, is amplified using both pair of primers (for methylated and unmethylated sequences). We analyzed three genes (p16, GSTP1and BRCA1), so we performed six PCR for every sample.

Detection on Agarose Gel:

The MSP products were analyzed by gel electrophoresys in 3% agarose gel stained with ethydium bromide and visualized under UV light.

Statistical Differential Methylation Analysis

From the *BRCA1* study, differentially methylated CpGs, with false discovery rate (FDR) cor-rected *p* values, between *BRCA1* mutant carriers and *BRCA1* wild type samples were identified via a multivariate logistic regression that was adjusted for age, batch and the presence of cancer.

RESULTS

The results agree with the purpose of this study, that is to show the correlation between the hypermethylation status and cancer and the presence of the promoter hypermethylation as a DNA marker for sensitive early tumour detection, allowing the monitoring of high risk healty patients and follow-up of surgical patients. Hypermethylation of gene promoters can occur early in the development of tumours. Consequently, hyper-methylated genes represent potential biological markers for early diagnosis, and could prove to be an useful tool. This is especially important in breast cancer, where early detection can incraise the chances of curative therapy but tools for early detection are still relatively limited[7]. Moreover, with exiting methods of detecting methylation, only minute quantities of biological material are required for screeningVisualized under UV light the results are shown in Table 1.

Table 1. Results of DNA methylation assay on 5' promotorial region of the selected genes.

	Healthy patients	Pre-CT patients	Post-CT patients
Subject (n)	7	28	28
Methylated P16	1 (7%)	17 (60,7%)	0
Unmethylated P16	7	28	28
Methylated BRCA1	1 (7%)	6 (21,4%)	0
Unmethylated BRCA1	7	28	28
Methylated GSTP1	0(0%)	21 (75,%)	1
Unmethylated GSTP1	7	28	28

DISCUSSION

Here we have provided several news lines of clinical evidence indicating that methylation profiles obtained in cells from women with a *BRCA1* mutation have the potential to indicate preventive breast cancer development many years in advance of diagnosis. Our findings also show that genes encoding developmental transcription factors integral for stem cell differentiation and biology are hypermethylated in women predisposed to breast cancer.

Our data suggest that the *BRCA1*-associated DNA methylated signature is a risk predicting signature rather than an early detection signature, because: 1) the DNA methylation signature was derived from WBCs in women with a known *BRCA1* status and was adjusted for cancer status (analysis included *BRCA1* carriers without cancer at the time of sample draw); 2) the time from sample draw to diagnosis had no dramatic impact on the strength of association between DNA methylated and potential for breast cancer development; 3) the signature was validated in two independent cohorts; 4) we observed a very strong overlap of CpGs associated with *BRCA1* mutation and CpGs indicating future breast cancer risk; and finally 5) the signature was also associated with invasive non-breast cancers.

The observation that the top ranked hypermethylated *BRCA1*-mutation associated CpGs are highly enriched for which we and others have previously shown to be an epigenetic hallmark of cancer tissue and which are among the earliest, if not the earliest, molecular changes in human carcinogenesis was an exciting finding because it fully supports recent data demonstrating that a *BRCA1* defect leads to retargeting of the other pathways and reduces cell differentiation[8].

Two key issues remain unclear. First, which factors lead to a *BRCA1*-mutation DNA methylated pattern in the absence of a *BRCA1* mutation? It is likely that a combination of risk factors or factors which we have not captured (for example, early life events, transgenerational inheritance, and so on) contribute to epigenetic modifications which are in common to those associated with *BRCA1* mutation. Second, is the *BRCA1*-mutation DNA methylated signature in White Blood Cells (WBCs) functionally relevant or just simply an indicator of breast cancer risk? The fact that the signature is indicative of breast cancer mortality would support the view that faint epigenetic misprogramming of immune cells may lead to general immune defects which in turn supports the development and proliferation of cancers. However, all these suggestions are highly provisional and need validation in further independent cohorts using well-defined subsets of patients[9].

There are limitations to this study. First, we analysed whole blood DNA or serum DNA representing whole blood DNA and not a specific subset of peripheral blood cells. Second, although we found some good preliminary evidence that DNA methylated profiles in buccal cells didn't predicting breast cancer risk (data not shown), we did not analyse buccal cells from *BRCA1* mutation carriers, nor did we have access to independent prospective buccal cell data.

In summary, our data highlight DNA methylated analysis as a promising tool to predict future breast cancer development. Future epigenome-wide studies should focus on using epithelial cells like buccal – or epithelial cells from the uterine cervix which are hormone sensitive and more likely to capture an 'epigenetic record' of breast cancer risk factors. Such studies are more likely to provide the level of specificity and sensitivity which is required for a clinically useful risk prediction tool[10].

CONCLUSIONS

Our DNA methylated assay performed from blood cells from *BRCA1* carriers is able to predict breast cancer risk years in advance of diagnosis. Our data further support the notion that DNA methylated modification on selected genes, even in unrelated tissues, is an early event associated with carcinogenesis.

Conflict of interest statement: The Authors declare that they have no conflict of interests.

REFERENCES

1. Silva J, Silva JM, Dominguez G, Garcia JM, Cantos B, Rodriguez R, Larrondo FJ, Provencio M, España P, Bonilla F. Concomitant expression of p16INK4a and p14ARF in primary breast cancer and analysis of inactivation mechanisms. J Pathol 2003; 199: 289-2897.
2. Huang TH, Laux DE, Hamlin BC, Tran P, Tran H, Lubahn DB. Identification of DNA methylation markers for human breast carcinomas using the methylation-sensitive restriction fingerprinting technique. Cancer Res 1997; 57: 1030-1034
3. Sinisi AA, Varriale E, Sangermano A, Lucarelli E, Pizzorusso M, Barletta O, Dell'Edera D. Hypermethylation of glutatione-s-transferasi (GSTP1) as plasmatic molecular biomarker for prostate cancer. WCRJ 2014; 1: e403.
4. De Monaco A, D'Orta A, Fierro C, Di Paolo M, Cilenti L, Di Francia R, Rational selection of PCR-based platforms for pharmacogenomic testing WCRJ 2014; 1: e391.

5. Rainone A, Troise A, Morelli CD, Berretta M, De Lucia L. Comparing methods for isolation of circulating-DNA (C-DNA). Implication of C-DNA as biomarker in the cancer. WCRJ 2014; 1: e38.

6. Huang TH, Perry MR, Laux DE. Methylation profiling of CpG islands in human breast cancer cells. Hum Mol Genet 1999; 8: 459-470.

7. Hu XC, Wong IH, Chow LW. Tumor-derived aberrant methylation in plasma of invasive ductal breast cancer patients: clinical implications. Oncol Rep 2003; 10: 1811-1815.

8. Zhuang J, Jones A, Lee SH, Ng E, Fiegl H, Zikan M, Cibula D, Sargent A, Salvesen HB, Jacobs IJ, Kitchener HC, Teschendorff AE, Widschwendter M. The dynamics and prognostic potential of DNA methylation changes at stem cell gene loci in women's cancer. PLoS Genet 2012; 8: e1002517.

9. Dalzovo L. About a "Cancer Survivor". WCRJ 2014; 1: e252.

10. Berretta M, Di Francia R, Tirelli U. Editorial – The new oncologic challenges in the 3rd millennium WCRJ 2014; 1: e133.