Insights in Breast Cancer

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Importance of Epigenetic Testing in Breast Cancer Patients [Version 1, Awaiting Peer Review]

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Introduction

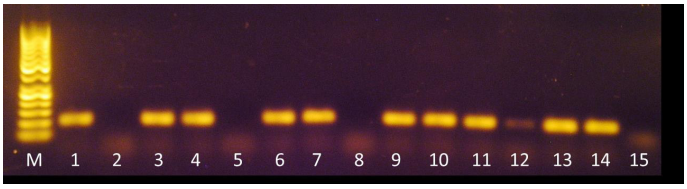
Frequency of breast cancer is increasing worldwide and is the most common cancer in women in most countries. The screening of mammary carcinoma is performed in the female population nationwide anywhere in the world. However, we have no available screening methods that would be effective and would meet the criteria of simplicity, non-invasiveness and financial difficulty. As is well known, the deregulation of epigenetic mechanisms in conjunction with genetic changes causes some genetic diseases, including cancer. Epigenetic mechanisms are spontaneous and reversible, they represent differences in DNA methylation and in the structure of chromatin. The number and pattern of methylated cytosines affects the function of the gene, low methylation leads to high activity, and vice versa. It plays an essential role during the process of differentiation and development of vertebrates, gene imprinting and X chromosome inactivation. In DNA isolated from malignant tumor cells, abnormal hypermethylation of CpG islands was observed in the promoter regions of genes, one of the major regulatory regions of the gene. In malignant tumors, transcription of tumor suppressor genes is also suppressed as a result of DNA methylation, and this affects cell cycle regulation. One of the options for targeting cancer treatment is to regain the gene expression by DNA-inhibition of methyltransferases. The constant position of abnormal CpG islands methylation in the tumor suppressor gene promoter region allows for a simpler detection strategy as is possible for many common mutations in cancer. This implies that the same primer combination could be used for all patients to detect tumor-specific methylation changes of the gene by means of a methylation-sensitive, high-resolution analysis of the melting curves. These promoter hypermethylation characteristics, along with the fact that all tumors have 1 or more hypermethylated loci, make these changes valuable as DNA markers for sensitive and early tumor detection. Thanks to the knowledge gained from gene expression regulation studies, it is possible to identify the specific causes of the disease and to develop therapeutic strategies on the basis of this, which could lead to the restoration of the normal epigenetic state [1-3].

Materials and Methods

Immunohistopathological classification including the tumor (pT), degree of differentiation (G), lymph node (pN) status, estrogen (ER) and progesterone (PR) receptor statuses, and HER2 gene amplification were determined by a qualified pathologist. The tumor type was evaluated on the basis of the WHO classification of tumors [4,5], the histological stage, and the classification was performed in accordance with the revised pTNM classification of AJCC and UICC [6]. The estrogen and progesterone receptor status was expressed as a percentage of positive reactive cells from the total number of captured cells. A positive ER or PR state was considered to be a case in which ≥ 1% of the cells were positive [7]. The HER2 amplification status was detected by immunocytochemistry (HerceptTest) and evaluated according to the criteria currently published by ACSO / CAP 2007 [8]. DNA for the purpose of the methylation analysis was subjected to deparaffinization from the paraffin sections of the tissue in the tube. The DNA extraction itself was performed by using DNeasy Blood & Tissue Kit (Qiagen, Germany), where we followed the manufacturer's recommendations. The method of the methylation-specific PCR (MSP) was performed on a MiniOpticon PCR Real-Time PCR system (Bio-Rad, Applied Biosystems, California, USA). For MSP, internal primers were used for both the methylated and non-methylated sequence of the *RASSF1A* gene. The MSP analysis allowed detection of the presence of methylation in genomic DNA by comparison with the commercially available methylated and unmethylated DNA standards (EpiTect, Qiagen, Germany). All statistical tests were performed using R software [9]. Statistically significant P values were equal to or less than 0.05 (P≤0.05)

Results

We found the association of the methylation status of the *RASSF1A* gene with histopathological parameters. The association of the methylation status of the *RASSF1A* gene with the tumor grade (G) was significant in all grades (P <0.0001 for G1, G2 and G3). Significant association was observed between stage 3 of ductal breast cancer and the positive methylation status of the *RASSF1A* gene (p <0.05). The metastatic status of the tumor stage (pT) was significantly associated with the methylation of *RASSF1A* gene (P <0.0001 for pT1 and pT2, P <0.05 for pT4). The relationship between the level of methylation and the type of breast cancer was strongly associated only in the ductal invasive carcinoma (DIC) (P <0.0001) samples for the *RASSF1A* gene. Presence or absence of methylation in *RASSF1A* promoter region was investigated by MSP in 116 samples of primary breast tumors, where we found out methylated alleles in 97 (83,6%) samples.



Observation 1: Result from electrophoresis in agarose gel shows representative results from MSP method. The presence of MSP products in lines 3,4,6,7,9,10,11,12,13,14 confirms presence of methylated alleles in promoter region of RASSF1A gene. The absence of MSP products in lines 5 and 8 confirms presence of unmethylated alleles in RASSF1A gene. Line 1 is methylated DNA standard and in line 2 is unmethylated DNA standard. Line "M" shows 100bp marker, and line 15 is negative control. Product size of RASSF1A gene is 198 bp.

Discussion

Recent studies show the diagnostic and prognostic potential of *RASSF1A* methylation. In relation to breast cancer, the incidence of *RASSF1A* methylation occurs in many independent studies in varying degrees. Honorio et al [10] demonstrated the presence of the *RASSF1A* promoter methylation in 65% invasive

breast cancer and 42% of the relevant ductal carcinoma in situ (DCSI). Burbee et al [11] recording 49% primary breast tumors with methylated *RASSF1A*. The largest occurrence of methylation of the *RASSF1A* promoter (95%) in breast cancer tissue was recorded by Yeo et al [12]. A recent study found a link between ER-positive and ER-negative status in relation to epigenetic changes in genes associated with breast cancer, including the *RASSF1A* gene [13].

Conclusion

Breast cancer is a heterogeneous disease with very different responses to therapy and varying lengths of survival. Overall is determined by histopathological criteria based on the size, level of invasiveness and infiltration of lymph nodes, and on the immunochemical characteristics of cell surface receptors, including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). In many cases, however, determining the degree of breast cancer is not sufficient to predict prognosis or response to treatment for the enormous heterogeneity of the disease. Therefore, new classifications approaches based on epigenetic patterns are now emerging. DNA methylation is a strong epigenetic biomarker, significantly more stable than RNA or proteins, and is therefore a promising target for developing new approaches to determining the diagnosis and prognosis of breast and other tumors. The aim of this study was to evaluate the presence of methylation of the *RASSF1A* promoter region and its potential relationship with the immunohistopathological characteristics of breast cancer. Our results suggest that hypermethylated gene loci may exhibit a methylated state during carcinogenesis, and in relation to the histopathological parameters, methylation appears to be an early tumor transformation event.

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