The Methylation Status of Retinoic Acid Receptor Beta 2 (RARb2) Gene and Breast Cancer Risk: A Case-Control Study

Joe Joseph¹, Manju P.A², Jaya Vinny Eapen³, Anu Yamuna Joseph⁴

ABSTRACT

Introduction: Breast cancer is a heterogeneous disease with very different therapeutic responses and outcomes. Aberrant hypermethylation of tumor-suppressor genes is found frequently in primary breast tumors and has been implicated in disease initiation and progression. Use of molecular markers such as DNA methylation as a diagnostic technique is recommended since methylation is a frequent event in breast cancer and can be detected at an early stage. The objective of this study was the analysis of variation in the DNA methylation patterns of RARb2 gene in the plasma DNA of breast cancer patients.

Material and Methods: DNA was isolated from the plasma of breast cancer patients and controls. The isolated DNA was modified by bisulfite treatment. The presence or absence of hypermethylation in RARb2 gene was analysed by conventional methylation specific PCR (MSP). The methylation pattern in the control and affected samples was obtained and statistical analysis was done to determine whether there is any association between hypermethylation of RARb2 gene and breast cancer in the samples under study.

Results: It was observed that 92% of the breast cancer samples showed hypermethylation whereas only 14% of the control samples showed hypermethylation in the RARb2 gene. Statistical analysis showed significant association between breast cancer and RARb2 methylation. The results showed that the methylation of RARβ2 was associated with susceptibility to breast cancer.

Conclusion: This study confirms the detection of hypermethylation of RARb2 in breast cancer patients. The findings point to the possibility of using RARb2 methylation as a molecular marker for the diagnosis of breast cancer.

Key-words: RARb2, Breast Cancer, Hypermethylation, Molecular Marker, MSPCR

INTRODUCTION

Breast cancer is the second leading cause of cancer death among women after lung cancer. Over 1.1 million women worldwide are diagnosed with breast cancer each year and more than 410,000 of them die from this disease. Breast cancer accounts for 10% of all new cancer cases and for greater than 1.6% of mortality in women globally. Breast cancer was found to be the most common cancer in urban women and second most common cancer in the rural women of India. A number of screening tests have been employed including clinical and self breast exams, mammography, genetic screening, ultrasound and magnetic resonance imaging. Mammographic screening for breast cancer uses x-rays to examine the breast for any characteristic masses or lumps. In developing countries, the extensive laboratory and clinical infrastructure required for mammographic screening and the high cost of mammography are major limiting factors in the diagnosis of breast cancer. Biopsies and tissue sampling require expert skill and tissue sample is usually removed surgically. New screening methods that could improve the detection would be highly beneficial for the timely diagnosis of breast cancer.

Cancer biomarkers are widely used for the early detection of cancers since they are measurable before overt cancer is clinically detectable. These molecular markers have high predictive accuracy, is easily measurable and reproducible, minimally invasive and acceptable to physicians. Epigenetic changes, such as DNA methylation is one of the most common molecular alterations in human neoplasia including breast cancer. DNA methylation as a molecular marker has numerous favorable characteristics. It is an early event in breast carcinogenesis. Since DNA methylation is stable and detectable by PCR, aberrations can be easily analyzed from small amounts of sample. Moreover, the studies showing high concordance between the epigenetic alterations found in primary tumor specimens and in plasma suggest methylation analysis as a potential non-invasive screen for early cancer detection. Hypermethylation of a number of tumour suppressor genes have been reported to be associated with breast cancer. Retinoic acid receptor beta-2 (RARb2) is known to have a critical role in the chemopreventive action of retinoids. It has been confirmed as a tumour suppressor gene whose reduced expression has been linked to many cancers. The role of RARb2 hypermethylation in breast cancer has been investigated in a number of studies. In 2000, Widschwendter et al. suggested methylation of the RAR-beta2 gene as an initial event in breast carcinogenesis. RARb2 methylation has been observed in in situ lesions of breast cancer.

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from both lobular and ductal cancers. Aberrant promoter methylation on RARb2 has been reported by Vasilatos et al. in 2009. Hypermethylation of RARb2 along with Rassf1A was reported by Yamamoto et al. in 2012.

**MATERIAL AND METHODS**

**Sample Collection**

In the present case-control study, fifty women from Kerala, India who were diagnosed with breast cancer were enrolled as cases and fifty healthy females were enrolled as controls. Informed consent was obtained from all the participants. Approval of research on human subjects was obtained from the Institutional Ethics Committee. After interviewing the participants, 3 ml blood samples were collected from them and stored at -80°C until further processing.

**Genomic DNA Extraction**

DNA was isolated from human blood plasma using GenElute blood genomic DNA kit (Sigma-Aldrich). 200µl blood plasma was treated with Proteinase K and DNA was extracted from these samples following manufacturer's protocol.

**Methylation specific-nested PCR**

Methylation specific PCR (MSP) was used to detect the presence or absence of hypermethylation in the RARb2 gene. Bisulfite modification is the process in which sodium bisulfite is used to determine the methylation patterns of DNA. Bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. Thus, bisulfite treatment introduces specific changes in the DNA sequence that depends on the methylation status of individual cytosine residues. The plasma DNA was modified using the EZ DNA Methylation-Gold kit (Zymoresearch). In MSP, methylated primers will anneal to those regions containing 5’ methyl cytosine while unmethylated primers will bind to bisulphite converted regions. Three sets of primers were used for MS-nested PCR.

**Stage I PCR**

PCR reaction was carried out in 20µl reaction volume, containing 1µl of the modified plasma DNA, 1.5mM MgCl₂, 0.2mM dNTP’s, 10 picomoles of each outer primers, 1 unit of Taq DNA polymerase and 1X Taq buffer. Cycling conditions consisted of an initial denaturation of 10 minutes at 95°C, followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. A final elongation of 72°C was given for 10 minutes. The PCR products were analysed on 2% agarose gel containing ethidium bromide.

**STATISTICAL ANALYSIS**

The significance of association of RARb2 hypermethylation between breast cancer and normal samples was determined using the chi-square test. Odds ratios (OR) and 95% confidence intervals (95% CI) were measured using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Statistical significance was assumed at the p < 0.05 level.

**RESULTS**

The mean age of the patients was 52.48 ± 12.05 years (range 31–70 years). 9 (18%) patients were aged below 40 years. RARb2 methylation was considered to be present if the samples gave amplification with the methylation specific primers. The RARb2 gene was considered unmethylated if the samples gave amplification only with the unmethylation specific primers and not with the methylation specific primers. When the fifty cancer samples were analysed by PCR, 46 samples gave amplification with methylation specific primers and very mild amplification with unmethylation specific primers. Four cancer samples gave amplification only with unmethylation specific primers indicating the absence of hypermethylation in these samples. Out of the 50 control samples, only 7 samples showed amplification with both methylation specific primers and unmethylation specific primers while 43 samples showed amplification only with unmethylation specific primers.

Out of the fifty cancer samples, hypermethylation was observed in 46 samples (92%) while it was not observed in 4 samples (8%). Among the control samples, only 7 samples (14%) showed hypermethylation while 43 samples (86%) did not show hypermethylation ($\chi^2 = 61.06, p<0.001$). The results show that the methylation of RARb2 was associated with susceptibility to breast cancer (OR = 70.64 95% CI 19.31 – 258.4 (Table 1)).

<table>
<thead>
<tr>
<th>RARb2 hyper methylation</th>
<th>Without Breast cancer</th>
<th>With breast Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count</td>
<td>Percent</td>
</tr>
<tr>
<td>Absent</td>
<td>43</td>
<td>86.0</td>
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<tr>
<td>Present</td>
<td>7</td>
<td>14.0</td>
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$\chi^2 = 61.06, p<0.001$

**Table-1:** Association of RARb2 hypermethylation with breast cancer
DISCUSSION

In this study the potential of hypermethylation as a molecular marker for the detection of breast cancer was analysed. Our study was focused on tumor suppressor gene RARb2. Reduced expression of RARb2 protein is a frequent phenomenon in many human cancers. A number of studies on breast cancer had shown the association between RARb2 hypermethylation and breast cancer.

Our study showed that all the patients who were recently diagnosed with breast cancer were highly methylated. Although most of the control samples did not show any methylation some samples showed faint methylation indicating that they might be partially methylated. This is in accordance with the previous reports where DNA methylation has been reported in non-malignant tissues [17]. Our study was conducted using DNA isolated from blood plasma. Plasma DNA has been previously used for genetic and epigenetic variation studies associated with cancer [18,19]. It has been proved that plasma contains DNA from malignant cells which is modified by hypermethylation and can be used as a molecular tool for the detection of methylation [20]. In this study, it was found that patients with breast cancer showed different methylation patterns when compared to the control population.

A number of studies have suggested that the methylation profiles of cancers may be ethnicity specific [21,22]. It has been proposed that that differences in exposure patterns among racial/ethnic subgroups might lead to differences in cancer susceptibility, irrespective of any intrinsic genetic differences between groups. Also, breast cancer prognosis was found to be considerably influenced by the ethnicity [23]. These findings advocate the necessity for the development of population specific molecular markers for the diagnosis and prognostic prediction of breast cancer. MSP of RARb2 using circulating DNA is a sensitive and specific technique and it may be used as a diagnostic tool for detecting breast cancer at an early stage. Development of novel biomarkers with diagnostic value will facilitate effective monitoring of the disease.

CONCLUSION

Breast cancer is a growing epidemic in many developing countries like India. Early diagnosis is crucial for the effective management of the disease. Hypermethylation of promoter region of RARb2 gene can be detected in plasma DNA during the early stages of breast cancer. This makes it a potential candidate to become a diagnostic tool. Since this study was done with limited number of breast cancer samples, it requires validation with a larger number of samples before accepting it as a screening method.

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