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

Joe Joseph[1], Manju P.A[2], Jaya Vinny Eapen[3], Anu Yamuna Joseph[4]

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[1] . Breast cancer was found to be the most common cancer in urban women and second most common cancer in the rural women of India [2].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[3]. 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 [4]. Epigenetic changes, such as DNA methylation is one of the most common molecular alterations in human neoplasia [5,6] including breast cancer[7].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 [8]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[9,10,11].The role of RARb2 hypermethylation in breast cancer has been investigated in a number of studies. In 2000, Widschwendter et al[12].suggested methylation of the RAR-beta2 gene as an initial event in breast carcinogenesis. RARb2 methylation has been observed in *in situ* lesions

[1]Associate Professor, Department of Medicine, Government Medical College, Ernakulam, [2]Assistant Professor, Department of Surgery, Government Medical College, Ernakulam, [3]Assistant Professor, Department of Biotechnology, Mar Athanasius college, Kothamanagalam, [4]Principal Scientist, Primordia Lifesciences, KRIBS-BIONEST, Kochi

Corresponding author: Dr. Manju P.A, Assistant Professor, Department of Surgery, Government Medical College, Ernakulam-683503, Kerala, India

How to cite this article: Joseph J, Manju PA, Eapen JV, Joseph AY. The methylation status of retinoic acid receptor beta 2 (RARb2) gene and breast cancer risk: a case-control study . International Journal of Contemporary Medical Research 2021;8(6):F1-F4.

DOI: http://dx.doi.org/10.21276/ijcmr.2021.8.6.5

BY-NC-ND

from both lobular and ductal cancers [13].Aberrant promoter metylation on RARb2 has been reported by Vasilatos et al,[14] in 2009. Hypermethylation of RARb2 along with Rassf1A was reported by Yamamoto et al[15], 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, bisufite 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[16].

Stage I PCR

PCR reaction was carried out in 20µl reaction volume, containing 1µl of the modified plasma DNA, 1.5mM $MgCl_2$, 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 95ºC, followed by 40 cycles of 94ºC for 30 seconds, 56ºC for 30 seconds and 72ºC for 30 seconds. A final elongation of 72ºC was given for 10 minutes. The amplifications were carried out in eppendorf master cycler personal with heated lid.

Stage II PCR

Two separate PCR reactions were done with two sets of internal primers for methylated and unmethylated regions of RARb2. Stage IIa PCR used methylation specific primers for amplifying the methylated region of the gene while stage IIb PCR used primers for amplification of the unmethylated regions of RARb2 gene. PCR reaction was carried out in 20µl reaction volume, containing 2µl of the stage I PCR product,1.5mM $MgCl_2$,0.2mM dNTPs,10 pico moles of each primer, 1 unit of Taq DNA polymerase and 1X Taq buffer. Cycling conditions consisted of an initial denaturation of 10 minutes at 95ºC, followed by 39 cycles of 94ºC for 30 seconds, 60˚C for 30 seconds and 72ºC for 30 seconds. A final elongation of 72ºC was given for 10 minutes. The PCR products were analysed on 2% agarose gel containing ethidium bromide.

STATISTICAL ANALYSIS

The significance of asscociation of RARb2 hypermethylation between breast cancer and normal samples was determined using the chi-squaretest. 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 umnethylation 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 (χ^2 =61.06, p<0.001). The results show that the methylation of *RARβ2* was associated with susceptibility to breast cancer (OR =70.64 95% CI 19.31 – 258.4 (Table 1).

RARb2 hyper methylation	Without Breast cancer		With breast Cancer		Odds (95% CI)
	Count	Percent	Count	Percent	
Absent	43	86.0	4	8.0	1
Present	7	14.0	46	92.0	70.64 (19.31 – 258.4)
$\chi2$ =61.06, p<0.001					

Table-1: Association of RARb2 hyper methylation 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, irrespectively 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.

REFERENCES

1. Parkin DM , Bray F , Ferlay J.Global cancer statistics. CA: A Cancer Journal for Clinicians, 2002; 55: 74-108
2. National Cancer Registry Programme, Indian Council of Medical Research. Leading sites of cancer. In, Consolidated Report of Population Based Cancer Registries 2001-2004, Incidence and Distribution of Cancer. Bangalore: Coordinating Unit, National Cancer Registry Programme (ICMR) 2006.8-30.
3. Kösters JP, Gøtzsche PC. Regular self-examination or clinical examination for early detection of breast cancer. Cochrane Database of Systematic Reviews. 2003(2).
4. Ramirez JL, Taron M, Balana C, Sarries C, Mendez P, de Aguirre I, Nunez L, Roig B, Queralt C, Botia M, Rosell R. Serum DNA as a tool for cancer patient management. Rocz Akad Med Bialymst. 2003;48:34-41.
5. Baylln SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. Advances in cancer research. 1997 Jan 1;72:141-96.
6. Bird A. The essentials of DNA methylation. Cell. 1992 ;70:5-8.
7. Müller HM, Widschwendter A, Fiegl H, Ivarsson L, Goebel G, Perkmann E, Marth C, Widschwendter M. DNA methylation in serum of breast cancer patients: an independent prognostic marker. Cancer research. 2003 ;63:7641-5.
8. Yang HJ, Liu VW, Wang Y, Chan KY, Tsang PC, Khoo US, Cheung AN, Ngan HY. Detection of hypermethylated genes in tumor and plasma of cervical cancer patients. Gynecologic oncology. 2004;93:435-40.
9. Yari K, Payandeh M, Rahimi Z. Association of the hypermethylation status of PTEN tumor suppressor gene with the risk of breast cancer among Kurdish population from Western Iran. Tumor Biology. 2016 ;37:8145-52.
10. Ivanova T, Petrenko A, Gritsko T, Vinokourova S, Eshilev E, Kobzeva V, Kisseljov F, Kisseljova N. Methylation and silencing of the retinoic acid receptor-β2 gene in cervical cancer. BMC cancer. 2002 ;2:1-7.
11. Xu XC, Ro JY, Lee JS, Shin DM, Hong WK, Lotan R. Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues. Cancer Research. 1994;54:3580-7.
12. Widschwendter M, Berger J, Hermann M, Müller HM, Amberger A, Zeschnigk M, Widschwendter A, Abendstein B, Zeimet AG, Daxenbichler G, Marth C. Methylation and silencing of the retinoic acid receptor-β2 gene in breast cancer. Journal of the National Cancer Institute. 2000;92:826-32.
13. Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, Sukumar S, Argani P. DNA methylation of RASSF1A, HIN-1, RAR-β, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. International journal of cancer. 2003;107:970-5.
14. Vasilatos SN, Broadwater G, Barry WT, Baker JC, Lem S, Dietze EC, Bean GR, Bryson AD, Pilie PG, Goldenberg V, Skaar D. CpG island tumor suppressor promoter methylation in non-BRCA-associated early mammary carcinogenesis. Cancer Epidemiology and Prevention Biomarkers. 2009 ;18:901-14.
15. Yamamoto N, Nakayama T, Kajita M, Miyake T, Iwamoto T, Kim SJ, Sakai A, Ishihara H, Tamaki Y, Noguchi S. Detection of aberrant promoter methylation of GSTP1, RASSF1A, and RARβ2 in serum DNA of

patients with breast cancer by a newly established one-step methylation-specific PCR assay. Breast cancer research and treatment. 2012;132:165-73.
16. House MG, Guo M, Iacobuzio-Donahue C, Herman JG. Molecular progression of promoter methylation in intraductal papillary mucinous neoplasms (IPMN) of the pancreas. Carcinogenesis. 2003;24:193-8.
17. Hoque MO, Feng Q, Toure P, Dem A, Critchlow CW, Hawes SE, Wood T, Jeronimo C, Rosenbaum E, Stern J, Yu M. Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. Journal of Clinical Oncology. 2006;24:4262-9.
18. Sozzi G, Musso K, Ratcliffe C, Goldstraw P, Pierotti MA, Pastorino U. Detection of microsatellite alterations in plasma DNA of non-small cell lung cancer patients: a prospect for early diagnosis. Clinical cancer research. 1999;5:2689-92.
19. Gonzalez R, Silva JM, Sanchez A, Dominguez G, Garcia JM, qi Chen X, Stroun M, Provencio M, Espana P, Anker P, Bonilla F. Microsatellite alterations and TP53 mutations in plasma DNA of small-cell lung cancer patients: follow-up study and prognostic significance. Annals of oncology. 2000;11:1097-104.
20. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer research. 2001;61:1659-65.
21. Mehrotra J, Ganpat MM, Kanaan Y, Fackler MJ, McVeigh M, Lahti-Domenici J, Polyak K, Argani P, Naab T, Garrett E, Parmigiani G. Estrogen receptor/progesterone receptor-negative breast cancers of young African-American women have a higher frequency of methylation of multiple genes than those of Caucasian women1. Clinical Cancer Research. 2004;10:2052-7.
22. Piyathilake CJ, Henao O, Frost AR, Macaluso M, Bell WC, Johanning GL, Heimburger DC, Niveleau A, Grizzle WE. Race-and age-dependent alterations in global methylation of DNA in squamous cell carcinoma of the lung (United States). Cancer Causes & Control. 2003;14:37-42.
23. Chlebowski RT, Chen Z, Anderson GL, Rohan T, Aragak, A, Lane D et al. Ethnicity and breast cancer: factors influencing differences in incidence and outcome. Journal of the National Cancer Institute. 2005; 97: 439-448.

Source of Support: Nil; **Conflict of Interest:** None

Submitted: 12-04-2021; **Accepted:** 07-05-2021; **Published:** 25-06-2021