Asian Journal of Biological and Life Sciences | Original Research

Circadian Rhythm and Breast Cancer Susceptibility - A study on PERIODIC3 Gene Polymorphisms in Breast Cancer

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Submitted : 15.06.2014 Accepted : 08.07.2014 Published : 30.08.2014

Abstract

Breast Cancer is one of the common cancers that is on the rise globally. Many risk factors have been implicated for the occurrence of the breast cancer. Recent studies show evidence towards a disruption in the circadian rhythm which could be a risk factor towards breast cancer mediated through the altered melatonin secretion which, in turn disrupts the reproductive hormones. PERIODIC 3 gene (Per 3) is one of the circadian rhythm genes that is being studied widely in several disease states. Various factors can disrupt circadian rhythm and alter normal nocturnal production of melatonin and hormonal changes influencing breast cancer aetiology. Our study sought to validate these findings in an Indian population with a case control study of 20 Breast Cancer cases and 10 non-neoplastic controls using breast tissue as our tissue of interest (in contrast to blood as in the Caucasian study). Results: Our study revealed a 55% of the samples with heterozygous alleles of the VNTR polymorphism of Exon 18 of the Per 3 gene amongst Breast Cancer tissue while the remaining 45 % were homozygous 4 repeat sequences. All ten controls that were analysed showed the presence of a 4 repeat homozygous configuration which was statistically significant ($p<0.05$). This study thus finds a statistically significant correlation between the presence of a heterozygous repeat sequence in Exon 18 of the Per 3 gene and breast carcinoma. However more studies with a larger sample size are required to confirm these findings.

Key words : Breast Cancer, Circadian Rhythm, PER3 gene

INTRODUCTION

Breast Cancer is one of the leading causes of cancer mortality in females and its incidence has been steadily increasing worldwide. [1] It is a disease of significant morbidity and mortality among adult females and research is ongoing on its genetic association and newer therapeutic possibilities.

Several of the body's physiological functions are regulated by the biological clock or the circadian rhythm. This has its master control in the suprachiasmatic nucleus in the hypothalamus and synchronised peripheral oscillators in various other tissues. These oscillators are controlled by a group of clock genes namely Period 1,2,3 and cryptochrome 1,2 which function in association with each other based on multiple complex transcription- translational feedback loops. These genes have been shown to regulate directly and indirectly cell cycle genes and thereby control cell proliferation, apoptosis and tumor suppression. As the period genes control several complex downstream regulators of the cell cycle and mediators of tumour suppression, a possible correlation between their mutation and breast cancer could throw open avenues to determine therapies to counteract or supplement the necessary downstream regulators in breast cancer patients[2]

Breast cancer has been shown to be associated with alterations in the circadian rhythm, first evidenced by decreased prevalence among blind people and increased prevalence among people who are exposed to more hours of light (pilots & night centre workers) [3]

Certain studies have shown correlation between mutations of these clock genes regulating the circadian rhythm and breast cancer particularly pointing to a variation in the PER 3 gene. Exon 18 of the Period 3 gene has been shown to have a variable number of tandem repeats- either a four repeat sequence or a 5 repeat one. The 5 repeat and heterozygous genotype have been associated with breast cancer. [4], [5], [6]. The number of studies available on this particular gene of interest and the association with breast cancer are few and none reported in an Indian setting. Thus this study was undertaken with the view that it could throw light on the association between PER 3 gene and breast cancer, thereby enabling formulation of newer cancer biomarkers or therapeutic modalities.

An attempt was also made to find an association if any between receptor positivity (Estrogen & progesterone receptor), HER2/Neu protein expression and the allelic pattern amongst malignant samples. The expression of these receptors and proteins in breast tissue has therapeutic and prognostic significance and any correlation between PER 3 allele patterns and their expression profile may be significant.

MATERIALS AND METHODS

Sample collection

A review of the records of the department of histopathology spanning the last five years yielded a total of 20 malignant and 10 non neoplastic samples which could be used for the purposes of our study. The slides of the stored samples were reviewed by an experienced pathologist to confirm presence of malignant breast tissue and then chosen for study. Cases of doubtful histopathology

and post chemotherapy/radiation samples were not used. The paraffin wax embedded tissue blocks were cut to 15 micron sections and de-paraffinised using standard techniques and collected in eppendorf tubes.

DNA extraction

DNA extraction of the samples was carried out by the tissue DNA extraction techniques standardized in our lab. DNA yield and concentration was assessed using Nano drop instrument.

Gene amplification by Polymerase chain Reaction

The sequence of interest in EXON 18 of the PER 3 gene (Gene sequence accession number AB047686.1 on NCBI)was amplified by Polymerase chain reaction using the following primers:-Forward primer of sequence -3' 5'ATGGCAGTG AGAGCAGTCCT & Reverse primer of sequence 5'ACCAGTTCTGGATGGGGATT-3'. Locus -1p36.23

EXON 18 of the PERIOD 3 gene has a polymorphism involving variable number of tandem repeats where a 54 base pair sequence is repeated either 4 times or 5 times. They were identified in our study as follows:-a) Homozygous 4-repeat allele represented by a single band of 295 base pairs on agarose gel in our study. b) Homozygous 5-repeat allele represented by a single band of 349 base pairs on agarose gel in our study. **c)**Heterozygous allele - represented by two bands of 295 & 349 base pairs on agarose gel electrophoresis in our study.

The amplified gene of interest was run on gel electrophoresis using 4 % agarose gel and band patterns studied.

Statistical Analysis

Statistical analysis of the observed data was done using Chi square test with Yates correction using SPSS 19.

RESULTS

There were totally 9 cases and 10 controls. The presence of the VNTR polymorphism is given in Table 1

Eleven of the twenty malignant samples had Estrogen Receptor, Progesterone Receptor and HER2/Neu protein expression status assessed. The results comparing the expression status with the allele type is displayed below in tables 2, 3 & 4. There was no significant difference among the cases and controls. (p>0.05)

Table 2: Comparison of allele type and Estrogen Receptor (ER) status

Per 3 allele	ER positive	ER negative	total
heterozygous	4	2	6
4 repeat homo	4	1	5
Total	8	3	

P value >0.05 ; Chi square test

Table 3: Comparison of allele type and Progesterone Receptor (PR) status

Per 3 allele	PR Positive	PR negative	total
heterozygous	4	2	6
4 repeat homo	3	2	5
Total	7	4	

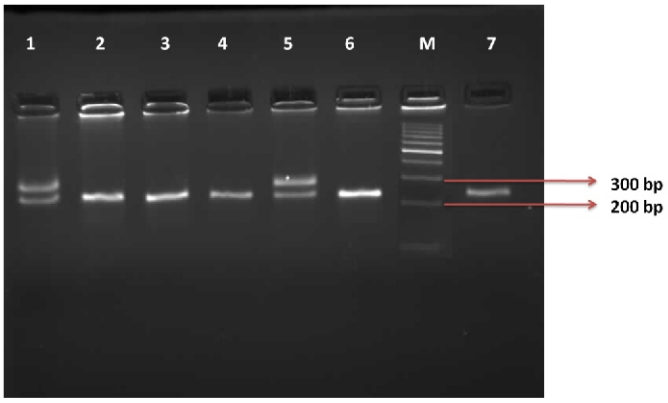
P value >0.05 ; Chi square test

Table 1 : Presence of VNTR repeats among cases and controls

Genotype	Cases	%	Controls	%
4 repeat	9	45	10	100
5 repeat	0	0	0	0
Heterozygous	11	55	0	0
Total	20	100	10	100

Comparison of allele type with Estrogen/Progesterone Receptor/HER2-Neu expression

Table 4: Comparison of allele type and HER2/Neu status			
Per 3 allele	HER2/Neu Positive	HER2/Neu Negative	Total
heterozygous	4	2	6
4 repeat homo	3	2	5
Total	7	4	
P value >0.05; Chi square test			



M – 100 bp DNA marker
Lanes 2,3,4,6,7 – Homozygous wildtype
Lanes 1,5– Heterozygous mutant

Fig 1. Gel picture of the PCR products showing the various alleles

These are the key observations that were made (Fig1)

a) Amongst the breast cancer cases - Eleven out of twenty samples showed bands suggestive of heterozygous alleles and nine were consistent with 4 repeat homozygous allele. No 5 repeat homozygous alleles were obtained.

b) Amongst the Non neoplastic cases, all ten samples were consistent with a 4 repeat homozygous allelic configuration.

c) No evident correlation could be made between Period 3 allele forms and oestrogen/progesterone Receptor, HER2/Neu protein expression status.

DISCUSSION

As per our study 55 % of the Malignant cases showed presence of heterozygous alleles of 4 and 5 repeat sequences, while the remaining 45% were homozygous 4 repeat sequence alleles. (Table1) No sample yielded a 5 repeat homozygous polymorphism. This is a very interesting finding in stark contrast to the study in Yale (2005) amongst a Caucasian population which demonstrated the high incidence of a 5 repeat homozygous allele 9.3% among cases and a 46 % incidence of 4-repeat homozygous allele and 44.5 % incidence of heterozygous alleles[3]. In our study the heterozygous allele seems to have a clear predominance over other allele types amongst the breast cancer samples a finding not demonstrated in the Yale study. This difference can be attributed to the following two factors

a) Racial variation Our study was amongst an Indian population while the Yale study was done in a Caucasian population.

b) Nature of sample - A key difference between our study and the study at Yale was the fact that ours utilized neoplastic breast tissue as the focus of study while theirs examined blood samples from patients.

Our investigation into the allele patterns of the non-neoplastic breast tissue was based on ten samples. Patients with a diagnosis of mastitis, breast abscess & breast hamartomas on whom an excision was undertaken were chosen. All ten samples proved to be 4 repeat homozygous alleles. This is in stark contrast to the study findings in the Zhu Y et al study which showed 47.7% - 4 repeat; 6.5 % - 5 repeat sequences and 45.8% - heterozygous alleles. Our study shows a 100% predominance of the 4 repeat sequences.

More than 10 samples could not be utilized for the following reasons:-

1. Presence of very small islands of breast lobules in the stored benign samples with high predominance of non specific tissue like inflammatory cells and fat. Several samples had no breast lobules in them and had to be rejected.

2. Limitation of number of non neoplastic samples available in the pathology department

Statistical analysis of the allelic distribution amongst the malignant and non neoplastic tissues reveals a statistically significant correlation ($p<0.05$) between the type of polymorphism and the outcome (malignant/control) as calculated by Chi square test with Yates correction. The association between a heterozygous PER3 genotype and Breast carcinoma appears to be significant. In comparison, the study by Zhu Y et al could not establish any statistically significant correlation between breast carcinoma and the polymorphism in the general study population. Only on segregating the population into pre and post menopausal could they establish a statistical difference in the allelic distribution.

On comparison of the allele type with the oestrogen & progesterone receptor, HER2/Neu protein expression status of the malignant samples, it was seen that there is no evidence of correlation between the two from this study. (p value was >0.05). However as the sample for analysis was low, further investigation into this particular association is warranted.

The overall picture that is gained at the end of this study is the statistically significant association between the heterozygous allelic form of the Period 3 gene and breast carcinoma. This study provides evidence to show that the heterozygous allele form of the Period 3 gene is a possible predisposition to breast Cancer. A distinct predominance of the heterozygous allele amongst the breast cancer cases (55%) compared to earlier studies is noted. Also significant is the total absence of the 5 repeat homozygous alleles amongst both cases and controls.

These findings suggest that the pattern of genetic polymorphisms in Exon 18 of the Period 3 gene in the Indian setting are quite different from those reported in the western population particularly in reference to breast cancer tissues. This

study thus calls upon more concerted efforts to further investigate the Period 3 gene in tissue samples obtained from breast cancer patients and compare it with results in normal breast tissue samples.

From this study it can also be concluded that there is no statistically significant association between Oestrogen/ Progesterone receptor/ HER2 neu status with a particular allele type amongst malignant samples.

Implications

This study thus brings to light the association of Period 3 gene with breast cancer and stresses the need to investigate this gene association in detail for diagnostic and therapeutic possibilities.

Future Directions

We wish to continue this study in a prospective format using both blood & tissue samples from patients to investigate this polymorphism as a potential blood marker for breast carcinoma.

ACKNOWLEDGEMENTS

ICMR-STS program for approving this project

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