

RESEARCH ARTICLE

Mutational Analysis of Prohibitin - A Highly Conserved Gene in Indian Female Breast Cancer Cases

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Abstract

Prohibitin (PHB) is a chaperone protein which is highly conserved evolutionarily. It shows significant homology with the *Drosophila* cc gene which is considered important for development and differentiation of *Drosophila melanogaster*. Investigations have revealed an involvement of PHB in cellular proliferation and development, apoptosis, signal transduction, mitochondrial function and regulation of the estrogen and androgen receptors. Therefore, we conducted the present study to analyze mutations in the highly conserved region in Indian female breast cancer patients. Conventional PCR-SSCP and Automated DNA sequencing were performed with a total of 105 breast cancer samples along with adjacent normal tissue. Of the total, 14.2% (15/105) demonstrated a mutation status of prohibitin observed in our study population. We identified a novel missense mutation (Thr>Ser), a novel deletion of T nucleotide in an intron adjacent to intron-exon boundary and a previously determined missense mutation (Val>Ala). A statistically significant correlation was obtained which suggested that prohibitin may be associated with tumor development and/or progression of at least some proportion of breast cancers.

Keywords: Prohibitin (PHB) - mutation - breast cancer - sequencing - PCR-SSCP - India

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Introduction

Breast cancer is one of the most common malignancies affecting woman worldwide with a mortality rate of more than one million per year (Coughlin et al., 2009) and also ranks second overall (Ferlay et al., 2010). There are many risk factors responsible in the development of breast cancer (Dumitrescu et al., 2005). Various genetic and environmental factors have been established as the causes of breast cancer (Vargo-Gogola and Rosen, 2007). Approximately, 5% of the breast cancer occurs due to germline mutation in BRCA1 and BRCA2 (Kuusisto et al., 2011). The remaining 95% is due to genetic changes that take place in a women's life and the genes which undergo sporadic mutation plays a major role.

Prohibitin is an evolutionarily conserved M_r 32,000 protein, the majority of which is found on the inner membrane of mitochondria (Snedden and Fromm, 1997). For the first time, it was shown that the microinjection of prohibitin mRNA into cell nuclei blocked S phase entry, while down-regulation of prohibitin via anti-sense stimulated cell entry into S phase (Nuell et al., 1991) demonstrating its ant proliferative property. Later the human homologue of rat Prohibitin gene was isolated and

mapped it to chromosome 17q12-21 (Sato et al., 1993). Prohibitin might have additional anti proliferative role as it was shown that 3' UTR of the PHB gene encodes a functional RNA that arrests cell-cycle proliferation between the G1 and S phases of the cell cycle (Manjeshwar et al., 2003). The chromosomal location of Prohibitin was found distal to the BRCA1 locus i.e. in a region that frequently shows loss of heterozygosity in breast and ovarian cancer (Nagai et al., 1994). Moreover it showed a significant homology to the Cc gene which was considered to be important for the vital growth and differentiation of *Drosophila melanogaster* (Eveleth and Marsh, 1986). Studies have revealed that prohibitin represses cell growth by modulating E2F transcriptional activity (Wang et al., 1999b) and further broad research found that prohibitin collaborates with chromatin remodeling molecules and regulates transcription (Zhang et al., 2007). Interestingly, it was found that there might be a functional relation between estrogen antagonists and prohibitin as a research have indicated that estrogen antagonists induced growth suppression at G1, which coincides with the phase of cell cycle arrest induced during prohibitin-mediated repression of cell cycle progression (Wang et al., 2004; Saeki et al., 2005). In addition to transcriptional repression, Studies

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revealed that prohibitin can also induce p53-mediated transcription by associating with p53 and enhancing its transcriptional activity through the process of upgrading p53 to bind to its consensus sites on the DNA, indicating that prohibitin may have dual functions in modulating transcription (Fusaro et al., 2003; Joshi et al., 2007).

Additional diverse functions of prohibitin were recently reported, which link this evolutionally highly-conserved gene to apoptosis (Chowdhury et al., 2007), to signal transduction through the MAPK pathway (Rajalingam and Rudel, 2005).

Materials and Methods

Biological Sample Collection

Cases of breast cancer were all females. The tissues collected did not compromise the availability of sufficient biopsy material for routine pathology and other tests performed as part of patient care. Prior informed consent was obtained from all patients after the approval from the ethics committee of Rajiv Gandhi Cancer Institute and Research Centre. Total of 105 breast cancer tissue samples and adjacent normal tissues not infiltrated by tumors as confirmed by pathologist were collected.

DNA extraction from tissue

High molecular weight DNA was extracted from fresh tumor and normal adjacent breast tissue samples using DNA Extraction kit (Qiagen, USA). The quality of the extracted genomic DNA was stringently checked by gel electrophoresis using ethidium bromide stained 2% Agarose.

PCR-SSCP Analysis

A pair of tumor and its corresponding normal DNAs were used for the analysis. Briefly PCR products of fourth exon were amplified using two primers (forward and reverse) in the introns flanking exon 4. The amplification was performed in 30 µl reaction volume containing 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTPs (dATP, dCTP, dGTP, and dTTP), 5 pmoles of each forward and Reverse oligonucleotide primers, 100 ng of tumor DNA and 1 U of Taq DNA polymerase.

The PCR condition were initial denaturation at 94°C for 5 min followed by 35 cycles with denaturation at 95°C for 30 sec, annealing at 66°C for 30 sec and extension at 72°C for 30 sec, which was extended for 7 minutes in the final cycle. Both positive and negative control was performed in parallel for each PCR reaction. Negative control reactions were performed without DNA template to exclude non specific amplification. The PCR product was electrophoresed on an ethidium bromide-stained 2% agarose gel in 1X TAE with 100 bp molecular weight marker as reference to check the presence of 378 bp amplified DNA sequence of interest in gel using Quantity one Software in Gel documentation system (Bio-Rad Laboratories, CA, USA) as shown (Figure 1). The high quality samples were assessed with SSCP analysis. Briefly 5µl of each PCR product was mixed with 5µl of formamide gel-loading buffer, heat denatured at 95°C in thermocycler for 5 minutes and immediately chilled

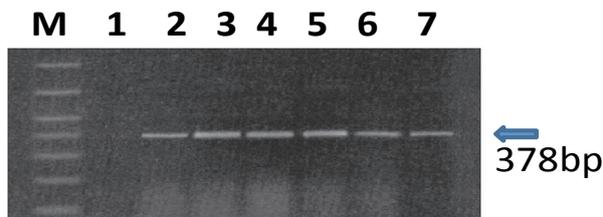


Figure 1. Amplified Gene Product (378bp) of Prohibitin Gene. Lane M: molecular marker of 100bp. Lane 1: negative control; Lanes 2-7: amplicons from the breast cancer tissue samples.

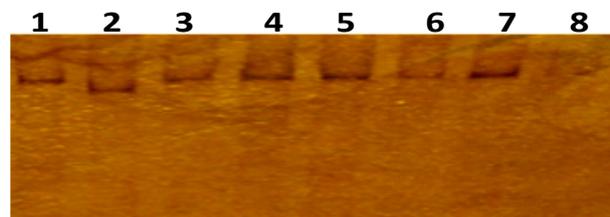


Figure 2. SSCP Pattern of Prohibitin Gene Which Shows a Variation or Shift in the Band at Lane 2 of the Breast Tumor Samples.

on ice for 10-15 minutes. 5µl of denatured PCR product were loaded on 1XTEMED gel. Electrophoresis was carried out at a constant voltage 16-18 hr (overnight) at optimized temperature. The gel was stained for 45 minutes in silver nitrate solution with constant shaking in a dark room. The silver stained gel was labeled and scanned for computer image analysis and documentation. Alteration in electrophoretic mobility of single strand DNA bands was analyzed in comparison to that of wild type and were noted as SSCP positive (Figure 2).

Sequencing

PCR products showing altered band mobility in SSCP were re amplified for sequencing. DNA sequencing was carried out at Scigenom labs, Cochin by using reverse primer sequence. The process of sequencing was carried out twice in order to exclude any contamination and or PCR artifacts.

Statistical analysis

Chi-square test was applied to compare the Prohibitin gene mutation(s) identified in our study with various clinico-pathological characteristics. The value $p > 0.05$ were accepted as statistically not significant. The analysis was done using GraphPad Prism 5.04 for windows, GraphPad software, Inc, La Jolla, CA 92037.

Results

Alteration in electrophoretic mobility of single strand DNA bands were analyzed in comparison to that of wild type. Samples that showed alteration were identified as SSCP positive (Figure 2) and then were sequenced to confirm the mutation and its type. Our study included a total of 105 Breast cancer tissue samples along with the adjacent normal tissue. Total of 15 samples showed mutations in the fourth exon and the Introns flanking it in the highly conserved gene Prohibitin. In the process of direct sequencing, we were able to detect a novel Missense

mutation (Thr>Ser) in the coding region harbored by five cases of breast cancer, moreover a deletion of T nucleotide near the exon-intron junction was also observed in six cases. During the course of our molecular analysis, we were also able to detect a single missense mutation that has been previously defined (Sato et al., 1992), harbored by 4 cases of the breast cancer (Table 1, Figure 3). Importantly, Prohibitin (PHB) gene sequence changes noted in the study were exclusively associated with the isolated DNA of the breast cancer cells; they were not observed in mammary epithelial cells isolated from the adjacent normal tissue.

Statistical analysis and correlations between mutational status of Prohibitin (PHB) with different clinical parameters like age, menopausal status, nodal status, Breast involved, tumor stage, ER status, Histological grades were assessed (Table. 2).

Table 1. Detail of Prohibitin Gene Mutation(s) in Female Breast Cancer Cases from India

Affected codon	Base change	AA Change	Mutation Effect	PD
123	ACA > TCA	Thr>Ser	Missense mutation -	-
88	GTC > GCC	Val>Ala	Missense mutation +	-
-	GTG/gtgagtga>GTG/gtgag-gaa		Deletion of t	-

*The bold nucleotides were mutant; lower case letters represent introns; upper case letters represent exons, **mutation effect: MS, Missense mutation; PD, previously described, +Yes; -No

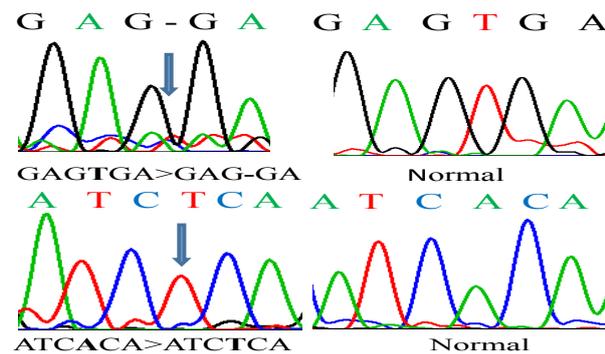


Figure 3. Representative Partial Electropherograms of Mutant (shown by arrow) with Normal Adjacent Forms.

Table 2. Mutation Pattern and Mutation Effect of Prohibitin Gene in Female Breast Cancer Cases from India

Variables		Total N (%)	Mutants M (%)	P value
Age	≥50	50 (22%)	11	<0.05
	<50	55(7.27%)	4	
Nodal Status	Involved	73(6.84%)	5	<0.01
	Not Involved	32(31.2%)	10	
Tumor Stage	Stage2(a+b)	47(23.4%)	11	<0.05
	Stage3(a+b)+4	58 (6.8%)	4	
Menopausal status	Pre	46(15.2%)	7	NS
	Post	59(13.5%)	8	
ER status	Positive	37(24.3%)	9	<0.05
	Negative	68(8.82%)	6	
Breast Involved	R	15(20.0%)	3	NS
	L	90(13.3%)	12	
Histological grade	WD	54(12.9%)	7	NS
	MD	23(13.0%)	3	
	PD	28(17.8%)	5	

Discussion

Previous genetics studies have revealed that the chromosomal location of prohibitin gene was mapped to position 17q21-q22, a region genetically linked to early-onset of breast cancer (Sato et al., 1992). Prohibitin was first linked to human cancers by the discoveries of prohibitin mutations in breast cancers (Sato et al., 1993).

Since, the initial report by Sato et al. (1992; 1993), four mutations were reported in total of 23 breast cancer patients, it gained the attention of researchers to investigate its possible critical functions and growth control activity in different set of populations. We examined the fourth exon since it is highly conserved in the *Drosophila Cc* gene, which was considered to be important for development and differentiation of *Drosophila melanogaster* (Eveleth and Marsh, 1986). Very importantly, it has been established that Prohibitin interact with Rb through a specific region in the exon 4 (Wang et al., 1999b). Other studies have also indicated that prohibitin and its co-repressors are required for the growth suppression induced by estrogen antagonists (Wang et al., 2004). We formulated our study as a population based study to check and analyze mutation(s) in the fourth exon and the intron flanking of the prohibitin gene in Indian female breast cancer patients. As to the best of our knowledge, the genetic mutational analysis in the prohibitin gene has not been yet analyzed in India and our designed study is first to report prohibitin gene mutation association with Indian female breast cancer cases.

We found a total 9/15 (60%) cases having Missense mutation (Thr>Ser and Val>Ala) that were present in different independent tissue samples of breast cancer, 6/15(40%) mutation in an intron region where samples harbored a deletion mutation (i.e., deletion of T nucleotide) just adjacent to the intron-exon boundary. Though the exact effect of the intronic mutation is unknown but it might play a role in an abnormal splicing and an abnormal gene product which in turn can lead to a loss of the potential activity of the prohibitin gene. In the course of our study, we were able to detect only one type of the previously determined Prohibitin gene mutation(s) as shown (Table 1). Interestingly, the previously determined missense mutation (Val>Ala) at codon 88 found in our study population has already been shown to lie in the Rb-binding domain of the Prohibitin gene (Wang et al., 1999b) which show the antiproliferative activity of Prohibitin.

Regulation of estrogen receptor (ER) activity by prohibitin has been explored earlier. Brg1 has been reported to be connected with the activation of ER α -mediated transcription (DiRenzo et al., 2000) and further it was found that prohibitin interacts with Brg1/Brm and represses ER α mediated transcription after the binding of estrogen antagonist to the ER α , by the mechanism requiring both Brg1 and Brm (Zhang et al., 2007). Research has also shown that the depletion of prohibitin using siRNA prevented tamoxifen-induced cell cycle arrest from the tamoxifen-sensitive breast cancer cells (Wang et al., 2004) exploring a powerful connection between Estrogen and Prohibitin. In addition to the above, we found a statistically significant correlation (P<0.05)

between mutation profile of Prohibitin with Estrogen receptor status. The ER positive patients harbored 24.3% (9/37) of the total mutations observed (Table 2).

Genetic changes in the form of point mutation, chromosomal translocation, and gene loss can affect key genes like tumor suppressor genes involved in cell proliferation, drug resistance, invasiveness and other malignant characteristics. In a very large study, it was found that the risk of localized breast cancer where the lymph nodes were not involved was significantly elevated among the women having the missense mutation of HER-2 gene (Roberta et al., 2001). In relation to above, We found a significant association between lymph node status and Prohibitin, where the cases in which the lymph node were not involved showed 31.2% (10/32) mutational profile of prohibitin. Moreover, a significant correlation was also observed between Stages of breast cancer and Prohibitin, the mutation were more frequent in the early stage 2 (a+b) accounting to 23.4% (11/47) (Table 2), suggesting that mutation in prohibitin may be associated with tumor progression, though broader research on large population scale is required for the establishment of successive connection between stages and lymph node status of breast cancer with the prohibitin.

According to the very recent Breast cancer facts and figures of 2011-2012 by American cancer society, the chances of breast cancer increases with the increasing age and 1 in 42 women of the age ≥ 50 could get cancer of breast due to many important genetic changes that occur as the age proceeds but on the other hand, we cannot ignore the genetic changes occurring earlier in life as there are also cases where the high penetrance gene like BRCA1, BRCA2 and ATM are linked to early onset of breast cancer (Julian et al., 1999; Mailliet et al., 2002). Here, We found a statistically significant correlation between the age and the mutational status of prohibitin and the breast cancer cases of the age ≥ 50 accounted to 22% (11/50) of the total number of mutation found (Table 2) exploring that prohibitin might be linked to late onset of breast cancer and may play a role as the age proceed.

Our data have provided evidence for the existence of naturally occurring mutation of Prohibitin gene with the female breast cancer cases. The recent implication of prohibitin in the mechanism of action of anti-estrogens-agents that are currently used therapeutically can open a new avenue between the anticancer drugs and prohibitin. The active property of prohibitin gene should be evaluated in the near future for at least in breast cancers because of its diverse role in apoptosis, growth suppression of the breast cancer cells and cell signaling.

These results suggest that the mutation in prohibitin gene may have a significant role for development and/or progression of at least some breast carcinomas. However, the role of prohibitin gene as a tumor suppressor is still controversial. Our study was done on a limited sample population and further large scale translational and clinical studies are required to look deep insight and extract the diverse function of prohibitin gene in breast and other cancers.

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