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BRCA1 Promoter Hypermethylationas an Early Diagnostic Tool for Breast Cancer

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Abstract: *Background:* Breast cancer is the second most common cancer among women after cervical cancer. As Cancer development and progression is dictated by chain of alterations in genes. Over the past few years, the Kashmir valley has witnessed a tremendous increase in the incidence of breast cancer in its unexplored ethnic population. The aim of present study was to find out the role of Promoter Hypermethylation of BRCA1 gene in Breast cancer patients. *Material Methods:* The DNA was extracted from all the samples and was modified using bisulphite modification kit. Methylation-specific polymerase chain reaction was used for the analysis of the promoter hypermethylation status of BRCA1 gene. *Results:* The epigenetic analysis revealed that unlike other high risk regions, Kashmiri population has a different promoterhypermethylation profile of BRCA1 gene as 68% of the cases showed BRCA1 promoter hypermethylation in comparison to 20% of the normal cases, the association of promoter hypermethylation with breast cancer and normal cases was found to be significant (P=0.0006). The frequency of BRCA1 promoter hypermethylation was found to be certainly higher in Stage III/IV (75%) compared to Stage I/ II (62%) but the difference was not statistically significant(P =0.0674). The frequency of promoter methylation was foundhigher (77.1%) in age group above 60- years) than ages below 60 years. *Conclusion:* These results suggest that BRCA1 aberrant promoter hypermethylation in Kashmiri population contributes to the process of carcinogenesis in breast cancer and is reportedly one of the commonest epigenetic changes in the development of breast cancer.

Keywords: Breast Cancer, BRCA1, Hypermethylation, Bisulphite Treatment

1. Introduction

The breasts are external symbol of beauty and womanhood in women; however cancer of the breast is responsible for the death of millions of women worldwide every year. Malignancy of the breast is one of the commonest causes of death in women aged between 40-45 years (Showkat, *et al*., 2013). The incidence of this disease is rising in many countries such as Japan and other developing nations and has become a genuine public health problem, with one woman in ten, developing it in her lifetime throughout the world. The incidence of breast cancer increases with age, being uncommon below the age of 32 years (Wernberg *et al*., 2009). Epigenetics changes, as DNA methylation is one of the most common changes of epigenetics events, covalent addition of the methyl group to DNA, which played an important role in driving tumorgenesis (Fang., *et al* 2012). The DNA methylation usually occurs in the CpG islands located in or near the promoter of over 70% of all genes. In breast cancer, hypermethylation occurs in BRCA1 gene's promoter leaded to the cancer development and progression (Ramezani., et al., 2012 and Phuong *et al*., 2014). Three main features of BRCA1 are thought to be important for its function.BRCA1 has been implicated in many cellular

functions including cell cycle regulation, DNA damage response, maintenance of genomic stability and recombination, amongstothers (Parvin *et al.,* 2004). Aberrant DNA methylation is now recognized as one of the most common molecular abnormalities in cancer and references therein). Thisepigenetic modification occurs at the cytosines of CpG dinucleotides, which often exist in clusters called CpG islands. Methylation of these sites in the promoter region of a gene can result in chromatin condensation and gene silencing. In cancer cells, aberrant methylation has frequently been reported in tumor suppressor genes, DNA repair genes and genes related to cancer metastasis and invasion.In the present study, we analyze the hypermethylationstatus of BRCA1, in breast cancer patients and normal controls in ethenic population of Kashmir valley.

2. Materials and Method

2.1. Sample Collection

100 surgically obtained breast tissue Samples among which 75 were breast cancer patients and 25 were normal samples were obtained from the Department of Surgery, of Shri Maharaja Hari Singh (S. M. H. S) hospital Srinagar in sterilized plastic vials containing 10% of normal saline and transported from the theatres to the laboratory on ice and stored at -80°C till further process. The information regarding gender, age stage, occupation and residence were collected from the record file of patients present in the hospital and histological grade for each sample was collected from the histopathological reports.The DNA was extracted by phenol chloroform method (Sam brook & Russell). All breast cancer patients included in the study were females, with the histopathological diagnosis of the breast cancer.

2.2. Qualitative and Quantitative Analysis of Genomic DNA

The integrity of the genomic DNA was examined by gel electrophoresis using 1% agarose gel. DNA in the gel was visualized with the help of gel doc system (Biorad). The quantity of DNA was determined by U.V. spectrophotometric method.

2.3. Bisulpite Treatment

Sodium bisulfite treatment converts unmethylatedcytosines to uracil and hence enabled to distinguish between the hypermethylated and non hypermethylated cytosine residues. DNA was modified by kit based method (EZ DNA Methylation^{TM} Kit) supplied by ZYMO RESEARCH. The modified DNA was stored at -20°C for further use.

2.4. Methylation Analysis

Methyl specific polymerase chain reaction (MSP): Amplification of the promoter region of the gene was carried out in Eppendorf Gradient Thermalcycler in a 25µl reaction mixture as shown in table 1. Information about primers is given in table 2.

Table 1. *Volume and concentrations of different reagents used in PCR.*

Reagent	Concentration	Volume
PCR MM (master mix)		12.5 µl
Forward primer	10 pmol/µl	1µl
Reverse primer	10 pmol/µl	1 µl
DNA sample	250 ng/µl	1µl
Deionised water		9.5 µl
Total volume		25 µl

Table 2. *Primers described by Herman (25) used and length of fragments obtained in MSP (Methylation Specific PCR).*

Nature of Primer	Primer sequence	
unmethylatedPrime (*BRCA1*)	Forward primer	5′ttggtttttgtggtaatggaaaagtgt3′
	Reverse primer	5′caaaaaatctcaacaaactcacacca ′
methylated primer(*BRCA1*)	Forward primer	5′tcgtggtaacggaaaagcgc3′
	Reverse primer	5′aaatctcaacgaactcacgccg3′

Gradient thermal cycler (Eppendorf) was programmed as in table: 3, to carry out the PCR amplification.

Table 3. *Thermal cycling conditions.*

Steps	Temperature °C	Time	Number of cycles
1. Initial Denaturation	95	5 min	1
2. Denaturation	95	30 sec	35
3. Annealing	56.9/60.4	30 sec	
4. Extension	72	30 sec	
5. Final extension	72	4 min	1
6. Hold	42	5 min	

After completion of PCR, the reaction products were run on 2% agarose gel, with ladder of 100 bp as marker, methylated and unmathylated bands were counted in all samples.

3. Statistical Analysis

The data obtained was statistically assessed by descriptive analysis, one-way ANOVA using SPSS (SPSS Software products, Marketing Department, SPSS Inc. Chicago, USA).

4. Results

4.1. Analysis of BRCA-1 Promoter Hypermethylation

Figure-1 showed DNA isolated from case samples with phenol-chloroform method (Sambrook et al., 1989).

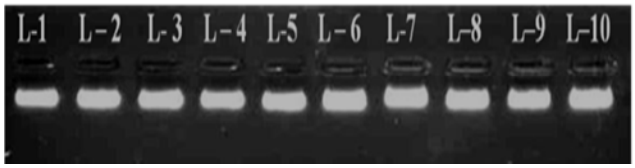


Figure 1. *Lane (L) 1-10 showing the isolated DNA of case samples, run on 1% Agarose gel.*

4.2. Relationship of Promoter Methylation of BRCA-1 Gene with Breast Cancer in Histopathologically Confirmed Cancer and Normal Cases

Methylation-specific PCR was done to examine the methylation status of the promoter region of BRCA-1gene figure 2 & 3. 68% (51/75) of the breast cancer tissue samples showed methylated BRCA-1promoter and 32% (24/75) of the cases however showed unmethylated BRCA-1promoter (Table 4). Almost all 80% (20/25) of the histopathologically confirmed normal tissue samples showed unmethylated BRCA- 1 promoter, except only in 20% cases (4/20) where BRCA-1promoter was found to be methylated (graph-1 and graph-2). The association of promoter methylation with breast cancer was evaluated by $\chi 2$ (Chi square) test, using SPSS soft weare and was found to be significant (P=0.0006).

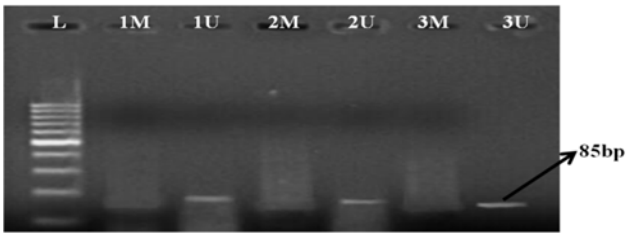


Figure 2. *MSP (Methylation Specific PCR) of histopathologically confirmed Normal Breast DNA Samples Run on 2% Agarose Gel.*

L- 50bp marker
Cases amplified with unmethylated primer, product size was 85bp.

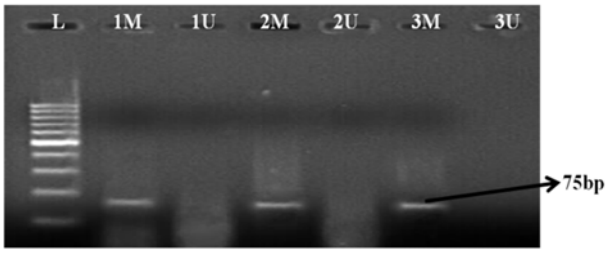
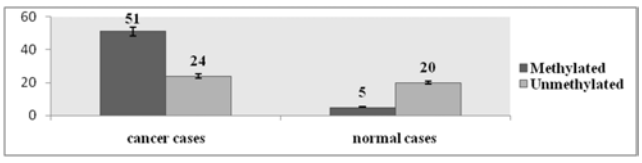


Figure 3. *MSP (Methylation Specific PCR) of Breast Cancer DNA Samples Run on 2% Agarose Gel. Methylated Product is of 75bp length and unmethyaletd is 85bp in length.*

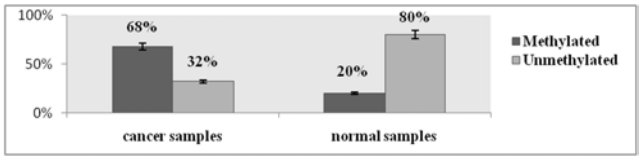
L – 50bp marker
Cases amplified with methylated primer, product size was 75 bp.

Table 4. *Number & Frequency of Methylated/Unmethylated Cancer &Normal Cases.*

Cancer cases (75)		**Frequency**
Methylated	51	68 % (51/75)
Unmethylated	24	32 % (24/75)
Normal samples (25)		Frequency
Methylated	5	20%(5/25)
Unmethylated	20	80%(20/25)



Graph 1. *Number of Methylated/Unmethylated Cancer & Normal Cases.*



Graph 2. *Frequency of Methylated/Unmethylated Cancer & Normal Cases.*

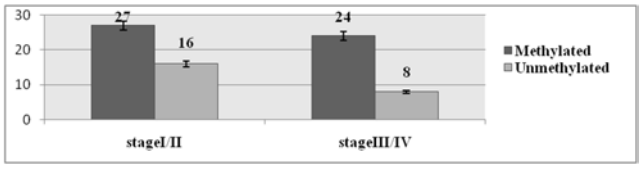
4.3. Relationship Between Promoter Methylation of BRCA-1 Gene and Selected Clinico-Pathological Parameters: These Parameters Included Clinical Staging and Age

4.3.1. Relationship of Promoter Methylation of BRCA-1 Gene in Stage I/ II and Stage III/IV

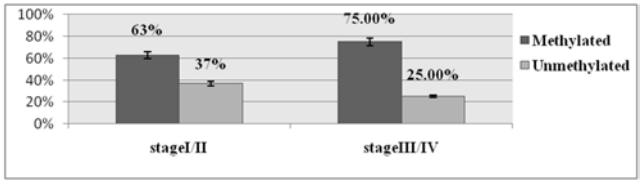
There were 43 breast cancer cases that were in Stage I and Stage II of the disease. Among these cases 27 cases were methylated and 16 cases were unmethylated. However, among 32 cases that were in Stage III and Stage IV of the disease, 24 cases were methylated and 8 cases were unmethylated (Table 5). When the frequency of *BRCA-1*promoter methylation was compared with clinical staging of the disease, *BRCA-1*promoter methylation was found to be certainly higher in Stage III/IV (75%) compared to Stage I/ II (62.7%) but the difference was not statistically significant (P =0.0674).

Table 5. *Number of Cases Showing Promoter Methylation &Unmethylation in Stage I/II & Stage III/IV during MSP Amplification Confirmed By 2% Agarose Gel Electrophoresis.*

Total number of cases (75)		
Stage I/II (43 cases)		
Parameter	Cases	Frequency
Methylated	27	62.7 % (27/43)
Unmethylated	16	37.2 % (16/43)
Stage III/IV (32 cases)		
Parameter	Cases	Frequency
Methylated	24	75 % (24/32)
Unmethylated	8	25 % (8/32)



Graph 3. *Number of Methylated &Unmethylated Cases of Different Stages of Breast Cancer Cases.*



Graph 4. *Frequency of Methylated &Unmethylated Cases of Different Stages of Breast Cancer Cases.*

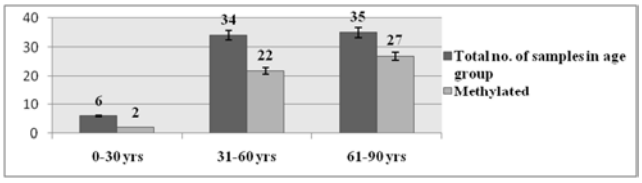
4.3.2. Relationship of Promoter Methylation in the Breast Cancer Cases and Normal Samples of Different Age Groups

Among 75 breast cancer cases, 6 belonged to age group (0-

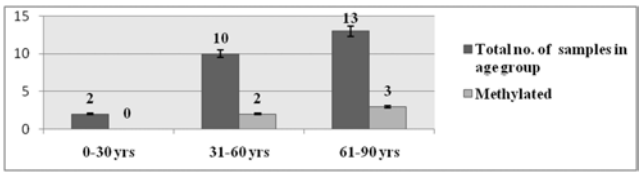
30 yrs), 34 belonged to age group (31-60yrs) and 35 belonged to age group (61-90yrs). However the number of methylated cases in each age group was 2, 22 and 27 respectively, as shown in Table 6 and graph 5 & 6. The frequency of promoter methylation was found 33.33% in age group (0-30 yrs), 64.7% in age group (31-60 yrs) and 77.1% in age group (61-90) yrs) as shown in Table 6 and grapg 7. Similarly among 25 normal cases, 2 belonged to age group (0-30 yrs), 10 belonged to age group (31-60 yrs) and 13 cases belonged to age group (61-90 yrs). However the number of methylated cases in each of the age groups were 0, 2 and 3 respectively (Table 6).The frequency of promoter Methylation was found 0% in age group (0-30yrs), 20% in age group (31-60yrs) and 23% in age group (61-90yrs) as shown in graph 7.

Table 6. *Methylation Pattern of Different Age Groups During MSP Amplification Confirmed By 2% Agarose Gel Electrophoresis.*

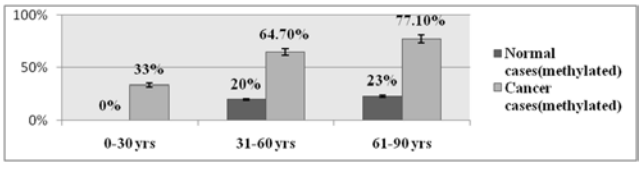
		0-30 years	31-60 years	61-90 years
Cancer cases(40)	Total	6	34	35
	Methylation	2	22	27
	Frequency	33.3%	64.7%	77.1%.
Normal Cases(20)	Total	2	10	13
	Methylation	0	2	3
	Frequency	0%	20%	23%



Graph 5. *Methylation Pattern of Different Age Groups in samples.*



Graph 6. *Methylation Pattern of Different Age Groups In Normal samples.*



Graph 7. *Frequency of Promoter Methylation of Different Age Groups intheHistopathologically Confirmed Cancer & Normal Cases.*

5. Discussion

Epigenetic mechanisms, such as hypermethylation of CpGIsland of promoter regions, have been proposed as a mechanism of gene inactivation in tumour cells **(**Esteller et al., 2002). Our study showed methylated *BRCA-1*association of promoter methylation between breast cancer and histologically confirmed normal controleswas found to be significant which was supported bythe findings of Catteau et al., 1999; Esteller *et al*., 2008; Niwa et al., 2000; Rice *et al*., 2000, but our results are incontradictic with the findings of Knudson et al., 1971, Knudson *et al*., 1985 as thy showed in their results that BRCA1 promoter hypermethylation is not involved in the breast cancer. Our results are showing that BRCA1 promoter hypermethylation was involved in breast carcinoma as an epigenetic change. Our study also supports the findings of Hanahan D. et al., 2000 as they studied that tumour of breast cancer patients showed promoter hypermethylation of BRCA1 gene, these results were similar to our studied reults, that in BRCA1 gene in breast cancer patients, there is an altrationsof methylation pattern, which may the causative agent for the breast cancer and is also in support of the findings of Friedman et al., 1994; Easton 1997, Castilla et al., 1994 they studied in their study that alterations in BRCA1 have been estimated to be responsible for about 50% of familial breast cancer. Our findings was also supported by several workers in different parts of the world in their studies reportes that aberrant promotor hypermethylationof BRCA1 could occur in breast carcinoma, as they observed during their studies that in some breast cancer cell lines and primary tumors. (Rice., *et al*, 18. Magdinier *et al*., 1998 and Catteau *et al*., 1999)

Promoter hypermethylation of BRCA1 gene in breast cancer patients in our study based on comparison to the worldwide researches and the mean of frequency of hypermethylated BRCA1 was 68% of the breast cancer tissue samples showed methylated *BRCA-1* promoter and 32% of the cases however showed unmethylated*BRCA-1* promoter. Almost all 80% of the histopathologically confirmed normal tissue samples showed unmethylated*BRCA- 1* promoter, except only in 20% normal cases where *BRCA-1*promoter was found to be methylated and was rather higher than Tapia et al. (2008) and Phuong Kim Truong et al. (2013), (19,20). Regarding to clinical parameters, in our study, When the frequency of *BRCA-1* promoter methylation was compared with clinical staging of the disease, *BRCA-1* promoter methylation was found to be certainly higher in Stage III/IV (75%) compared to Stage I/ II (62.7%) but the difference was not statistically significant (P =0.0674). The frequency of promoter methylation was found 33.33% in age group (0-30 yrs), 64.7% in age group (31-60 yrs) and 77.1% in age group (61-90) yrs)which was in support of the findings of Phuong Kim Truong et al. (2013), Hora Loghmani et al. (2014) as in there studies there was no any significant differences between the hypermethylated BRCA1 promoter gene and the patient ages, tumor grades and stages.

Our study confermes that BRCA1 promotor region was specific characteristic of breast cancer patients in ethenic population of Kashmir valley, but in more additional studies are needed to understand the nature of association of promotor hypermethylationin breast cancer, so the epigenetic methylation may be used as early diagnostic and proganostic tools in breast cancer patients.

6. Conclusion

This study has interestingly revealed that promoter region hypermethylation status of mismatch repair gene *BRCA1* shows a significant increase in Breast cancer patients of Kashmiri origin as compared to controls. This became more apparent when the data for hypermethylation was interpreted taking Clinical stage into consideration and it was seen here that stage III/IV shows higher frequency of promoter region hypermethylation as compared to stage I/II which was earlier reported in literature Further it has been seen that also patients of above 60 years of age shows high frequency compared to below 60 years of age and breast carcinogenesis is a stepwise process of the accumulation of genetic and epigenetic abnormalities. It is clear that promoter hypermethylationis important for multistep process though genetic changes in the progression of breast carcinogenesis. Our study has supplemented the steadily growing list of genes inactivated by promoter hypermethylation in breast carcinoma and has provide not only new insights into the molecular basis of the diseases but also list of interesting candidate genes for the development of molecular markers which might contribute to the improvement of diagnosis. From our results it was confermed that BRCA1 promotor region was specific characteristic of breast cancer patients in ethenic population of Kashmir valleywas associated with breast cancer risk.

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