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Comprehensive Mutational Spectra of Epithelial Mesenchymal Transition Markers-SOX4, EpCAM, CK19 and their Impact of Methylene Tetrahydrofolate Reductase C677T Gene Polymorphism Associated Risk Factor Modified by BRCA1/2 and TGF-βR1 Genes in Pre/Post Menopausal Cases of Breast Cancer

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ABSTRACT

In tumorigenesis, epithelial mesenchymal transition (EMT) markers such as cytokeratin 19 (CK19), epithelial cell adhesion molecule (EpCAM) and SRY-Box Transcription Factor 4 (SOX4), play a significant role during metastasis in breast cancer patients. Folate metabolism is regulated by methylenetetrahydrofolate reductase (MTHFR) enzyme, a crucial factor for the synthesis of macromolecules (DNA, RNA) along with their epigenetic regulation during differentiating tumor cells. Genetic mutations are a characteristic feature of cancer cells, and DNA copy number variations (CNVs) show the genetic susceptibility. The present study has been designed with the aim to carry out the comprehensive analysis of EMT marker genes and their DNA CNVs to determine tissue specific genetic heterogeneity. Frequency of breast cancer gene 1/2 (BRCA1/2) mutation was also evaluated to determine the "risk" between familial and sporadic cases of breast cancer. Simultaneously, MTHFR C677T gene polymorphism was also studied to evaluate genotype frequency (CC & TT) in homozygous and heterozygous (CT) conditions in such cases. Also, we studied the role of transforming growth factor beta receptor 1 (TGF-βR1) as signal transducing regulator factor during progression of breast cancer metastasis. Genomic DNA was isolated from peripheral blood samples, after confirmation of clinical diagnosis of breast cancer cases (n = 159) and age matched controls. Polymerase chain reaction (PCR) analysis was carried out to evaluate the frequency of CK19, EpCAM, and SOX4 gene mutations along with over expression or under expression using specific primers. Amplification-refractory mutation system (ARMS) Polymerase chain reaction was used to assess the frequency of MTHFR (C677T) genotypes (CC, TT & CT) and their allele (C/T) frequency.

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PCR products were analyzed on agarose gel (1.5%) and individual bands were characterized after ethidium bromide staining for DNA CNVs. ELISA kits were used for hormone assay-estrogen, progesterone and follicular stimulating hormone from the serum to determine endocrine dysregulation. In the present study CK19 gene shows highest frequency (50.14%) of mutation followed by decreasing trend was observed in SOX4 (38.09%) and EpCAM (28.57%), respectively. Besides, this up-regulation (over expression) and the calculated values of O.R (19.65) and (2.64) with confidence interval (C.I) varying from 19.8 - 1.08 and 34.07- 0.23 showing significantly ($p < 0.05$) in EpCAM and SOX4. Similarly, DNA CNVs shows highly significant ($p < 0.001$) differences in SOX4 while, again decreasing trends were observed in EpCAM and CK19. The frequency of BRCA1 gene mutation frequency was observed (9.0 %) and statistical analysis showing significant ($p < 0.01$) differences in DNA CNVs and EMT markers. Hormone profile also show significant ($p < 0.05$) difference between estrogen and progesterone. MTHFR gene polymorphism showing 15.15% frequency of CT genotype in heterozygous condition with changes in ‘C’ (0.075%) and ‘T’ 0.924 allele frequency. Interestingly, statistical analysis shows significant correlations coefficient between CNVs of MTHFR with EMT markers.

Positive correlation between EMT markers and their DNA CNVs of MTHFR gene C677T polymorphism was observed that impact on C>T genotype increase risk for the disease progression. BRCA1/2 mutation modulates hormone dysfunction in premenopausal cases of breast cancer patients that increases tissue-specific genetic susceptibility either through TGF-βR1 signaling to confirm transition in metastasis or unknown factors.

KEYWORDS

EMT markers; MTHFR C677T; CNVs; BRCA1/2; TGF-βR1; Breast cancer

INTRODUCTION

The etiopathology of breast cancer (BC) is highly complex in nature and regulated by a variety of pathways including endocrine dysfunction (pre/post menopause), socioeconomic (nutrition) status based on folate - metabolism. Genetic factors involving chromosomal instability, p53 gene regulation, transforming growth factors (TGF), tumor necrosis factor (TNF) and epigenetic modification play an important role in disease progression during metastasis. Despite the advancement of technology, the mortality rate is still alarming perhaps either due to failure of early diagnosis of the patients or the patients reached the clinicians at an advanced (metastasis) stage [1].

World health organization shows that around 2.26 million new incidences of breast cancer were reported worldwide [2].

Interestingly, the incidence of breast cancer gene (BRCA1/2) is prominent in developed countries and differs prominently between age groups and ethnicity (race).

There is a tremendous increase of breast cancer in Asian subcontinent (24%) including in China, Japan, and Indonesia [3]. However, recent data have shown that most of the women are surviving with BC after mammography screening, conventional histopathology techniques and with adjuvant therapy. Hence, there is only option left and become challenges to the scientist is for early detection and timely management of the disease. Women having BRCA gene mutation are at high risk of developing BC in lifetime, and maximum risk (70%) reaches by attaining the age of 70. BRCA1/2 are tumor suppressor genes and mapped on chromosome 17q21 and 13q12-13 locus that causes large number variants resulting truncated protein is produced. The molecular biology of breast cancer is based on the

mutational frequency of BRCA1 and BRCA2 genes varies in different population with age and ethnic group of the BC patients. The frequency of BRCA1/2 mutation is 2% to 6% and BRCA1 is slightly higher than BRCA2 and lifetime risk for breast cancer increase 45%-80% as carriers. In African Americans 25% BRCA1 mutations are frameshift, 38% missense and 13% nonsense, while, BRCA shows three distinct mutations of BRCA1185delAG, BRCA1538incC and BRCA2 6174 del in Ashkenazi Jewish population [4-7]. Besides, BRCA1 gene mutation has also been associate with colon, endometrial, pancreatic and prostate cancer, while BRCA2 mutation also increased risk in pancreatic and prostate cancer, while BRCA2 gene mutation act as carrier for developing risk in gall bladder, stomach and melanoma [8,9]. Hereditary factors for BC are unique features in term of age, pathological conditions and prognosis. Hence, clinicians suggest investigation of BRCA1 and BRCA2 gene mutation for high-risk patients becomes essential because of significant role in familial cancers patients as carriers.

However, recent evidence suggests that methylenetetrahydrofolatereductase (MTHFR) gene C677T polymorphism, and epithelial mesenchymal transition (EMT) markers - markers cytokeratin 19 (CK19), epithelial cell adhesion molecule (EpCAM) and SRY-Box Transcription Factor 4 (SOX4) play a relevant role in variety of tumors [10-12]. Folate metabolisms play a vital role for several biological processes followed by synthesis of DNA, DNA repair, and methylation. The deficiency of folate induces chromosome breaks and followed by disruption of DNA repair process due to uracil disincorporation into DNA strands in cancer patients [13]. MTHFR gene is mapped on chromosome 1p36.3, play a crucial role in regulation of cellular metabolism to maintain equilibrium for intracellular concentration of de-novo methionine and homocysteine during conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. MTHFR gene C677T polymorphism has been associated in variety of cancer patients and their enzymatic activity varies either in homozygous (TT) or heterozygous (CT) condition [14-17]. However, the frequency of genotypes and their allele (C/T) varies under different conditions such as age, ethnicity, family history, socio economic conditions, professional life such heavy-duty drivers, and environmental factors (exposure with toxic fumes of chemicals like pesticide [14]. Hence, it becomes essential to identify the association between breast cancer patients with hormone profile changes (dysfunction) associated risk factor of the disease during tumor progression.

The mechanism of transformation from epithelial to mesenchymal cells during metastasis has not been defined clearly in breast cancer patients, but three specific circulatory molecules -CK19 and EpCAM is epithelial in origin and circulates in peripheral blood of the cancer patients [18,19]. Third, SRY-Box Transcription Factor 4 (SOX4) marker belongs to gene family of Sry-related high-mobility group box (SOX) (SRY- related High Mobility Group (HMG box) region associated for cell -growth, diversification and progression during malignancies [20]. CK19 is released from apoptotic tumor cells in variety of cancer and elevated or over expression (mRNA) was observed in both primary and metastatic lesion of breast cancer patients [21,22]. EpCAM, a transmembrane protein and involved in cell adhesion. Over expression is observed in both breast as well as ovarian cancer [23-25]. *In-vitro* analysis of SOX4 expression also involves controlling expression of EMT-related genes by activating CXC chemokine receptor-7 (CXCR7) transcription [26]. The proliferation of BC cells are hormones responsive to estrogen including MCF7 cell lines and play an important role in therapeutic due to either positive or negative hormone receptor [27].

During metastasis of breast cancer and uncontrolled proliferation from epithelial to mesenchymal tissue, transforming growth factor beta receptor 1 (TGF-βR1)

gene signaling is a relevant factor to regulate cell differentiation and cell proliferation either in autocrine or paracrine fashion in breast cancer patients. TGF-βR1 is rich in serine/threonine mediated signaling pathway to involve in invasion of extracellular matrix formation for surrounding differentiating cells during metastasis [28].

Present comprehensive mutational spectra have been designed with the aims to evaluate the frequency of mutation of CK19, EpCAM and SOX4 genes and their correlation to MTHFR C667T gene polymorphism, DNA copy number variations to determine genetic heterogeneity. The study was further extended to evaluate the frequency of germ line BRCA1 and BRCA2 gene mutation, endocrine dysfunction and TGF-βR1 signaling mediated tissue specificity and susceptibility, besides determine the "risk factors" in pre and postmenopausal cases of breast cancer patients.

MATERIALS AND METHODS

Preclinically diagnosed total cases (n = 159) and age match controls (n = 105) before receiving any adjuvant chemotherapy were included in the present study. The samples were collected randomly over a period of 2 years and sample size was selected to reach a significant conclusion based on the study. Family history of the patients was noted at the time of sample (blood & tissue) collection, such as, clinical stage, side of the breast affected, lifestyle, geographical location, family history of the disease, and environmental exposure to the drugs, that may help to assess risk factors for the occurrence of disease. Samples were collected in sterilized condition after written informed consent either from the patient or family member of the patients. The study has been approved by Institutional Research Committee and Institutional Ethical Committee of AIIMS-Patna.

Isolation of DNA, RNA and Selection of Primers

Genomic DNA was isolated from 2.0 ml of blood samples using Promega Kit, USA following manufacturers protocol. The isolated blood samples were dissolved in Tris-EDTA buffer (pH 8.0) and quantified using nanodrop spectrophotometer. The samples were diluted to bring the final concentration of 1.0 ug/ul and kept in -20°C till further analysis of EMT markers (CK 19, EpCAM and SOX4) use. The primers for MTHFR C677T and EMT markers EpCAM, CK-19 and SOX4 were designed using online primer designing tools (NCBI Primer Blast). The tetraplex primer-based real-time polymerase chain reaction Real Time-Polymerase Chain Reaction (RT-PCR) assay was performed for genotyping of MTHFR-C667T, moreover, mismatch was incorporated at second position of 3' end of both allele specific primer to enhance the sensitivity and specificity and further primer were selected. These group of tetra primer Amplification-refractory mutation system (ARMS PCR) using SYBR Green based on melting-point analysis was part of the strategy to distinguish single nucleotide polymorphisms (SNP) of MTHFR allele between wild type and mutant i.e., CT/TT in homozygous and heterozygous condition. The specific forward/reverse primers for each gene with optimized annealing temperature and cycling conditions are depicted in Table 1.

MTHFR C677T Gene Polymorphism using ARMS PCR

The ARMS-PCR technique, a highly sensitive and reliable technique, is used for the analysis MTHFR C677T gene polymorphism. The assay was performed by RT-PCR (BioRad, USA) with following cycling condition: initial denaturation at 95°C for 10 minutes, followed by 30 cycles of amplification steps (95°C for 10 s, 58°C for 10s, 72°C for 10s), final elongation at 72°C and followed by melt curve analysis as detailed documented earlier [29]. Melting curves were constructed by lowering the temperature to 65°C and later increasing the temperature by 0.2°C/s to 98°C for measuring the change fluorescence consistently.

Further the PCR product was used on 1.5% of agarose gel and bands were visualized on GelDoc system (BioRadTM) to evaluate again by appearing the genetic heterozygosity between C/T alleles based on Tm values shift from C→T allele observed in the banding pattern during gel electrophoresis.

Types/Genes	Forward and Reverse Primer Sequence (5’→3’)	Anne. Temp (°C)	References
CK19	F- 5’-ATTCCGCTCCGGGCACCGATCT-3’	60.2	Balducci et al., 2005
	R- 5’-CGCTGATCAGCGCCTGGATATGCG-3’		
TGFBR1	F- 5’-TTTCGCCTTAGCGCCCACTG -3’	56	Wang et al., 1996
	R- 5’- GAAGTTGGCATGGTAGCCCTT-3’		
BRCAI	F- 5’-GGTTGGCAGCAATATGTGAA-3’	57	Chan et al., 1999
	R- 5’-GCTGACTTACCAGATGGGACTCTC-3’		
BRCA2	F- 5’-AGCTGGTCTGAATGTTCGTTACT-3’	57	Chan et al., 1999
	R- 5’-GTGGGATTTTTAGCACAGCTAGT-3’		
MTHFR C667T	F- 5’TGTCATCCCTATTGGCAGGTTACCCCAAA-3’	58	Saxena et al., 2016
	R- 5’CCATGTCGGTGCATGCCTTCACAAAG-3’		
	C-poly 5’GGCGGGCGGCCGGGAAAAGCTGCGTGATGATGAAATAGG3’		
	T-allele-5’GCACTTGAAGGAGAAGGTGTCTGCGGGCGT-3’		
SOX4	F-5’-GGTCTCTAGTTCTTGCACGCTC-3’	57.2	Zafarnejad et al., 2010
	R-5’-CGGAATCGGCACTAAGGAG-3’		
EpCAM	F-5’ATTCCGCTCCGGGCACCGATCT3’	58.7	Alowaidi et al., 2018
	R-5’CGCTGATCAGCGCCTGGATATGCG3’		

Table 1: PCR strategy and primers with annealing temperature for EMT marker analysis in breast cancer patients.

Characterization of EMT Markers in Breast Cancer Patients

The comprehensive analysis of EMT markers CK19, EpCAM and SOX4 genes mutation frequency were evaluated using PCR with highly specific forward and reverse set of primers either in terms of over expression (up-regulation), or down-regulation. The complete disappearance of band (bp) considers as gene mutation (null), characterized on 1.5% agarose gel electrophoresis and individual bands were visualized under GelDoc system The DNA copy number variations (CNVs) for the EMT marker genes were determined using the inbuilt ImageLab software. The PCR reaction contains 5 µl of 5X Go Taq buffer, 1 µl of primer set, 1.25 µl dNTPs, 0.2 µl Taq DNA polymerase, 50 ng of template DNA and further volume was maintained by nuclease free water. The PCR cycling condition was initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 45s, annealing temperature based on specific target gene mentioned in table 1 for 30s and extension at 72°C for 1minute, final extension at 72°C for 7 minutes. The PCR products were separated on 1.5% agarose gel and intensity of individual bands were observed under gel documentation system (BioRad ImageLab™ software inbuilt) to determine the DNA CNVs of each gene in cancer cases and controls.

Statistical Analysis

The frequency of EMT gene frequency (%) and their significance p-values were calculated using chi-square test between cases and controls. The frequency of DNA CNVs of genes was evaluated using ImageLab software of BioRad, frequency was evaluated using Mann Whitney U test, while correlation between CNV of genes was evaluated using Pearson’s Coefficient Correlation analysis. Graphs were plotted in Graph pad Prism (Version 8.0) and SPSS® (Version 26, IBM Chicago, USA) was used for evaluation of statistical significance. All tests were two-tailed and $p < 0.05$ was considered as statistically significant.

RESULTS

Identification of CK19, EpCAM and SOX4 Markers in Breast Cancer Patients

PCR based comprehensive analysis was carried out to evaluate the mutational spectra either over expression (upregulation) or suppression (down regulation) or

complete disappearance of band (null) of EMT markers genes - CK19, EpCAM and SOX4 in two different types of samples (blood & tissue biopsy) collected from the breast cancer patients along with controls to determine the tissue specific genetic heterogeneity using specific set of forward & reverse primers in table 1. The detailed findings of spectrum of EMT gene mutation (null), up regulation (over expression) or down regulation (under expression) are documented in table 2A for blood samples, where the decreasing trends of mutation i.e. complete disappearance of band (null) of EMT markers - CK19 (50.00%), SOX4 (38.09%) and EpCAM (28.57%) were observed as depicted in Figure 1. The calculate values of odd ratio of CK19 (17.33) with C.I at 95% interval varying between 171.66 -1.75 and SOX4 the O.R (14.33) with C.I varying between 17.66 -1.75, statistically showing the significant (p <0.05) differences between breast cancer cases and controls.

Gene	Mutations	Number and (%) Frequency		C.I at 95%		O.R.	p-value
		Cases	Control	Upper	Lower		
CK-19	Mutation (Null)	9 (50.00)	1 (7.14)	171.66	1.75	17.33	0.0045**
	Upregulation	4 (22.22)	2 (14.29)	11.7	0.23	1.64	0.6217
	Downregulation	5 (27.77)	5 (35.71)	1.91	0.05	0.3	0.1904
EpCAM	Mutation (Null)	4 (28.57)	0	(-)	(-)	(-)	(-)
	Upregulation	1 (0.07)	12 (85.71)	198.56	1.08	19.65	0.0001***
	Downregulation	9 (64.24)	2 (14.2)	13.65	1.21	1.81	0.005***
SOX4	Mutation (Null)	8 (38.09)	1 (0.07)	17.66	1.75	14.33	0.05**
	Upregulation	8 (38.09)	10 (71.29)	34.7	0.23	2.64	0.05**
	Downregulation	5 (23.80)	3 (21.42)	3.76	0.05	0.7	0.09

Table 2A: PCR based analysis to determine mutation frequency of EMT markers CK19, EpCAM and SOX4 genes from blood samples in breast cancer patients. Note: *Significant differences (p <0.05) were observed between cases and controls using two tailed x2- test.

Genes	Type of Mutations	Number and (%) Frequency		C.I at 95%		O. R.	p-values
		Cases	Control	Max	Min		
CK-19	Mutation (Null)	1 (11.11)	0 (0.0)	0	0	0	0
	Upregulation	2 (22.44)	1 (33.3)	11.7	0.23	1.64	0.6217
	Downregulation	6 (66.6)	2 (66.6)	1.81	0.25	0.3	0.0342*
EpCAM	Mutation (Null)	0 (0.0)	0 (0.0)	0	0	0	0
	Upregulation	7 (77.7)	1 (33.33)	154.76	2.87	23.23	0.0267*
	Downregulation	2 (22.44)	2 (66.6)	123.99	0.67	13.22	0.576
SOX4	Mutation (Null)	1 (11.11)	0 (0.0)	0	0	0	0
	Upregulation	6 (66.66)	2 (66.6)	39.7	0.73	5.64	0.05**
	Downregulation	2 (22.44)	1 (33.33)	3.76	0.05	0.7	0.687

Table 2B: PCR based analysis of EMT markers-CK19, EpCAM and SOX4 genes from biopsy (tissue) samples in Breast Cancer Patients. Note: *Significant differences (p <0.05) were observed using x2- test between cases and controls.

Sample Type	Genes	Mean ± S. D		S.E.		Significance p-values
		Cases	Control	Cases	Control	
Blood	CK19	1697.73 ± 258.59	1963.83 ± 302.52	317.14	193.71	0.01*
	EpCAM	1687.95 ± 191.22	2062.25 ± 262.21	264.33	86.98	0.002**
	SOX4	1282.56 ± 191.22	1834.08 ± 260.63	378.12	188.68	0.00001***
	MTHFR	1328.67 ± 171.22	1734.08 ± 120.63	255.43	167.22	0.05*
Biopsy Tissue	CK19	2372.83 ± 104.52	1856.12± 158.59	124.14	113.71	0.05*
	EpCAM	2448.66 ± 172.21	1714.83 ± 165.22	138.33	86.98	0.05*
	SOX4	2025.05 ± 220.63	1646.93 ± 121.22	177.12	188.68	0.05*

Table 3: Statistical analysis showing significance mean copy number variations of EMT markers in blood and biopsy (tissue) in breast cancer patients. Note: *Significant differences (p <0.05) were observed using x2- test between cases and controls.

Interestingly, again, the calculated values of upregulation (over expression) of O.R (19.65) with C.I at 95% interval (198.56-1.08) and O.R (2.64) with C.I at 95% (34.70 - 0.23) in EpCAM and SOX4 showing significance (p <0.05) difference in EpCAM and SOX4, respectively, between cases and controls. EpCAM showing frequency (28.57%) of down regulation with calculated values of O.R (1.81) and C.I at 95% intervals are varying 13.65-1.21, showing statistically significant (p <0.05) difference in breast cancer patients when compared with controls after using two tailed chi-square test. Table 2B showing calculated values of upregulation (over expression) of O.R (0.30) with varying confidence interval (C.I) at 95% is 1.81-0.25 for

CK19, EpCAM the value of O.R (13.22) with C.I vary at 95% (123.99-0.67) and the value of O.R (5.6) with vary C.I varying at 95% (39.70-0.73) for SOX4 showing statistically significance (p <0.05) in biopsy samples with respect to controls.

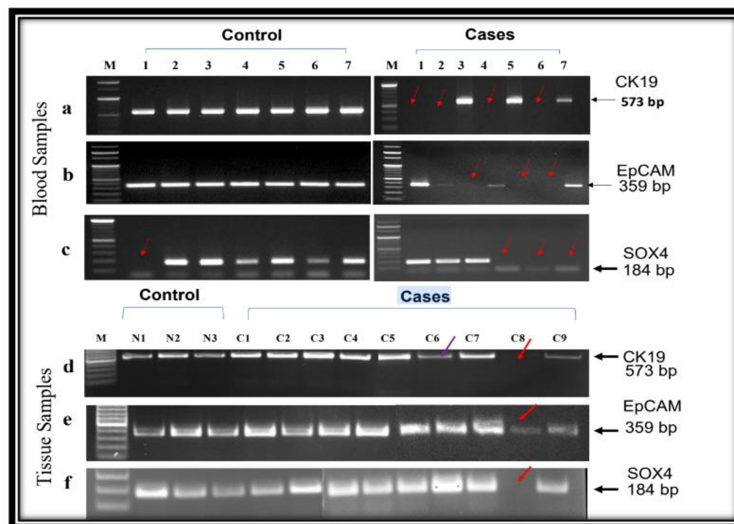

Figure 1: Representation of the PCR based analysis of CK19, EpCAM and SOX4 genes on agarose (1.5%) gel, stained with ethidium bromide and bands were visualized and characterized on GelDoc system in breast cancer and normal samples. **(A)** down and upregulated expression along with complete disappearance of band was observed in several cancer samples denoted by arrow head **(B)** represent the band of PCR product of CK19 in control samples **(C)** represent the downregulation, upregulation and complete disappearance of band of EpCAM in cancer samples **(D)** EpCAM expression in control samples **(E)** represent upregulation, downregulation and complete disappearance of SOX4 band in cancer patients **(F)** represent the SOX4 PCR band in control samples.

Table 3 shows the comprehensive data analysis of mean ± S.D and S.E values for DNA CNVs in both (blood & tissues (biopsy) samples in breast cancer patients and controls. The calculated values of mean for SOX4 (1282 ± 191) with S.E (378) for blood and 2535.30 ± 191 and S.E (150) for tissue (biopsy) samples statistically showing significant variations i.e. highly significant (p <0.001) difference was observed in blood, while in tissue showing decreasing trend (p <0.05) with respect to controls. Similar, significant differences (p <0.5) were also observed for EpCAM and CK19 in both blood and biopsy (tissue) samples.

MTHFR C677T Gene Polymorphism in Breast Cancer Patients

Methylene tetrahydrofolate reductase (MTHFR C677T) genes polymorphism was evaluated using allele refractive mutational system (ARMS) PCR in breast cancer and controls by specific sets of tetraplex primers (Table 1). The frequency of MTHFR genotypes varying in homozygous CC (31.45%), TT (22.31%) and heterozygous conditions CT (46.24%) in breast cancer patients. Moreover, Hardy-Weinberg equilibrium analysis showing individual frequency of 'C' and 'T' allele is 0.924 and 0.0758 in disease condition (cancer patients) as compared to controls. Moreover, MTHFR C677T gene polymorphism showing significant (p <0.05) difference in cases with respect to controls. Figure 2 & Figure 3 showing MTHFR C677T gene polymorphism, a rare genotype (TT) variant in homozygous condition and showing severity of the disease. Moreover, favourable correlation of C677T genotype with reduced folate intake (OR = 2.31, 95% CI: 1.27-3.80) appears to back this impression. The DNA CNVs were also observed in MTHFR C677T gene polymorphism and calculated values of mean ± S.D 1328 ± 171 with S.E 255 in breast cancer cases compare with controls values 1734 ± 120 (S.E 167.3) shows significant (p <0.05) differences in blood samples only (Table 3).

Further, correlations coefficient analysis was also evaluated between the DNA CNVs of MTHFR C677T and individual EMT marker gene CK19, EpCAM, and SOX4. Figure 2 (Bar diagram) showing mean ± s.d DNA CNVs with two different colors (red = breast cancer patient), blue (controls) and EMT marker gene in both blood and biopsy (tissue) samples with respective controls to calculate (p = values) to determine significance difference. Apparently, bar (red) diagram showing CK19 and EpCAM showing equal values and down regulated for SOX4 in biopsy samples as compared to controls (red bar), but decreasing trends were observed CK19, EpCAM and SOX4 in blood (blue). The highest mean ± S.D (2535 ± 191.22) value was recorded for SOX4 in biopsy samples as compared to controls (1211.73 ± 79.07), while, for CK19 (2518 ± 451) and EpCAM (2503 ± 586) gene showed significantly (p <0.5) down regulated in breast cancer cases (biopsy) as compared to controls. Similarly, MTHFR C677T showing (p <0.05) significantly down regulation and their calculated mean ± S.D (1328.67 ± 171.22) values, when compared CK19 and EpCAM with controls (1734.08 ± 120.63 (blue) in blood samples (Table 3).

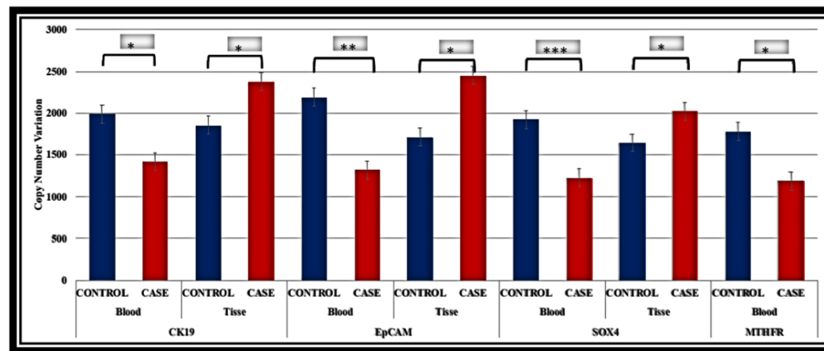

Figure 2: Bar diagram showing the significant difference in CNV of CK19, EpCAM, SOX4 and MTHFR C677T genes in breast cancer cases (red) compared to the normal (blue) samples. Statistical analysis showing the level of significance using Mann Whitney U- test (*p <0.05, **P <0.005, ***P <0.0005).

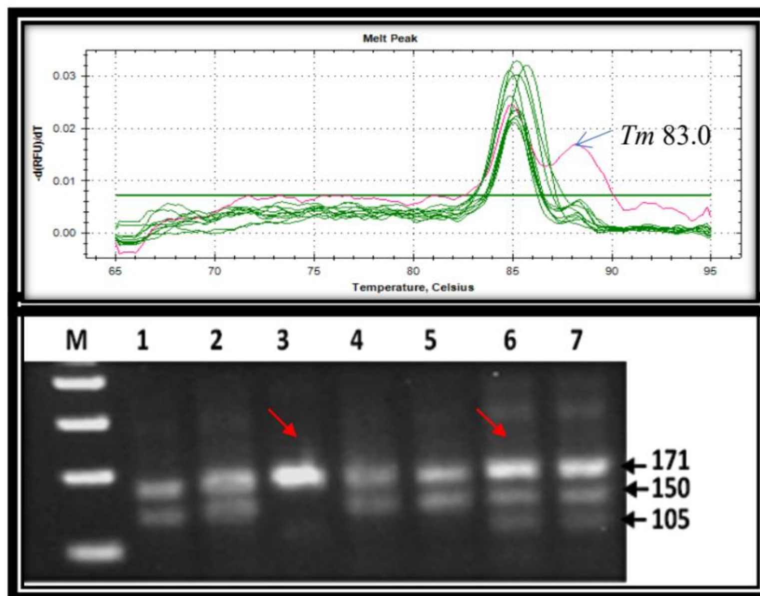

Figure 3: A&B): ARMS -PCR analysis of MTHFR C677T gene polymorphism showing melt peak shifting (Tm) values from 82°C (wild type CC genotype) to 83°C (Figure 3A) and, confirm the point mutation (C →T) as shown in Figure 3A. Lane 6 and 7 (arrow) showing in heterozygous condition (CT genotype) increase "risk" factor, while rare TT genotype (lane-3) in homozygous condition showing lethality for the growing cell (Figure 3B). Lane-1,2,4 & 5 showing wild type (CC genotypes) in homozygous condition in breast cancer patients.

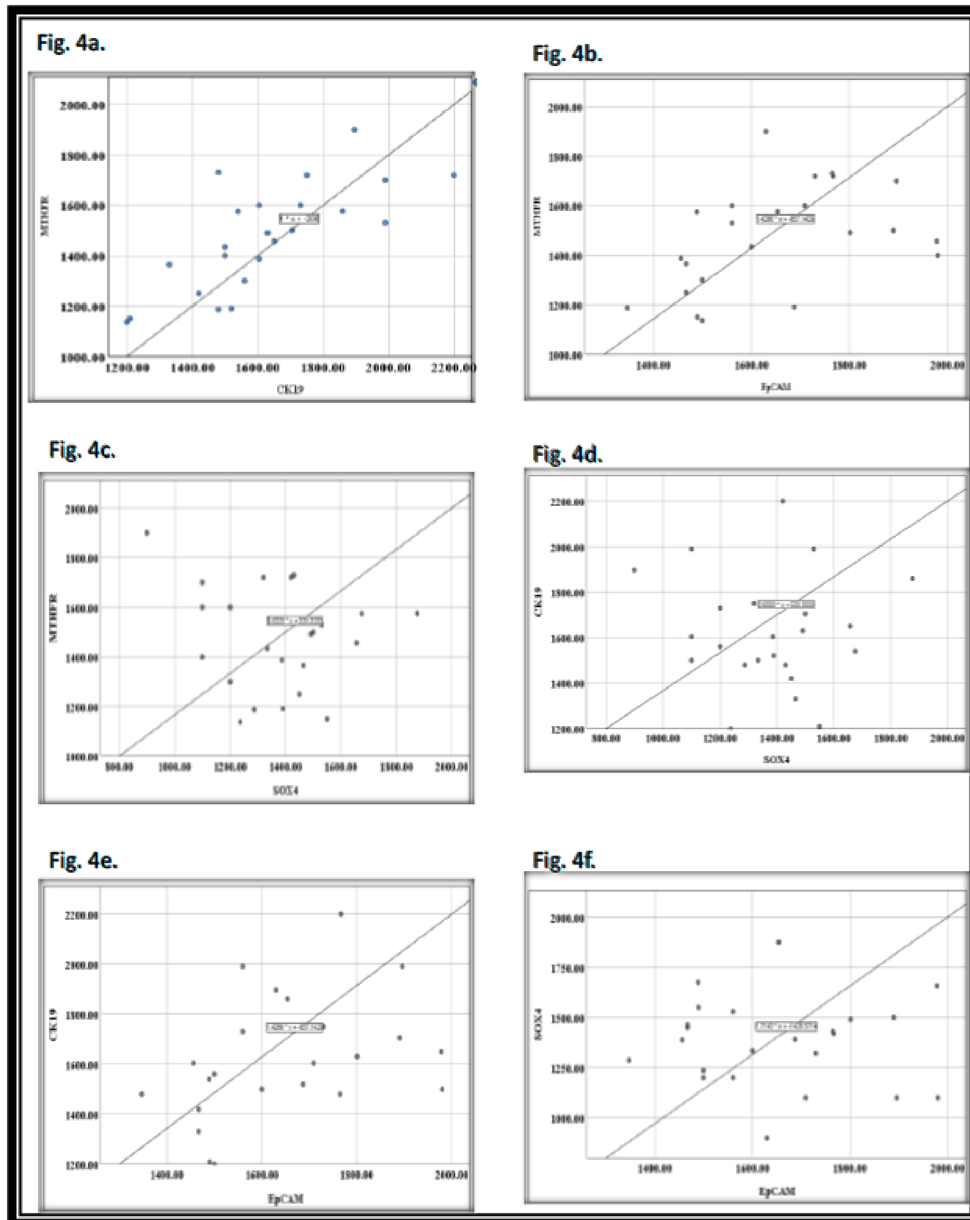

Figure 4: Statistical analysis of the correlation coefficient between CNVs of MTHFR, CK19, EpCAM, SOX4 genes in breast cancer patients from blood samples.

Association of CNVs between MTHFR C677T and EMT Markers

The study was further extended to evaluate correlation (r - value) of copy number variations of MTHFR C677T gene with CK19, EpCAM and SOX4 in breast cancer patients shows significant (r = 0.432, p <0.05; r = 0.334, p <0.05; r = 0.389, p <0.05) positive association using Spearman's rank correlation in age (pre menopause) matched controls as shown in Figure 4A - Figure 4C. Table 4 showing significant (p <0.05) calculated values of correlation between EpCAM (CNVs) with SOX4 and CK19 (r = 0.448, p <0.05; r = 0.567 as depicted in Figure 4D & 4E). The association between CNVs of SOX4 and CK19 were observed during disease progression (r = 0.645, p <0.05) in blood samples (Figure 4F). The calculated values for correlation coefficient showing significant association between EMT markers CK19, EpCAM and SOX4 genes and CNVs (Figure 5A - Figure 5C) in biopsy (post

menopause) samples from breast cancer patients. Similarly, EpCAM and CK19 showing strong positive correlation with statistically significant (p <0.001) differences but lacking between SOX4 and CK19 (Table 4).

Table 5 showing the endocrine evaluation of three hormones i.e., estrogen and progesterone showing significant differences (p <0.05), while follicular stimulating hormone (FSH) showing lack of significance (p >0.05) difference between cases and controls. Moreover, the study was further extended to evaluate the correlation coefficient (r - value) between CNVs of three EMT markers (CK19, EpCAM and SOX4) with hormone level of age matched premenopausal breast cancer patients showing positive correlation between CK19 with FSH (r = 0.673; p <0.033), while, negative correlations were observed in estrogen and progesterone (r = -0.321; p <0.035 and r = -0.646, p <0.05). Similarly, the CNVs of EpCAM again showed positive correlation with estrogen, and progesterone, (r = 0.564, p <0.05; r = 0.345, p <0.05), and FSH showed negative correlation (r = -0.765, p <0.001). Similarly, the CNVs of SOX4 showed positive correlation with estrogen, and progesterone (r = 0.442. p <0.03; r = 0.364, p <0.05; r = 0.325, p <0.05). MTHFR C677T gene CNVs were calculated and correlated with estrogen, progesterone, and FSH (r = 0.322. p <0.05; r = 0.462, p <0.05; r = 0.347, p <0.05) as shows significance as shown in table 4.

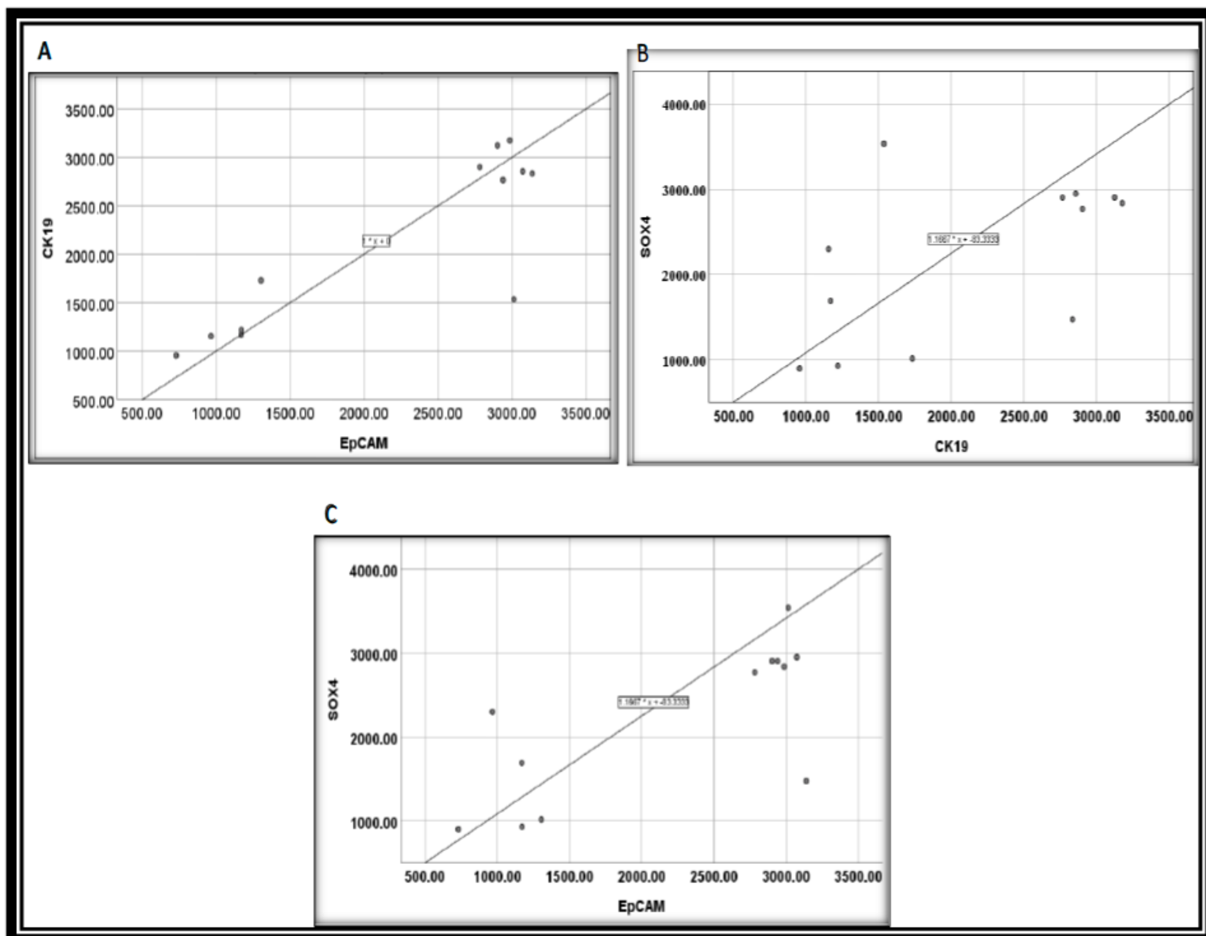

Figure 5: Superman's coefficient correlation analysis showing statistical significance difference between EMT genes (CK19, EpCAM & SOX4) and DNA CNVs in breast cancer patients from biopsy (tissue) samples.

Parameters		MTHFR	CK19	EpCAM	SOX4	ER	PR	FSH
MTHFR	r	(-)	0.432	0.334	0.389	0.322	0.462	0.347
	p	(-)	0.05	0.05	0.05	0.05	0.05	0.05
CK19	r	0.432	(-)	0.567	0.645	-0.321		0.673
	p	0.05	(-)	0.05	0.05	0.035	0.646	0.033
							0.05	
EpCAM	r	0.334	0.567		0.448	0.564	0.345	0.342
	p	0.05	0.05		0.05	0.05	0.05	0.05
SOX4	r	0.389	0.645	0.448	(-)	0.442	0.364	0.325
	p	0.05	0.05	0.05	(-)	0.03	0.05	0.05

ER: Estrogen, PR: Progesterone, FSH: Follicle-Stimulating Hormone

Table 4: Correlation analysis between MTHFR, EMT markers and hormone in breast cancer patients.

Hormone Types	Mean ± S.D.		S.E.		C.I at 95% Interval		p-Value
	Case	Control	Case	Control	Min	Max	
Estrogen	31.1 ± 27.62	105.4 ± 74.99	8.73	23.72	38.89	99.53	0.01*
Progesterone	6.5 ± 4.66	37.1 ± 51.68	1.47	16.34	3.51	40.03	0.03*
FSH	18.7 ± 27.64	3.3 ± 2.41	8.74	0.76	1.31	20.67	0.09

*Significance difference (p <0.05) was observed using x2- test between cases and controls.

Table 5: Statistical analysis showing hormone profile analysis in breast cancer patients and controls.

BRCA1 and BRCA2 Gene Mutation in Breast Cancer Patients

PCR based analysis of BRCA1 and BRCA2 gene mutation was carried out using specific set of forward & reverse primers on agarose (1.5%) gel in breast cancer patient and controls as shown in Figure 6.

Data of mutation was including either complete disappearance of band treated as (null) or upregulation (over expression) and down regulation (under expression), when compared with their respective controls. The statistical analysis showing the highly significant (p <0.001) values with mean ± S.D (1379 ± 578.3) in breast cancer cases and 1842 ± 309.20 (controls).

Parameter	Types	CK19	Sox4	EpCAM
CK19	r	N/A	0.553	0.869**
	p		0.465	0.0001
SOX4	r	0.553	N/A	0.743**
	p	0.465		0.006
EpCAM	r	0.869**	0.743**	N/A
	p	0.0001	0.006	

Table 6: Spearman's Coefficient Correlation analysis showing significance between copy number variations in CK-19, EpCAM and SOX4 genes. Note: *Significant p-value ** represent p<0.05, r= Correlation Coefficient, P-value.

Genes	Type of Mutations	Frequency (%)		C.I. at 95%		O.R.	p-value (Chi-square test)
		Case	Control	Max	Min		
BRCA1	Upregulation	3 (15.8%)	14 (56.0%)	3.94	0.33	0.124	0.0129*
	Downregulation	14 (87.5%)	11 (44.0%)	2.16	0.54		
	Mutation (Null)	2 (10.5%)	0	0	0		
BRCA2	Upregulation	17 (81%)	17 (68.0%)	2.2	0.57	4	0.093
	Downregulation	2 (9.5%)	8 (9.5%)	4.71	0.24		
	Mutation (Null)	2 (9.5%)	0	0	0		
TGFβRI	Upregulation	10 (55.6%)	3 (50%)	12.68	0.96	1.25	0.813
	Downregulation	8 (44.4%)	3 (50%)	4.58	0.93		
	Mutation (Null)	0	0	0	0		

Table 7: Statistical analysis showing the frequency of BRCA1/2 and TGF βR1 gene mutations in breast cancer patients. Note: *Significance difference(p<0.05) was observed using x2- test between.

The observed frequency of BRCA1 gene mutation is 9.0% and calculate values of O.R (0.12) with C.I at 95% intervals vary 3.94 - 0.33 for up regulation (over expression) that showing statistically significant (p <0.5) difference with respect to controls. The calculated value of O.R (0.02) with C.I at 95% interval vary 2.16 - 0.54 for down regulation and showing lack of significant differences in BRCA1. Apparently, the bar diagram (Figure 7) showing higher mean ± S.D values between CNVs of BRCA1 and BRCA2 gene mutations as shown in black bar for BRCA2.

Similarly, the calculated values of BRCA2 gene mutation are 9.50% in breast cancer cases with observed mean ± S.D (1794.8 ± 667) and 1531 ± 216.21 values for controls again showing statistical significance differences (Table 6 & Table 7).

Gene	Mean ± S.D.		S.E.		Significance P value
	Cases	Control	Cases	Control	
BRCA1	1379.8 ± 578.3	1842 ± 309.20	126.19	61.81	0.001**
BRCA2	1794.8 ± 667.50	1531 ± 216.21	142.25	43.2	0.0493*
TGFβR1	1991.66 ± 240.96	2005.6 ± 188.99	56.79	77.16	0.897

Table 8: Statistical analysis showing the mean ± S.D values for CNVs of BRCA1/2 and TGF βR1 gene in breast cancer patients. Note: *Significance difference(p<0.05) was observed between cases and controls.

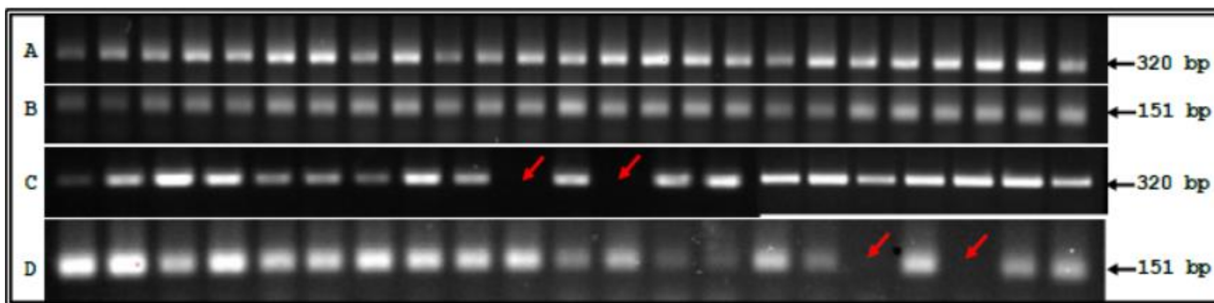

Figure 6: Identification and characterization of BRCA1 and BRCA2 gene mutation using PCR based analysis in breast cancer patients; **A)** BRCA1 control; **B)** BRCA2 control; **C)** BRCA1 cases, **D)** BRCA2.

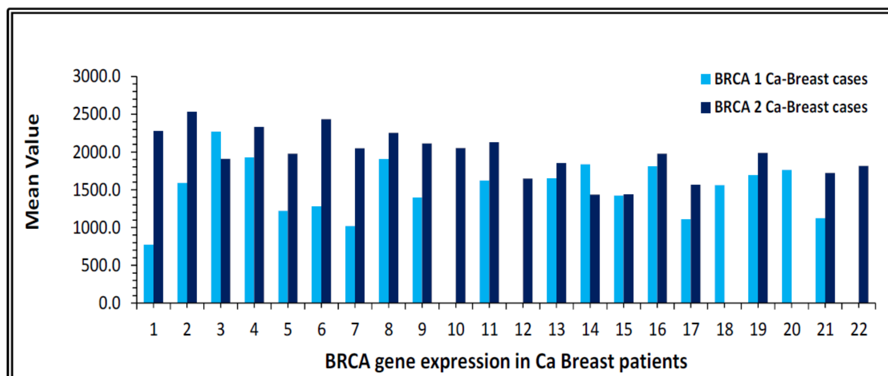

Figure 7: Bar diagram showing DNA copy number variations in BRCA1 (blue) and BRCA2 (black) gene in breast cancer patients.

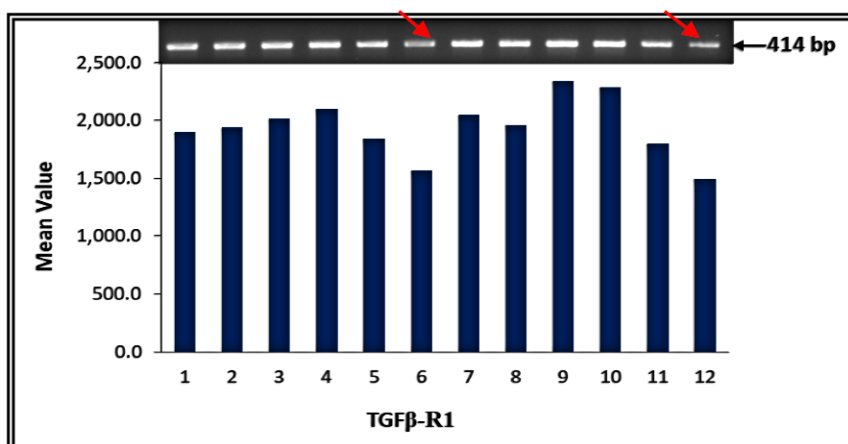

Figure 8: PCR based analysis of transforming growth factor βR1 gene on 1.5% agarose gel and band (414bp) and bands were visualized after ethidium bromide on GelDoc system. Bar diagram showing the DNA copy number variations in breast cancer patients.

PCR based analysis of TGF-βR1 (414bp) was carried out in random selected cases with respect to controls, showing higher frequency for up regulation when compared to down regulation (under expression) in breast cancer cases as shown in Figure 8 (arrow). Apparently, bar diagram showing lack of significance (p >0.05) difference with respect to controls and the calculated values of mean ± S.D for copy number variations observed in cases (1936.55 ± 2920 and (1951.22 ± 59.9) controls. Further, statistical analysis was carried out to evaluate the frequency of up regulation (55.6%) and down regulation (44.4%) with confidence interval varying 12.68-096 and 4.58-0.93, respectively (Table 7 & Table 8).

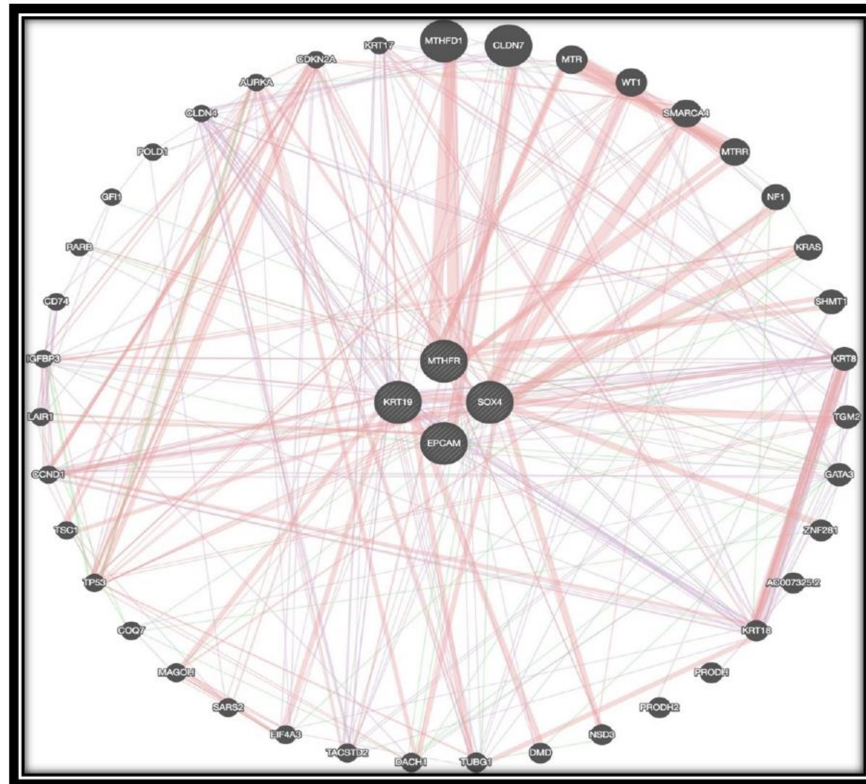

Figure 9: Represent the interaction network of MTHFR C677T gene and EMT Markers (SOX4, EpCAM, and CK19), approximately, more that 74 % of genes were enriched in physical interaction and represented by orange line. In co-expression, 8% of genes were enriched (violet colour lines), and 2.87% of genes were enriched in genetic interaction parameters (green lines).

In-Silico- Bioinformatics Based Analysis of Interaction Between MTHFR C677T and EMT Markers CK19, EpCAM and SOX4 Genes

A curiosity has been arising to identify the interaction between DNA CNVs of MTHFR gene C677T and EMT marker genes CK19, EpCAM & SOX4 gene markers, that showing positive correlation using bioinformatics analysis-gene mania webtool. The parameters were kept as default and interaction of all four genes were analyzed In-Silico. More than 50 genes were enriched in the interaction network. Based on physical interaction parameter, the MTHFR gene showed interaction with MTHFD1, TP53, MTR, KRAS, MTRR, SHMT1, SOX4, CK19 genes and EpCAM gene showed interaction with CLDN7, LAIR1, TP53, KRAS and SOX4. MTHFR gene analysis with SOX4 gene and KRT19 (CK19) gene showed physical interaction (protein) with large number of WT1, SMARCA4, NF1, KRAS, SHMT1, GATA3, NSD3, DACH1, TP53, TSC1, CDKN2A and IGFBP3, CCND1, MAGOH, EIF4A3, TUBG1, DMD, KRT8, TGM2 gene, respectively. Further, network was stratified based on the genetic interaction (DNA/RNA) of MTHFR and EMT markers that showed the enrichment in case of SOX4

(KRAS, SHMT1, GATA3, MAGOH, RARB, DACH1) and EpCAM gene (DACH1, ZNF2B1, KRAS and IGFB3), while MTHFR and CK19 doesn't have any genetic interaction. Further, we also evaluated the network on the basis of co-expression parameter of interaction and observed SOX4, EpCAM and KRT-19 genes were interacting with each other along with several other shared genes (TACSTD2, CLDN4, KRT18, KRT8, CLDN7, CDKN2A, IGFBP3, WT1), but MTHFR C677T showing lack of significant interaction in co-expression of genes as shown in Figure 9.

DISCUSSION

World Health Organization says that the incidence of BC increasing 2% per year in world and becomes the most common diseases of women. Globally, the higher incidences were observed in American and European women as compared to Asia. Etiopathology of BC is highly complex due to involvement of multiple factors including lifestyle and environment. Simultaneously, genetics, epigenetic factors play an important role for differential increase of incidence of BC in women belongs to different geographical regions. More than 90% of cases of BC are sporadic in nature and only 10% cases are familial (hereditary). Authors have designed a comprehensive study based on mutational spectra in blood (premenopausal) and biopsy samples to determine tissue specific genetic heterogeneity after using EMT markers CK-19, EpCAM, SOX4 and MTHFR gene C677T polymorphism and DNA CNVs and their correlation. Moreover, our study significantly reveals that higher frequency of mutation (null), over expression (upregulation) and down regulation of EMT markers were observed in cancer patients as compared to the controls. Significantly, higher mutation (50%) frequency of CK19 gene, followed by subsequently decrease in EpCAM and SOX4 genes (28.57% & 38.09%, respectively) in breast cancer patients confirm the progression of disease (i.e. premetastatic to metastatic stage). Moreover, correlation of CNV of MTHFR gene with EMT marker (CK19, EpCAM, and SOX4) showed significant association, confirm the "risk factor" for the women suffering from the disease. Endocrine dysfunction plays an important role during the onset of disease by significant association with estrogen and progesterone to confirm the tissue specific genetic susceptibility between pre and postmenopausal case of BC either due to mutation of BRCA1/2 genes or discordance of TGF-βR1 gene signalling during metastasis.

Association of MTHFR Gene Polymorphism in Breast Cancer Cases

The differential frequency of MTHFR gene C677T polymorphism of CC, CT and TT genotype, suggests that CT genotype increase risk of the disease in heterozygous condition due to significant ($p < 0.05$) variation between cancer patients and controls. Further, statistical analysis shows significant ($p = 0.038$) values for TT genotype, rare mutation in homozygous condition, suggesting the severity of the disease increase several folds with respect to controls. Our observation for a stronger inverse association in breast cancer risk among women with the TT genotype is in line with previous study that revealed the higher frequency of CT genotype increase risk for developing cancer as compared to normal [30,31]. In Iranian population, MTHFR C677T polymorphism study showing lack of significant association probably small sample size, while several studies illustrated that positive correlation was observed between MTHFR 677 C>T polymorphism and associated risk factor for the women belongs to early onset 25 years to 44 years age group [32,33]. The women have good socioeconomic conditions and maintaining equilibrium of serum folate have low risk of developing cancers with better survival. Several studies have been carried out by the same author shows that increase frequency of CT genotypes in heterozygous condition increase risk factor for developing in variety of tumours like pancreatic tumors, gall bladder, and carcinoma of cervix perhaps due to low folate intake in the diet

[11,12,34]. Bihar is a poor state with low socioeconomic status that might be the important factor to increase risk in cancer patients. Our outcomes established that the MTHFR polymorphisms showed strong linkage and genetic instability in due to substitution of nucleotide from cysteine to thymidine (C>T) followed by change of amino acid Alanine22Valine due to ‘point mutation.

Role of EMT in Breast Cancer Cases and its Correlation with MTHFR Gene Polymorphism

In addition, the mutational analysis and DNA CNV analysis EMT markers -CK19, EpCAM and SOX4- reveals that highly significant value was observed in SOX4 in blood samples of BC patients, suggesting increased risk during early onset of diseases with increase tissue specific genetic susceptibility in individual women suffering from disease like BC. In addition, variation in the frequency in the expression (over expression /down regulation) of EMT markers was significantly correlated due to the dysregulation of endocrine hormones (ER, PR and FSH) may induce early onset of disease (early age group) in the cases of premenopausal to post-menopausal i.e. transition to metastatic stage. Further analysis of the data also supports the findings of correlation of variance, suggests that the CNVs of MTHFR, CK-19, EpCAM, SOX4 and endocrine hormone profile (estrogen or progesterone) of patients are working in synergetic way to upbringing of disease progression in metastatic stage or use of two different tissues (blood & biopsy). Overall, our comprehensive study on EMT markers could stratify the cancerous and non-cancerous patients due to linkage with genes (BRCA and TGF).

Association of BRCA1/2 in the Cases of Breast Cancer

The etiopathology of breast cancer is highly complex because more than 90% cases are sporadic in nature, only 5-10% cases are familial (hereditary). The most common inherited genes are BRCA1, BRCA2, CDH1 and p53 that increase the genetic susceptibility in the incidence of breast cancer. BRCA1 and BRCA2 are tumor suppressor genes mapped on chromosome 17q21 and 13q12, containing 24 and 27 exons, respectively, including exon1 containing noncoding region for both BRCA1/2. The frequency of gene mutation of BRCA1/2 varies in different population and in general population the frequency of BRCA1/2 gene mutation vary between 2%-6%, while the frequency of BRCA1 increase up to 10% in Jewish population [4,35,36]. Present study shows higher frequency (9%-9.5%) of BRCA1/2 gene mutation due to either endocrine dysregulation or MTHFR C677T genetic heterogeneity. Although, the frequency of BRCA1 gene mutation including frame shift, missense or rearrangements was recorded between 25%-37% in African American population [37]. The large genomic rearrangements (LGRs) between BRCA1 and BRCA2 gene leads to the origin of pseudogenes sequences or pretermination codons during homologous recombination followed by non-functional truncated protein [38].

Dysregulation of TGF-βR1 Gene in the Cases of Breast Cancer and Correlation between TGF-βR1 Gene EMT Markers and MTHFR

In tumor biology, TGF-β plays a central role in regulating cellular proliferation and differentiation with other molecules like interleukins through either autocrine or paracrine signalling during carcinogenesis. TGF-β also regulates stem cell proliferation and differentiation along with modulation of SOX4 in cancer cells. Present study shows lack of significant association in breast cancer, suggesting either due to tissue specific genetic heterogeneity of EMT markers or loss of genetic material of BRCA1/2 gene mutation. However, TGF-βR1 gene expression has been associated to decrease the tumorigenicity by modulating either up regulation (over expression) or down regulation with other genes [39]. Present study shows significantly over expression of SOX4 gene, suggesting aggressiveness of BC patients either

independently or synergetic fashion with other genes like EpCAM, CK19 and MTHFR gene C677T polymorphism.

Moreover, the MTHFR C667T polymorphism and dysregulated CNV of EMT markers showed significant correlation in breast cancer cases. Moreover, MTHFR C677T and EMT markers together point toward the tissue specific susceptibility due genetic variation (mutation of BRCA1 and BRCA2) in BC patients, and their possible use as genetic biomarker during disease progression. Hence, there is need to develop techniques for early detection by identification and characterization of risk factors of the disease that may help the clinicians for better therapeutic management by timely assessment.

CONCLUSION

The present study has been concluded with the remark that: 1) EMT markers (CK19, EpCAM and SOX4) may help for early diagnosis of the patients, 2) Over expression of stem SOX4 gene confirm the transitional event towards metastasis during tumorigeneses, 3) Variation in the frequency of BRCA1 and BRCA2 genes may be due to genetic heterogeneity of MTHFR C677T polymorphism or discordance between endocrine dysfunction in pre/postmenopausal cases, suggesting failure of DNA mismatch repair mechanism and activation of truncated protein that might be involved to trigger tumorigenesis, and 4) Bioinformatics analysis confirm the genetic and physical interaction with MTHFR gene along with several other genes during early event to later stages (metastasis) of cancer.

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ETHICS APPROVAL

This study respected the Helsinki principles for research and the ethical guidelines of our institution.

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AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on request from the corresponding author.

AUTHOR CONTRIBUTIONS

AKS executed experiment designed, implementation, validated results, proof reading, funding, and publishing of article; PS &VS help for clinical diagnosis, providing samples (Blood & Tissue), S and MT, helped data analysis, and finalization of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Study was approved by Institute ethical committee of AIIMS Patna (AIIMS/Pat/IRC/2020/610), and informed consent form was dually signed by patients.

PATIENT CONSENT FOR PUBLICATION

Patients consent form was dually signed by patients involved in study.

COMPETING INTERESTS

There are no competing interests among the authors.

REFERENCES

1. Petrone I, Bernardo PS, Dos Santos EC, et al. (2021) MTHFR C677T and A1298C polymorphisms in breast cancer, gliomas and gastric cancer: A review. Genes 12(4): 587.
2. Parkin DM, Bray F, Ferlay J, et al. (2005) Global cancer statistics, 2002. CA: A Cancer Journal for Clinicians 55(2): 74-108.
3. Momenimovahed Z, Salehiniya H (2019) Epidemiological characteristics of and risk factors for breast cancer in the world. Breast Cancer: Targets and Therapy (11): 151-164.
4. Malone KE, Daling JR, Neal C, et al. (2000) Frequency of BRCA1/BRCA2 mutations in a population-based sample of young breast carcinoma cases. Cancer 88(6): 1393-1402.
5. King MC, Marks JH, Mandell JB (2003) Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. Science 302(5645): 643-646.
6. John EM, Miron A, Gong G, et al. (2007) Prevalence of pathogenic BRCA1 mutation carriers in 5 US racial/ethnic groups. JAMA 298(24): 2869-2876.
7. Struewing JP, Hartge P, Wacholder S, et al. (1997) The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. New England Journal of Medicine 336(20): 1401-1408.
8. Kadouri L, Hubert A, Rotenberg Y, et al. (2007) Cancer risks in carriers of the BRCA1/2 Ashkenazi founder mutations. Journal of Medical Genetics 44(7): 467-471.
9. Murphy KM, Brune KA, Griffin C, et al. (2002) Evaluation of candidate genes MAP2K4, MADH4, ACVR1B, and BRCA2 in familial pancreatic cancer: Deleterious BRCA2 mutations in 17%. Cancer Research 62(13): 3789-3793.
10. Li Z, Zhang J, Zou W, et al. (2021) The methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism is associated with breast cancer subtype susceptibility in southwestern China. Plos One 16(7): e0254267.
11. Saxena AK (2023) Disconcordance Between stem cell Oct4, Sox2, Sox4 and epithelial mesenchymal transition CK 19, EpCAM markers in circulating tumor cells-MTHFR C677T gene variant increase risk factor in pancreatic tumors. Journal of Cancer Medicine & Metastasis 6(1): 1-9.
12. Saxena AK (2022) Non-synonymous variants of methylene-tetrahydrofolate reductase c677t gene polymorphism showing discordance with KRAS mutation in circulating tumor cells of hepatocellular carcinoma-a rare case report. Clinical Oncology 6(9): 1-5.
13. Ebara S (2017) Nutritional role of folate. Congenital Anomalies 57(5): 138-141.
14. Cortese C, Motti C (2001) MTHFR gene polymorphism, homocysteine and cardiovascular disease. Public Health Nutrition 4(2b): 493-497.
15. He L, Shen Y (2017) MTHFR C677T polymorphism and breast, ovarian cancer risk: A meta-analysis of 19,260 patients and 26,364 controls. OncoTargets and Therapy 10: 227-238.
16. Levine AJ, Siegmund KD, Ervin CM, et al. (2000) The methylenetetrahydrofolate reductase 677C→ T polymorphism and distal colorectal adenoma risk. Cancer Epidemiology Biomarkers & Prevention 9(7): 657-663.
17. Niu YM, Deng MH, Chen W, et al. (2015) MTHFR C677T gene polymorphism and head and neck cancer risk: A meta-analysis based on 23 publications. Disease Markers 2015: 1-11.
18. Ulrich CM, Kampman E, Bigler J, et al. (1999) Colorectal adenomas and the C677T MTHFR polymorphism: Evidence for gene-environment interaction?. Cancer Epidemiology Biomarkers & Prevention 8(8): 659-668.
19. Songun I, Litvinov SV, Van de Velde CJH, et al. (2005) Loss of Ep-CAM (CO17-1A) expression predicts survival in patients with gastric cancer. British Journal of Cancer 92(9): 1767-1772.
20. Moreno CS (2020) SOX4: The unappreciated oncogene. In Seminars in cancer biology 67: 57-64.

21. Keyvani S, Karimi N, Orafa Z, et al. (2016) Assessment of cytokeratin-19 gene expression in peripheral blood of breast cancer patients and breast cancer cell lines. Biomarkers in Cancer 8: BIC-S38229.
22. Orafa Z, Karimi N, Keyvani S, et al. (2022) Quantitative CK19 biomarker detection in breast cancer cell lines. Journal of Medicine and Life 15(2): 188-195.
23. Litvinov SV, Bakker HA, Gourevitch MM, et al. (1994) Evidence for a role of the epithelial glycoprotein 40 (Ep-CAM) in epithelial cell-cell adhesion. Cell Adhesion and Communication 2(5): 417-428.
24. Schmidt M, Hasenclever D, Schaeffer M, et al. (2008) Prognostic effect of epithelial cell adhesion molecule overexpression in untreated node-negative breast cancer. Clinical Cancer Research 14(18): 5849-5855.
25. Went P, Vasei M, Bubendorf L, et al. (2006) Frequent high-level expression of the immunotherapeutic target Ep-CAM in colon, stomach, prostate and lung cancers. British Journal of Cancer 94(1): 128-135.
26. Zhang J, Xiao C, Feng Z, et al. (2020) SOX4 promotes the growth and metastasis of breast cancer. Cancer Cell International 20(1): 1-11.
27. Patnayak R, Jena A, Rukmangadha N, et al. (2015) Hormone receptor status (estrogen receptor, progesterone receptor), human epidermal growth factor-2 and p53 in South Indian breast cancer patients: A tertiary care center experience. Indian Journal of Medical and Paediatric Oncology 36(02): 117-122.
28. Ding Z, Yang HW, Xia TS, et al. (2015) Integrative genomic analyses of the RNA-binding protein, RNPC1, and its potential role in cancer prediction. International Journal of Molecular Medicine 36(2): 473-484.
29. Saxena Ajit K, Gupta RK, et al. (2016) ARMS-PCR based SNP analysis of MTHFR C677T allele using syber green in pancreatic tumor. British Journal of Medicine and Medical Research 11(12): 1-6.
30. Gao CM, Tang JH, Cao HX, et al. (2009) MTHFR polymorphisms, dietary folate intake and breast cancer risk in Chinese women. Journal of Human Genetics 54(7): 414-418.
31. Kaya EF, Karakus N, Ulusoy AN, et al. (2016) Association of the MTHFR gene C677T polymorphism with breast cancer in a Turkish population. Oncology Research and Treatment 39(9): 534-538.
32. Hedayatizadeh-Omran A, Alizadeh-Navaei R, Toghani-Hulari F, et al. (2017) Association between MTHFR (C677T) gene polymorphism with breast cancer in Northern Iran. World Cancer Research Journal 4(2): e876.
33. Omran MH, Fotouh BE, Shosha WG, et al. (2021) Strong correlation of MTHFR gene polymorphisms with breast cancer and its prognostic clinical factors among Egyptian females. Asian Pacific Journal of Cancer Prevention 22(2): 617-626.
34. Kennedy DA, Stern SJ, Matok I, et al. (2012) Folate intake, MTHFR polymorphisms, and the risk of colorectal cancer: A systematic review and meta-analysis. Journal of Cancer Epidemiology 2012: 1-24.
35. Roa BB, Boyd AA, Volcik K, et al. (1996) Ashkenazi Jewish population frequencies for common mutations in BRCA1 and BRCA2. Nature Genetics 14(2): 185-187.
36. John EM, Miron A, Gong G, et al. (2007) Prevalence of pathogenic BRCA1 mutation carriers in 5 US racial/ethnic groups. JAMA 298(24): 2869-2876.
37. Zhang S, Royer R, Li S, et al. (2011) Frequencies of BRCA1 and BRCA2 mutations among 1,342 unselected patients with invasive ovarian cancer. Gynecologic Oncology 121(2): 353-357.
38. Saxena AK, Singh V, Aprajita KA, et al. (2019) Stop codons of TGF βRI gene modulate the functional activity of 3D structure and their genetic susceptibility in the case of wilms' tumour. Journal of Cancer Science and Therapy 11: 251-255.
39. Akhurst RJ (2004) TGFβ signaling in health and disease. Nature Genetics 36(8): 790-792.