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Research Article

Discordance in the Frequency of Epithelial - Mesenchymal Transition Markers Sox4, EpCAM and CK19 Gene Expression in Circulating Tumor Cells in Variety of Cancer Patients - Significant Correlation with p53 and MTHFR C677T Gene Polymorphism

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Abstract

Circulating tumor cells (CTCs) are the rich source of 'liquid biopsy' for early diagnosis in variety of tumors and 3'-5' methylene tetrahydrofolate reductase (MTHFR C677T) polymorphism, play a relevant role to evaluate the 'risk factor' in during progression of disease. Present study has been designed with the aims to identify the gene-expression of epithelial - mesenchymal transition (EMT) markers such as Sox4, CK 19 and EpCAM in circulating tumor cells isolated from breast, colorectal, gall bladder and Wilm's tumors as early diagnostic markers. DNA copy number variations (DNACNV) were also evaluated to assess the genetic susceptibility of individual tumor bearing patients and correlates with tumor suppressor gene (p53) during angiogenesis. MRHFR C677T gene polymorphism was also includes to assess the genetic heterogeneity of CT genotype and "risk factor" of the disease. Peripheral blood samples were collected from clinically diagnosed cases and CTCs were isolated from lymphocytes cultures for the isolation of. DNA and RNA, quantified by nanodrop spectrophotometer and gene-expression of EMT markers were characterized by RT-PCR and DNA CNVs by densitometry analysis using in build software of the Gel Doc system. . Interestingly, over-expression of Sox4, in breast and gall bladder cancer, suggesting to confirm CTCs movement and invasion to the neighbouring tissue and induce metastasis. Findings also reveals that complete disappear of band 359bp and 573bp of EpCAM and CK19 respectively, were observed in breast, gall bladder, and Wilms's tumors consider as mutation, suggesting tissue-specific transition of the markers during progression of disease. ARMS-PCR was used to evaluate the frequency of genotype, CC, TT and CT variation of MTHFR C677T polymorphism, where, the Tm values between GAPDH (89.50) and 86.50 in breast cancer, gall bladder shift significantly confirming the genetic heterogeneity followed by risk factor of the disease. p53 gene showing significant ($p < 0.05$) down - regulation only in gall bladder tumor suggest to increase genetic susceptibility towards carcinogens.

Keywords: CTCs; Breast Cancer; Wilm's Tumor; CNVs; p53; MTHFR Polymorphism

Introduction

In women, breast cancer is highly prevalent disease in the world. Etiopathology is highly complex due to involvement of both genetic and epigenetic factor during disease progression. Several conventional techniques have been established such as histopathology, cytochemistry, polymerase chain reaction (PCR) and flowcytometric analysis that raised conflicting issues such as time consuming, cell-number, survival and lack of sensitivity for early diagnosis [1]. Recently, the scientists have developed advanced technique based on circulating tumors cells (CTCs), a highly sensitive techniques based on early prognosis followed by diagnosis of primary tumors "biomarker" and becomes relevant for clinicians during chemotherapeutic regime. The metastasis is main cause of mortality in cancer patients due to invasion of CTCs to the neighbouring organs or tissues. The role of epithelial mesenchymal transition (EMT) markers- Sox4, EpCAM and CK19 regulating metastasis during tumor progression has not been defined clearly. However, CTCs are the rich source of "liquid biopsy" and act as an "information centre" for early diagnosis, during management in variety of tumors. Recent studies have documented that KRAS mutant develop resistant in wild-type genotypes after chemotherapy in primary or metastatic stages in variety of cancer like colorectal, liver and lungs [2].

The identification and characterization of CTCs are very difficult task because of several factors including sensitivity, (~1%) quantity, and genetic heterogeneity in population [2,3]. The collection of blood sample (5-10 ml) from their tumor bearing patients raises the question of ethics, obtained from single patient or if pooled the samples for the isolation of CTCs using Ficoll's method and charac-

terization of EMT markers - Sox4, EpCAM and CK19) during early diagnosis is poorly understood [4]. Several efforts have been developed to improve the techniques for both quality and quantity of cell number to maintain sensitivity for the clinicians to develop better management to the tumor bearing patients. The characteristic feature of CTCs shows adaptive niche or "tumor seeds" before infiltration or invasion to the neighbouring distant organs like breast, lymph nodes, liver, kidney and nervous system during metastasis.

Folate metabolism play an important role in transformation from normal to malignant cell during intracellular conversion of 5, 10 methylene tetrahydrofolate reductase (MTHFR) to 5-methyltetrahydrfolate maintaining *de-novo* equilibrium of methionine and homocysteine. MTHFR C677T gene is assigned on chromosome-1p36.3 and their polymorphism has been assigned in variety of cancer as well as non-malignant conditions either in homozygous or in heterozygous condition [5]. Hence, it's become essential that such genetic factor to correlate in differential gene-expression of EMT markers in variety of tumor bearing patients. Efforts have been developed to improve the quality and quantity of CTCs with maintaining sensitivity using cell-culture engineering, after minimal use of sample (blood 0.5ml) from clinically diagnosed patients. The sensitivity was characterized by EMT markers - Sox4, EpCAM and CK19 and these markers are highly conserved in nature, extensively used during morphogenetic transformation [6]. Sox4 belongs to the member of SRY high mobility transcription factor that encodes proteins are responsible to decide fate of cell- differentiation and their expression after activation of oncogenes mediating TGF-β signalling [7]. Similarly, epithelial cell adhesion molecule (EpCAM), cytokeratin (CK19 is also known as mesenchymal EMT makers of cell surface of stem cells and their expression diverges during cell invasion during progression of the disease before or after metastasis [8]. Present study, has been designed with the aims to evaluate differential gene-expression of EMT markers in CTCs isolated from four different cancer patients to determine tissue-specific genetic heterogeneity, and further determine "risk factor" using MTHFR C677T gene polymorphism either alone or with p53 mediated signalling in a synergistic manner.

Materials and Methods

Subjects

The peripheral venous blood (1.0 ml) was obtained in a sterile EDTA vial from clinically diagnosed breast cancer patients (n = 28) with age-matched control, ,gall bladder, colon and Wilms tumor patients after informed consent. Study was approved by Institute ethical committee of AIIMS, Patna (AIIMS/Pat/IRC/2020/610), and informed consent form was dually signed by patients.

Isolation of circulating tumor cells by Ficoll's gradient method.

Peripheral blood lymphocytes (PBL) were cultured using RPMI-1640 media supplemented with 10% foetal bovine serum, phytohemagglutinin-M (PHA), and antibiotic solution consist of streptomycin and penicillin. After Ficoll-Paque density gradient centrifugation at 400g for 30 minutes, a ring is formed between upper and lower layers. Cells were isolated from the ring consist of CTCs, washed twice with 10 ml RPMI-1640 media and centrifuge at 300g for 10 min followed by resuspend in 1.0 mL of RPMI-1640 media and stored at -20 °C till characterization and identification of CTCs [9,10].

Isolation of RNA and cDNA Synthesis.

CTCs were fixed in TRIZOL (Invitrogen, Life Technologies, USA) for isolation of total RNA according to manufacturer's instruction. After isolation of mRNA, quality was checked on 1.5% agarose gel electrophoresis, and quantification was carried out by Nanodrop (Thermo Scientific USA) instrument at 260nm. The 2µg of total spectrophotometer RNA was used for cDNA using reverse transcriptase (20U/µl) with Oligo (dT) and random primer (cDNA Kit, Promega, USA) in a total volume of 20 µl followed by incubation of 5 min at 25 °C, 1 hr for 42 °C and complementary products were used for characterization of EMT markers - Sox 4, EpCAM and CK 19 using PCR.

Characterization of EMT Markers – Sox 4, EpCAM and CK-19.

In order to detect the pattern of comparative analysis and sensitivity of epithelial-mesenchymal transition (EMT) markers in CTCs in different cancers were identify and further characterize by PCR/RT-PCR procedure. Table 1, showing the specific set of forward and reverse of primers sequences after confirmation from NCBI (BLAST/http://blast.ncbi.nlm.nih.gov.) of Sox4, EpCAM and CK-19 markers were used for the identification of CTCs. PCR reaction was achieved in a 25µl mixture containing 5x Green GoTaq PCR reaction buffer, dNTPs Mix (10 mM), 1µl each of 10 pmol of CTCs specific primer i.e. forward and reverse, 0.2µl of GoTaq DNA polymerase (5U/µl). The template (cDNA-1µg) is mix with reaction mixture before performing PCR. The reaction profile was different for each of the CTC's marker i.e. carried out for 35 cycles comprising, initial denaturation at 95°C for 5 minutes. There are three EMT markers showing different PCR stratifies like Sox4 denaturation at 95°C for 30 seconds, annealing at 57.2°C for 30 seconds, elongation at 72°C for 30 seconds, followed by final elongation at 72°C for 8 minutes. Similarly, for EpCAM and CK-19, the denaturation at 95°C for 45 seconds, annealing at 60.2°C for 30 seconds and elongation at 72°C for 1 minute, followed by final elongation at 72°C for 7 minutes as shown. The amplified products were characterized on 1.5% agarose gel electrophoresis and individual's bands were visualized on Gel Doc systems (Bio-Rad USA) for densitometry analysis to enumerate DNA copy number variations using inbuilt software system of gel Doc (Bio-Rad USA).

Analysis of MTHFR C677T gene polymorphism.

Folate-metabolism play an important role during cancer progression and regulated by methylenetetrahydrofolate reductase (MTHFR C677T) polymorphism to assess the genetic heterogeneity followed by "risk factors" of the disease. Allele refractive mutational system (ARMS- PCR) is highly sensitive and reliable technique to

Table 1: RT-PCR based analysis showing different set of forward and reverse primers for the characterization of epithelial-mesenchymal transition markers in circulating tumor cells and primers for MTHFR C677T genes polymorphism for breast, colon, gall bladder and Wilms's tumor.

S. N.	Type of Genes	Forward/Reverse Sequences used for PCR analysis	Annl. Temp. (°C)	Size (bp)	Ref.
1	p53	F 5′-TGA AGT CTC ATG GAA GCCAGC-3′	60	279	(11)
		R 5′-GCT CTTT TTC ACC CAT CTACAG-3′			
2	SOX4	F GGTCTCTAGTTCTTGCACGCTC	57.2	183	(12)
		R CGGAATCGGCACTAAGGAG			
3.	EpCAM	F- 5'-GCCAGTGTACTTCAGTTGGTGC	58.7	359	(13)
		R- 5'-CCCTTCAGGTTTTGCTCTTCTCC			
4	CK 19	F- 5'-ATTCCGCTCCGGGCACCGATCT	60.2	573	(14)
		Reverse CGCTGATCAGCGCCTGGATATGCG			
5	MTHFR C667T	F- 5'-TGTCATCCCTATTGGCAGGTTACCCCAAA	58.0	171	(15)
		R- 5'-CCATGTCGGTGCATGCCTTCACAAAG			
		CpolyGGCGGGCGGCCGGGAAAAGCTGCGTGATGATGAAATAGG		150	
		T allele GCACTTGAAGGAGAAGGTGTCTGCGGGCGT		105	

evaluate SNP analysis using specific primers (tetra plex) as details of primer are documented in table 1. The primers were designed and confirmed after BLAST (http:// www.ncbi.nlm.nih.gov/blast) to determine specificity. These tetra primer selected for ARMS - PCR of MTHFR C677T genotypes i.e. CC (wild type), TT (rare mutant, disease causing) either in homozygous or CT (mutant) heterozygous condition using SYBR green. The reaction mixture consists of a total volume of 20µl containing 10µl of SYBR Green PCR master mix, 1.0 µl of each primer per reaction, 1µg of cDNA, and distilled water was used for RT- PCR analysis. RT-PCR protocol initially consist of initial denaturation step (95˚C for 5 min) was followed by amplification and quantification steps repeated for 40 cycles (95˚C for 20 seconds, 58˚C for 30 seconds, 70˚C for 30 seconds) with a single fluorescence measurement at 530nm. Melting curves (*Tm)* were constructed by lowering the temperature to 65°C and later increasing the temperature by 0.2˚C/s to 98˚C to measuring the change of fluorescence consistently. After obtaining *Tm* values, a plot was developed between fluorescence versus temperature (dF/dT) for the amplification of candidate gene products. PCR products were further analysed to reconfirm the findings on 1.5% agarose gel electrophoresis by evaluating the appearance of additional band consist of 105bp, confirming genetic heterozygosity (CT genotype), where, the nucleotide cytosine change to thymidine and confirming "point mutation".

Statistical analysis.

Data were collected from four different type of cancer patients and values were calculated in terms of mean ± SD to observe the significance difference (p value) between cases and controls. SPSS (7.0 version software, USA) and two tailed chi- square test was used for DNA copy number variations with respect to controls.

Results

PCR based analysis showing variations in the intensity of p53 gene expression (279bp), after using 1.5% agarose gel electrophoresis as shown in Figure -1A. In case of gall bladder, the gene-expression apparently showing significant down-regulated (lane-3) as compared to control (lane-1). This finding was further confirmed that showing significant ($p < 0.05$) differences in DNA copy number variation as shown in bar diagram (Figure -1B, lane-3) with respect to controls. Figure 2A-D (arrow) showing the differential expression of epithelial mesenchymal transition markers genes, Sox4, EpCAM and CK19 either complete disappearance of band consider as mutation or over expression (up regulation) or (under expression) down regulation in CTCs isolated from four different type of tumors. CTCs showing complete disappearance (mutation) in CK19 (573bp) and EpCAM (359bp) as shown in figure - 2A, lane-5 and 6 in breast cancer patient (arrow), but lack of changes in Sox4. Similarly, in gall bladder, the expression of CK19 showing down-regulation and mutation of EpCAM (Figure .2B, lane4, arrow). Further, down-regulation of EpCAM was observed in colorectal case (Figure .2C, lane 4) and mutation of CK19 (573bp) and EpCAM (359bp) were observed in Wilms tumor. Statistical analysis showing significant correlation ($p < 0.05$) in CK19 and EpCAM. Interestingly, Sox4 showing lack of significant differences in all four different tumors.

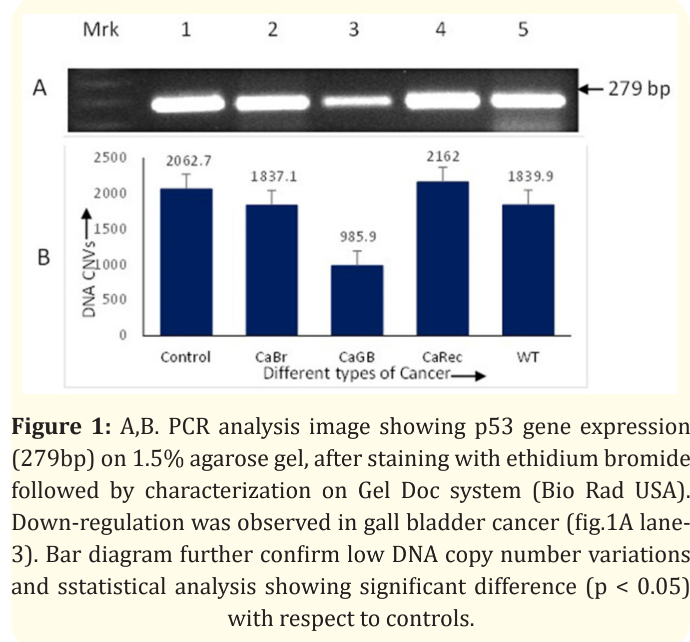

Figure 1: A,B. PCR analysis image showing p53 gene expression (279bp) on 1.5% agarose gel, after staining with ethidium bromide followed by characterization on Gel Doc system (Bio Rad USA). Down-regulation was observed in gall bladder cancer (fig.1A lane-3). Bar diagram further confirm low DNA copy number variations and sstatistical analysis showing significant difference ($p < 0.05$) with respect to controls.

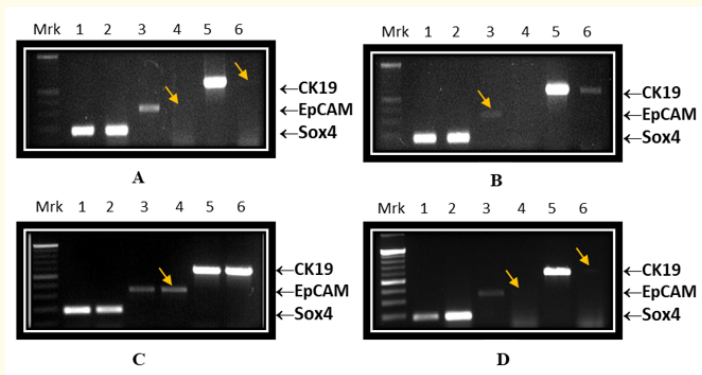

Figure 2: A-D. PCR based analysis showing differentialtional expression of epithelial mesenchymal transition markers - Sox4, EpCAM and CK19 in circulating tumor cells from four differential type of tumors - breast cancer (A), gall bladder (b), colorectal cancer (c), and Wilms tumor (D).

Table 2 showing the comprehensive data of DNA copy number variations of EMT marker gene in CTCs, in four different tumors. The mean ± s.d values of cases 209.5 ± 41.8 and control (886.3 ± 99.14) in EpCAM and CK19 927.3 ± 13.4 (case) and 279.9 ± 36.65 (controls), showing significant ($p < 0.05$) difference as also appears in bar diagram (Figure -3A, B and C).The mean ± s.d values between case 1847.4 ± 675.5 and controls 1487.4 ± 120.38 showing lack of significant differences of Sox4, and also shown in bar diagram. Figure -3D, apparently showing the down regulation of Sox4 and mutation of CK19 and EpCAM (arrow), when compare with controls in the case of Wilms' tumor.

Table 2: DNA copy number variations showing significant difference (p<0.05) in epithelial- mesenchymal transition marker in different tumor patients with their respect to controls

Types	SOX4		EpCAM		CK-19	
	Control	Case	Control	Case	Control	Case
Breast Cancer	1357.53	1556.81	1010.66	0	2310.73	0
Gallbladder Cancer	1640.46	1757.88	896.14	0	2966.38	858.30
Colorectal Cancer	1437.6	1260.5	769.6	837.9	2754.5	2850.9
Wilm's Tumor	1513.9	2812.8	868.8	0	3165.9	0
Mean ± S.D.	1487.4 ± 120.38	1847.0 ± 67.50	886.3 ± 99.14	209.5 ± 41.95	2799.4 ± 36.6	927.3 ± 13.4
S.E.	60.19	337.75	49.57	209.48	183.26	672.36
p-values	0.33		0.02*		0.04*	

*Showing significant ($p < 0.05$) difference with respect to controls.

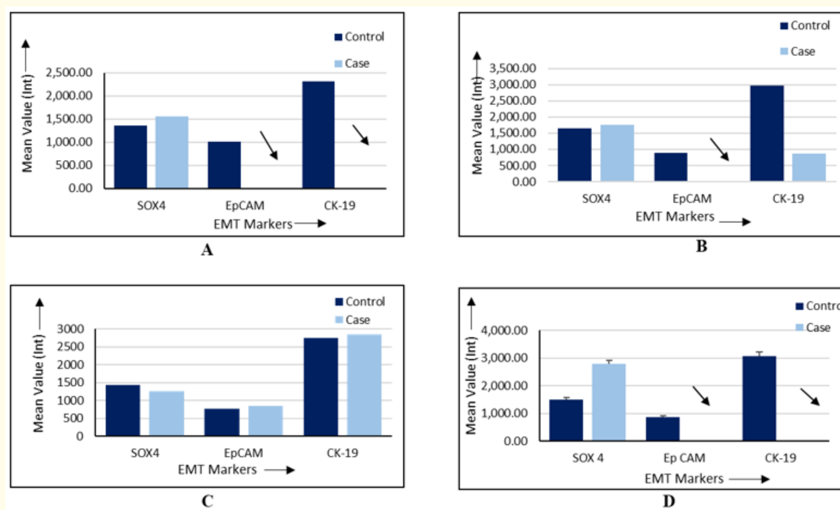

Figure 3: A-D. Bar diagram showing DNA copy number variations of epithelial mesenchymal transition markers - Sox4, EpCAM and CK19 in circulating tumor cells isolated from four different type of tumors - breast cancer (A), gall bladder (b), colorectal cancer (c), and Wilms tumor (D). The complete disappearance of band considers as mutation as shown by arrow (→) in all the cancer patients except, colorectal cancer.

MTHFR C→T gene polymorphism analysis was carried out using ARMS-PCR, a highly sensitive technique for genetic heterogeneity between cases of breast, colon, gall bladder and Wilms tumor and Tm values among were compared with GAPDH, consider as genomic controls. The observed *Tm* values showing significant difference after shift from 89.00 (case) to 86.00 in control in breast cancer patient again *Tm* values shift from 89.50 to 86.50 and 88.00 to 85.50 between cases and controls, in gall bladder and colon cancer, respectively as shown in figure 4A, B and C to confirm the genetic heterogeneity, where, the nucleotide cysteine is substituted to thymidine (C → T) of MTHFR gene polymorphism to confirm point mutation. In the case of Wilms tumor, *Tm* values shift from 86.00 (control) to 85.50 (case), showing lack of significance (Figure 4D) differences with respect to controls, suggesting other than folates like p53 might have influence in the development of tumor progression either alone or with environmental factor like arsenic. However, the genetic susceptibility in Wilms tumor is difficult to predict because of multiple factors other than EMT markers, TGFβ1, p53 and MTHFR C677T variants increase “risk factor”, but fetal-maternal factor can not be ignored.

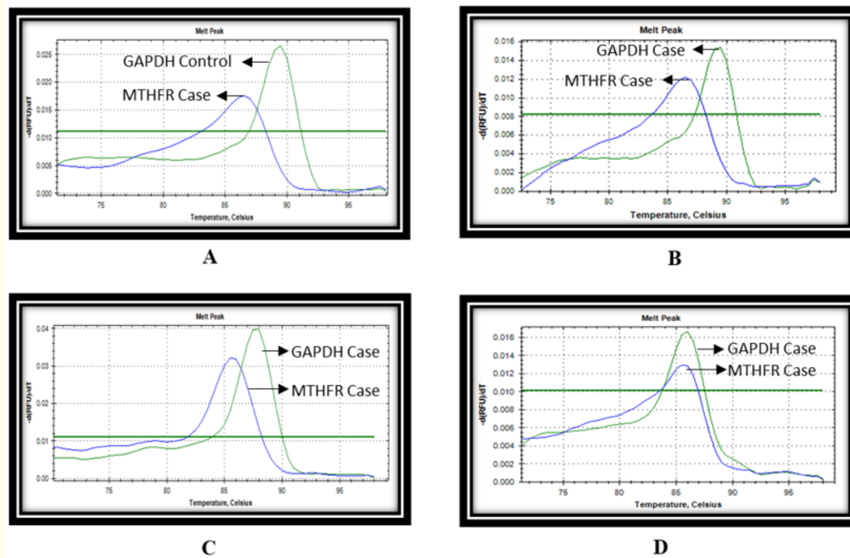

Figure 4: A-D. MTHFRC677T gene polymorphism showing genetic heterogeneity in circulating tumor cells isolated in variety of four different types of tumors. Significant difference was observed in Tm values with respect to GAPDH as controls in breast cancer (A), gall bladder (B), colon cancer (C), but showing lack of significance in Wilm’s tumor (D).

Discussion

Epidemiological study reveals that breast cancer leads to second highest incidence of mortality in the women population [16]. Although, the causative factor associated with the breast cancer remains unclear, it has been hypothesized that multiple factors such as hepatitis infection, life-style including dietary factors like alcohol and contaminated food are the major “risk factor” associated with the progression of disease [17]. Large number of invasive and non-invasive techniques are available to diagnose the cancer patients including histopathological, mammography, radiological and immunocytochemistry techniques having time consuming, and lack of sensitivity for early diagnosis of the cancer patients. Hence, there is need to develop new minimal non-invasive technique based on CTCs, isolated from the breast cancer patients and compared the same with three other tumor bearing patients like gall bladder, colon cancer and Wilms tumor based on cell- culture techniques using Ficoll’s gradient methods [9]. The sensitivity of CTCs was evaluated using epithelial-mesenchymal transition markers - CK19, EpCAM and Sox4 in terms of either mutation i.e. complete disappearance of band or over-expression (up-regulation) or under-expression (down- regulation) using specific primers. DNA copy number variations of EMT markers showing tissue-specific genetic susceptibility followed by invasion of CTCs in neighbouring tissues (gall bladder or Wilms tumor) and confirmation of metastasis during progression of disease. The genetic heterogeneity of genotype (CT) regulating folate-metabolism of MTHFR C677T gene polymorphism confirm the “risk-factor” of disease in variety of patients [9].

The most interesting finding is the variation between CTCs of breast cancer patient and Wilms’ tumor showing mutation of CK19 and EpCAM (Figure 2A and D) as compare to gall bladder and colorectal cases (Figure 2B and C). This may be either due to tissue specific susceptibility towards unknown environmental mutagen or differences in pre/post metastatic stages during tumor progression. In the case of breast cancer, the Sox-4 expression showing almost similar pattern to gall bladder, and colorectal cancer patients, except over expression in Wilms tumor, suggesting involvement of transforming growth factor beta R1 (TGFβR1) mediated signalling pathway during angiogenesis, further confirm by DNACNVs (Figure 3D).Bar diagram of DNA copy number variation showing lack of specific pattern in breast , gall bladder and colorectal cancer except that confirm mutation that might have either increase the genetic susceptibility (Figure 3A,B and C) or fail to maintain pluripotency during progression of disease [18].

Data of the present findings of CTCs in breast cancer cases are quite relevant and shows significant genetic alterations i.e. complete disappearance of CK19 and EpCAM, leading to increase aggressiveness during metastasis. Earlier study shows that variation in the frequency of EMT gene expression in variety of cancer tissue may be either different tissue specific susceptibility or interaction with vimentin- due to a marker of metastasis [19]. Although, the sensitivity of EpCAM is also observed in gall bladder and Wilms' tumor (mutation), confirming the transition of epithelial cells to mesenchymal followed by invasion of CTCs to neighboring cell. In colon cancer, there is lack of definite pattern of EMT maker gene expression (Figure .3C), probably either due to poor supply of CTCs to invade target site (tissue specificity) or unfavorable environment for the proliferation of malignant cell. The findings also confirm the genomic instability during tumorigenesis. Genetic heterogeneity of MTHFR C677T gene polymorphism showing significant shift of *Tm* values with respect to controls in breast, gall bladder and colon cancer as depicted in Figure -4 A, B and C, except in Wilms tumor, showing lack of significance changes in *Tm* values, suggesting that only 20% cases show genetic heterogeneity based on individual tissue specific susceptibility.

Conclusion

The variable frequency of epithelial - mesenchymal transition markers due to loss of polarity and identity of epithelial cells results showing aggressiveness in tumor cells. These cells detached from primary tumor and invade to the neighboring tissue of extracellular matrix and circulates in blood vessels to reach distinct tissue to induce secondary tumorigenesis. The variation in gene-expression of EMT markers in different tumors are due to:- 1) tissue-specific genetic heterogeneity, 2) p53 gene mutation and over- expression of Sox4 promotes metastasis, 3) genetic heterogeneity of MTHFR C677T polymorphism reveals risk-factor based on genetics-susceptibility. Differential expression of CK19, EpCAM and Sox4 may be used as "biomarker" for early diagnosis in tumor bearing patients.

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Conflict of Interest

There is no conflict of interest between authors.

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