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Differential expression of the vimentin gene modulated by MTHFRC677T polymorphism in circulating tumor cells isolated from breast cancer patients

A.K. Saxena[1], Shalini[1], V. Singh[2] and P. Singh[3]

[1] Human Cytogenetic and Molecular Genetics Laboratory, Department of Pathology/Lab Medicine, India Institute of Medical Sciences, Patna, Bihar, India
[2] Department of Burn & Plastic Surgery, India Institute of Medical Sciences, Patna, Bihar, India
[3] Department of Radiation Oncology, India Institute of Medical Sciences, Patna, Bihar, India

Corresponding author: A.K. Saxena
E-mail: draksaxena1@rediffmail.com

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ABSTRACT. Globally, breast cancer a common disease affecting women. The epithelial cells lose their characteristic features and gain mesenchymal properties during disease progression. During transition, the intermediate filament changes status from keratin to cell-adhesive molecules. Vimentin, a glycoprotein constitutes an intermediate filament of mesenchymal cells to maintain tissue architecture. The role of a tumor suppressor gene (p53) and methylenetetrahydrofolatereductase (MTHFR C677T) polymorphism becomes essential to explore the mechanism of isoform expression of vimentin in circulating tumor cells (CTCs) of breast cancer patients. We examined the frequency of vimentin gene expression along with isoforms and their correlation with p53 and MTHFR C677 polymorphism in CTCs. Blood samples were collected from clinically diagnosed cases of breast cancer (n=38) and age matched controls. Short term lymphocyte cultures were prepared for isolation of CTCs. cDNA was synthesized followed by PCR. PCR products

were separated with agarose gel electrophoresis. There was an additional 250bp band, besides a conventional band of 323bp, with differential expression in CTCs. Variable frequency of over-expression was observed in two different bands in 31.3% (323bp) and 18.8% in isoforms (250bp) of the CTCs. Similarly, down-regulation varying between 43.8% (323bp) and 25.05% (250bp), while 25% cases showed a mutation of 323bp in CTCs. Data was further analyzed to calculate an95% confidence interval with odds ratio varying from 10.85 to 0.62 and 3.95 to 0.76 for over and under expression, respectively showing significant differences with respect to controls. MTHFR polymorphism showed higher (*Tm*) values (6.36) in the cases, where the mutation was observed as compared to those cases showing isoforms (5.59). The p53 gene mutation (273bp) appeared in nineteen out of thirty eight cases of breast cancer patients with a positive correlation between vimentin isoform expression and MTHFR polymorphism, suggesting a risk factor for this disease.

Key words: Breast Cancer; Circulating Tumor Cell; Gene Expression; Vimentin

INTRODUCTION

Breast cancer is the highly prevalent disease among women**.** The incidence varies in different age groups and there is a hundred-fold increase at 45 years (Ivaska et al., 2007). During progression of the disease, management requires surgical intervention followed by radiation or chemotherapy either alone or in combination. Etiopathology involves epithelial cells losing their basic features and gaining mesenchymal properties, the event commonly known as epithelial-mesenchymal transition (EMT) complex. To accomplish this, a large number of intermediate protein molecules play a significant role such as cytokeratin (CK19) and cell surface adhesive (EpCAM) including vimentin to maintain the tissue architecture during disease progression. Hence, these molecules i.e., biological markers, becomes beneficial for clinicians during therapy.

Vimentin, a mesenchymal marker consisting of transmembrane glycoprotein and constituting an intermediate filament (Ifs) provide networking for adherence between two cell-junctions (hemidesmosomes) for maintaining the cellular integrity and signal transduction (Dumitrescu et al., 2005) Vimentin gene expression varies in breast cancer during transition of epithelial to mesenchymal cells (EMT). The over-expression of vimentin shows cell-migration, invasion and aggressiveness of mesenchymal cells in nineteen out of thirty eight cases of breast cancer patients (Benazzouz et al., 1999). During cancer progression, epithelial cells lose their polarity and detach from the primary tumors, invading blood vessels through the extra cellular matrix, where they rest, called seeds of “cancer cells”. As soon as possible to get favorable environment these seeds reach neighboring tissues or organs and proliferate. The methylenetetrahydrofolatereductase (MTHFR) gene is assigned on chromosome 1q36.3 and C677T (rs1801133) allele shows polymorphic variation in variety of diseases including in circulating tumor cells (CTCs) of

cancer patients. MTHFR (C677T) gene polymorphism play an important role during DNA synthesis in differentiation cells and to maintain folate pool, therefor, becomes an essential component to determine genetic heterogeneity and assess the risk factor in breast cancer patients (Saxena et al., 2020; Saxena et al., 2021; Saxena, 2022). Tumor suppressor gene (p53) play a relevant role in tumor cell growth and proliferation through cell-signaling mechanism and curiosity has been developed to know, how mRNA expression of isoforms of vimentin are modulated either by p53 or MTHFR C677T gene polymorphism. During disease progression, epithelial-mesenchymal transition shows three characteristic features- 1) loss of epithelial cell or adhesive molecules, 2) *de novo* expression of actin, and 3) increase the process of cell migration and invasion to the neighboring (Yang et al., 2001). The expression of vimentin is highly selective, cytoskeleton protein constitutes intermediate filaments (IFs) of mesenchymal cells, and their functional role is cell-cell adhesion of epithelial cells followed by subsequent maintaining tissue architecture (Kokkinos et al., 2007). The over- expression of vimentin was observed during cell migration, reduced or absent, if the cells are from epithelial in origin (Gilles et al., 2004). Vimentin gene expression occurs in variety of cell-types including fibroblast, endothelial, macrophage, neutrophils and lymphocyte (Evans, 1998). The over expression of vimentin was also observed in auto immunity and rejection of graft during tissue engineering (Carter et al., 2005). The aberrant or over-expression was observed during migration and invasion of cancer cell, but reduced during coupling with epithelial cells (Gilles et al., 2003; Korsching et al., 2005). The mRNA expression of vimentin gene is highly sensitive towards signal transduction mediating molecules like Sox4 and transforming growth factor beta (TGFβ) and has not yet been confirmed by alternative splicing. To explore the mechanism and involvement of differential expression of isoform origin in circulating tumor cells (CTCs) from breast cancer patients might be give new insight knowledge of aggressive behavior of mesenchymal cells and phenotypic changes. Therefore, the present study was designed to evaluate the mutation frequency of vimentin and DNA copy number variations (DNACNVs) in CTCs isolated from breast cancer patients. The study was further extended to evaluate genetic heterogeneity of folate metabolism using MTHFR C677T gene polymorphism and to correlate with p53 gene to assess risk factors for this disease.

MATERIAL AND METHODS

Blood (1.0 ml) samples were collected, after clinical diagnosis of breast cancer patients and women of the same age group considered as controls (n=38) from the department of Radiation Oncology in All India Institute of Medical Sciences, Patna, Bihar, after informed conscent either from the patient or guardian. Samples were collected in sterile EDTA vials and stored at 4°C, till further study. The present study is approved by the Institute Ethical Committee (IEC) No. /AIIMS/Pat/IRC/2020/610 of the AIIMS, Patna.

Isolation of Circulating Tumor Cells using Ficoll's Gradient Method

Blood samples (1.0 ml) were used for short- term lymphocyte culture (72 hours) using RPMI-1640 media, supplemented with 10% fetal bovine serum, phytohemagglutinin (PHA), and antibiotic solution consist of streptomycin & penicillin. Peripheral blood mononuclear cells (PBMC) ring was isolated after Ficoll-Paque density gradient

centrifugation at 400g for 30 min. The PBMC were washed twice with 10 ml RPMI-1640 and again centrifuge at 300g for 10 min followed by resuspend in 1.0 mL of media, stored at -20°C, till further characterization of CTCs using the standard protocol of the laboratory (Saxena, 2022).

Isolation of mRNA and cDNA Synthesis

Total mRNA isolated from CTCs, after fixation in TRIzol and quality of mRNA was checked on 1.5% agarose gel electrophoresis, followed by quantification using nanodrop spectrophotometer at 260nm. The 2µg of total RNA was reverse transcribed into 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 for 5 min at 25°C, 1.0 hr for 42°C , and the complementary products were characterize by vimentin gene as biomarkers of mesenchymal cells using specific forward and reverse primers Vim-FP-5'-GACAATGCGTCTCTGGCACGTCTT-3'; Vim-RP-5'-TCCTCCGCCTCCTGCAGGTTCTT-3', and p53 primers F 5′-TGA AGT CTC ATG GAA GCCAGC-3′; R 5′-GCT CTTT TTC ACC CAT CTACAG-3′ consist of 273bp with annealing temperature 60°C, after confirmation of sequences from NCBI (BLAST/http://blast.ncbi.nlm.nih.gov.). The 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 using PCR. The cycle conditions for the CTC's marker vimentin were as follows, 35 cycles comprising, initial denaturation at 95°C for 5 minutes, followed by denaturation 95°C for 45 seconds, annealing at 60.0°C for 1.0 minutes and elongation at 72°C for 45 seconds, followed by final elongation at 72°C for 8 minutes. Similarly, p53 gene was amplified with cycle conditions, 35 cycles comprising, initial denaturation at 95°C for 5 minutes, followed by denaturation 94°C for 30 seconds, annealing at 60.0°C for 1.0 minutes and elongation at 72°C for 1.0 minutes, followed by final elongation at 72°C for 8 minutes. The amplified products were characterized on 1.5% agarose gel electrophoresis and visualized by Gel Doc systems after ethidium bromide staining. After visualization of band, densitometric analysis were performed for enumeration of DNA copy number variation (DNACNV) of individual bands on the same Gel Doc system (Bio Rad USA) software. Tetra primer were selected for ARMS - PCR for the study of MTHFR C677T genotypes i.e. CC (wild-type), TT (rare mutant, disease causing) either in homozygous or CT (mutant) heterozygous condition. 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 to make up the volume. In brief, protocol consist of denaturation step (95°C for 5 min) is followed by amplification 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 (Saxena, et al. 2006). After obtaining *Tm* values, a plot was constructed between fluorescence versus temperature (dF/dT) for the amplification of candidate gene products. PCR products were further analyzed on agarose gel electrophoresis by evaluating the

appearance of additional band consist of 105bp, confirming heterozygosity (C → T) of MTHFR C677T gene polymorphism.

Statistical Analysis

Data was analyzed using SPSS statistical software version 22.0 (SPSS Inc. Chicago, IL, USA). The data was analyzed individually and calculated values of mean +/- s.d, confidence interval at 95 % observed followed by odd ratio (O.R) to evaluate differences between upper and lower limits. Chi square (χ^2) -test was selected to evaluate the level of significance i.e. $p<0.05$ value considered as statistically significant between cases and controls.

RESULTS

The PCR products were showing variable mRNA expression of vimentin in CTCs from breast cancer patients, after characterization and visualization of individual bands (323bp) and 250bp (isoforms) on 1.5% agarose gel electrophoresis, stained with ethidium bromide use as fluorescence dye on gel Doc system as shown in Figure 1. The mutational spectrum analysis showing variety of findings that includes either complete disappearance of band (323bp) in eight cases, considered as "mutation" in CTCs of breast cancer patients. Table 1 showing the comprehensive variable mRNA expression of vimentin and isoforms. Data was analyzed statistically, showing significant ($p<0.05$) difference and over-expression was observed in eight cases of CTCs for 323bp and seven cases for isoform (250bp) with calculated value of C.I. varying between 10.85-0.62, with an odds ratio of 1.5. Similarly, down regulation was observed in ten cases for 323 bp and four cases for (250bp) with observed C.I. values varies between 3.95-0.76 with respect to controls. Interestingly, Figure 2 (bar diagram) also shows significant differences in vimentin gene expression of isoform (red bar) between under and over expression in CTCs of breast cancer patients. No significant differences has been observed in both DNACNVs of vimentin gene mutation and under expression .

Table 1. RT-PCR analysis showing differential expression of the vimentin gene in circulating tumor cells isolated from breast cancer patients.

Gene Name	Types of Mutation	Frequency		95% C.I.		O.R.	p-Value
		323 bp	250 bp Isoform	Upper limit	Lower limit		
Vimentin (n=26)	Over Expression	8	7	4.18	2.60	1.97	0.019*
	Under Expression	10	4	23.26	2.21		
	Mutation (Null)	8	0	NA	NA		

*Statistical analysis showing a significant ($p<0.05$) difference compared to controls

The DNA copy number variations (DNACNVs) showing two different pattern of vimentin mRNA gene expression between 323bp and 250bp (isoforms) in CTCs of breast cancer cases as depicted in Figure 3. Apparently, the highest expression of vimentin gene (323bp) was observed in the case number 4, 8 and 13 as shown in blue colour bar, while, few cases showing completely disappearance (red bars) of mRNA isoforms (250bp) in

CTCs (arrow). However, our data support that mRNA gene expression of vimentin apparently showing under expression (down regulation) and no mutation in the isoforms of vimentin in the following case bar no. 3, 5, 6, 79, 10, 14, 15 and 16 of CTCs.

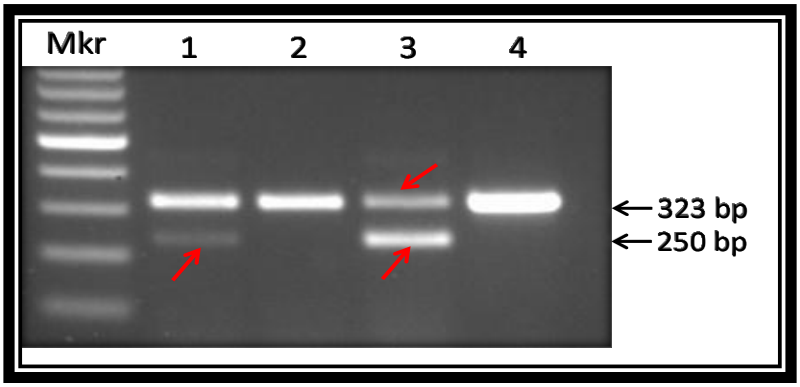

Figure 1. RT-PCR based analysis of the vimentin gene showing differential expression of isoforms of 250bp as shown in lane-1 & lane-3 (arrow) and lane-2 showing lack of mutation (323bp) with respect to the control (lane-4). The PCR products were characterized on 1.5% agarose gel electrophoresis followed by ethidium bromide staining and bands were visualized with the GelDoc system (BioRad USA).

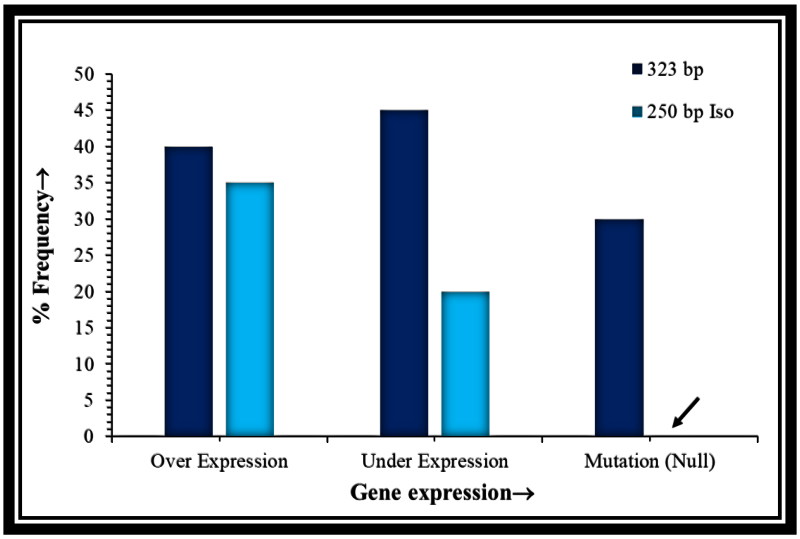

Figure 2. Bar diagram showing DNA copy number variation of the Vimentin gene showing differential expression of isoforms of 250bp as shown in lane-1 & lane-2 (light blue) and lane-3 showing mutation (arrow) after densitometry analysis of individual bands using inbuild software in the GelDoc system (BioRad USA).

The C677T gene polymorphism in the MTHFR gene in CTC showing genetic heterogenicity and significant variation of *Tm* values between cases 1 (6.36), and case 2 (5.58). Case 1 had complete mutation of vimentin gene, while case 2 showing 323bp band as well as an isoform of 250bp. GAPDH gene, used as genomic control.

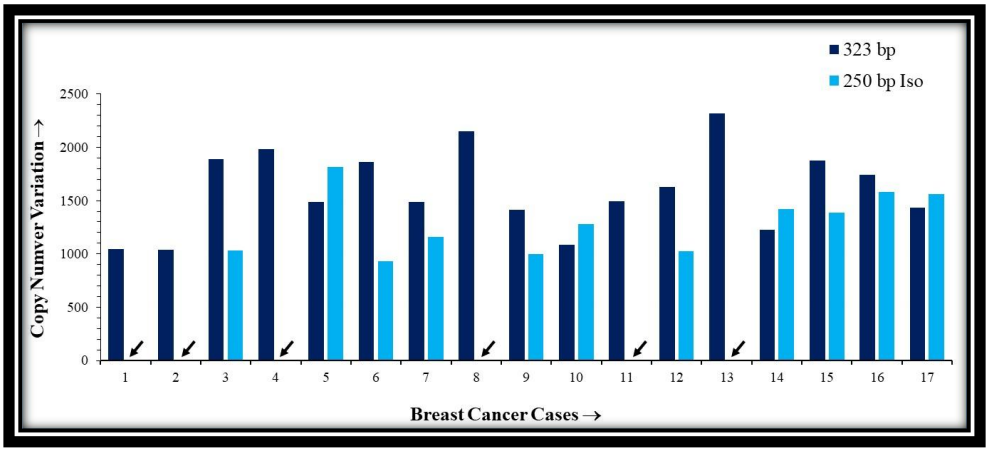

Figure 3. Bar diagram showing individual variations in DNA copy number variations of the vimentin gene (dark blue) and their isoforms (light blue color) in circulating tumor cells isolated from breast cancer patients.

In the present study, p53 gene mutation was also included in the same group of the breast cancer patients that showing disappearance of 273bp band in 19 out of 38 cases exhibiting mutation.

DISCUSSION

In women, the incidence of breast cancer is quite common due endocrine dysfunction, failure of early diagnosis and timely management is required, becomes essential to reduce the incidence of breast cancer cases. Etiopathology is complex due to involvement of both genetic and epigenetic factors. In recent trend of diagnosis i.e. based on CTCs for early diagnosis by characterization of epithelial mesenchymal transition markers-Sox4, EpiCAM and Vimentin (Ankeny et al., 2016; Saxena, 2022). Vimentin is a mesenchymal marker and shows aggressive behaviour due to phenotypic changes in the tumor cells during progression of disease at metastatic stage (Zhou et al., 2010). The findings of the DNACNVs of vimentin mRNA gene expression varying either in alone (323bp), or with isoform (250bp) perhaps due to different genetic susceptibility of CTCs or different pathological grading of breast cancer tissue. However, the mRNA expression of vimentin gene is not independent, and mediated though signaling of Sox4, a pluripotent stem cell epithelial-mesenchymal transition marker during disease progression. The mutation of vimentin in eight cases of breast cancer might be involved increasing "risk factor" of developing aggressive behavior of mesenchymal cells as data was depicted in Table 1. DNA profile study of vimentin gene showing lack of significant variation neither in up regulation (over-expression) nor down- regulation (under expression) of vimentin gene mRNA isoforms confirming not only the sensitivity, but regulating mesenchymal transition phenomenon during metastasis, reporting first time variable expression in CTCs) of breast cancer patients. However, vimentin gene expression could be used as an important biomarker by the clinicians during the onset of tumor and early diagnosis. The isolation of CTCs is highly sensitive in an individual to characterize during metastasis because of low population (>1%) that require either Sanger sequence or next-generation sequencing. Simultaneously, another technique to detect CTCs is based on Nano Velcro platform using anti-EpCAM-coated nano-substrates in conjunction microfluid mixture to improve capture of CTCs (Vang et al., 2011; Cheng et al., 2020). Present study is simple, well established in our laboratory, based on tissue

engineering with minimal use of blood samples (0.5ml). The sensitivity and quality of CTCs were well established, after characterization of EMT markers (Sox4, EpCAM & CK19) in variety of tumors like hepatocellular carcinoma, acute lymphoblastic anemia and pancreatic tumors (Saxena, 2022).

ARMS-PCR (amplification refractive mutational system) is highly sensitive procedure for the study of genetic heterogeneity, used for MTHFR C677T gene polymorphism. In CTCs isolated from breast cancer patients showing higher *Tm* values (6.36) in those cases where mutation of vimentin (300bp) was observed, as compared to those cases showing isoforms (5.59) and GAPDH, act as genomic controls due to tissue specific genetic susceptibility and also suggesting either due to genetic heterogeneity or "point mutation", where the nucleotide substitution from cysteine to thymidine (C→T) followed by changes in amino acid alanine is replaced by valine, resulting in decrease of folate concentration up 50% in tumor cell as depicted in Figure 4. High expression of MTHFRC677T gene increases during migration of tumor cells and involved in remodeling of the extracellular matrix during metastasis either in the presence of epithelial cell adhesive molecule or cytokeratin (Ozenf et al., 2013; Liew and Gupta, 2014) . The mutation of p53 (273bp) in 19 cases of breast cancer patients, might have increased the genetic susceptibility of mRNA expression of vimentin gene isoform during transition of mesenchymal cells in CTCs, confirming the angiogenesis in the patients. Earlier study also shows that isoforms of Nanog, associated to the neural tube defects patients and failed to regulate pluripotency during organogenesis (Saxena et al., 2013). Although, the exact role of these isoforms is still not known, but certainly regulates cell-proliferation during progression of the disease (metastasis) through cell-signaling molecules due to p53 gene mutation (Malkin et al., 1994). In human p53 gene regulates variety of biological process including DNA damage, cell-kinetic, apoptosis including post translational evet during protein synthesis (Hafner et al., 2018). Earlier, study reveals that new variant of p53, modify the 3D structure followed by functional activity in tumor that might have also increased the "risk factor" during progression of the disease, if mode of action is same for two different tissues (Saxena et al., 2022).

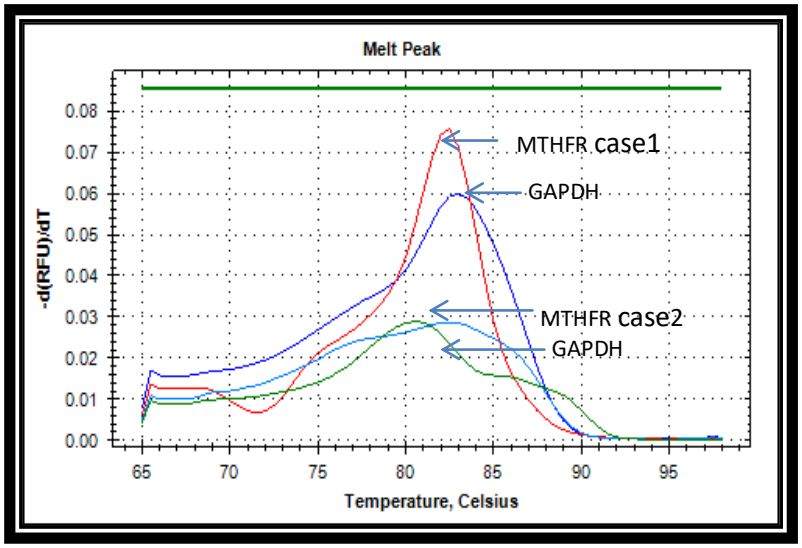

Figure 4. MTHFR C677T gene polymorphism showing genetic heterogenicity in circulating tumor cells showing significant variation of *Tm* values between case 1 (6.36), and case 2 (5.58), with complete disappearance of the vimentin gene (323 bp), considered a mutation and case 2 showing isoforms (250bp) when compared with GAPDH as genomic controls.

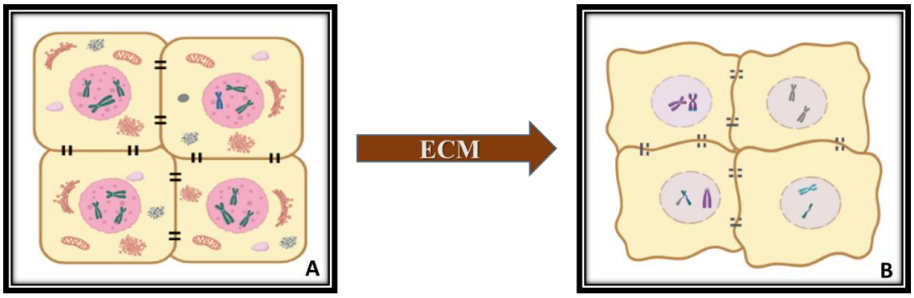

Figure 5. Illustration of the transformation of normal mesenchymal cells (A) with a well defined outer membrane attached with cell adhesive molecules between two cells (arrow) and B showing aggressive behavior of mesenchymal cells with irregular phenotypic appearance either due to loss of the content of extracellular molecules due to (ECM) like cell-adhesion or vimentin or β-keratin expression, and down regulated during metastasis in circulating tumor cells isolated from breast cancer patients. Intermediate filaments of vimentin also showing changes or loss in a network that involves cell-junction adherence (arrow).

CONCLUSION

CTC data confirmed that differential expression of vimentin gene mRNA isoforms is associated with the aggressiveness of mesenchymal cells during epithelial mesenchymal transition either alone or in synergy with p53 or MTHFR C677T polymorphism, suggesting vimentin could be used as an additional prognostic biomarker for early detection in sporadic breast cancer patients. However, there is a need to incorporate more samples size to validate these findings.

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AUTHOR'S CONTRIBUTIONS

AKS executed experiment designed, implementation, validated results, proof reading, funding, PS,VS helped with clinical diagnosis, and Shalini helped with data analysis and illustration of the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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