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Genetic Heterogeneity of MTHFR C677T Gene Polymorphism Increase "Risk Factor" Using Circulating Tumor Cells (CTCs) in Acute Lymphoblastic Anemia - A Rare Case Report

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ABSTRACT

BACKGROUND

Circulating tumor cells (CTCs) are used as an important genetic biomarker for early diagnosis. Several techniques have been enumerated to characterize the CTCs number and their sensitivity due to poor survival. Most of the approach is based on cytology or immunochemistry or even PCR for the diagnosis of acute lymphoblastic leukemia (ALL). In the present case study of ALL become quite important due to transformation of malignant cells and proliferation of progenitor cell in bone marrow, blood finally to extramedullary organs (liver and spleen). Present study is carry out to establish genetic link by developing karyotypes and to assess the "risk factor" using MTHFR C677T gene polymorphism after isolation and characterization of CTCs from proband of ALL family.

METHODS

CTCs, isolated from cultured blood (0.5ml) samples of Acute lymphoblastic Leukemia (ALL) and their sensitivity were characterized by epithelial to mesenchymal transition (EMT) markers -SOX4, EpCAM and CK19 using two different protocols with Ficoll's or without Ficoll's gradient methods. The GTG banded karyotypes were developed using short term lymphocyte cultures. The genomic DNA was isolated from CTCs and ARMS RT-PCR technique is used to determine the genetic heterogeneity of MTHFR C677T gene polymorphism.

RESULTS

The most interesting findings reveals that 30% cells showing 47, XX +Trisomy-21 in metaphase. There are no differences in the sensitivity of CTCs after adapting two different procedures. After isolation of genomic DNA, MTHFR C667T gene polymorphism the is used to determine genetic heterogeneity, where Tm values shift significantly between control (87.00) and

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Acute lymphoblastic Leukemia case (86.50), due to point mutation and cytosine change into thymidine followed by the substitution of amino acid alanine in to valine confirming the increase of "risk factor" of the disease.

CONCLUSION

Acute lymphoblastic Leukemia is a complex genetic disorder where genetic (karyotypes) and epigenetic factors (genetic heterogeneity) based on folate metabolism work together in synchronized manner which confirm to increase "risk factor" of the disease in heterozygous condition.

KEYWORDS

Circulating tumor cell; Acute lymphoblastic Leukemia and MTHFR C677T polymorphism; Trisomy-21

INTRODUCTION

In human, the circulating tumor cells (CTCs) play an important role for cancer management of solid tumors as noninvasive device. The number and sensitivity of CTCs are quite low (~1%) in cancer patients [1,2]. The CTCs shows heterogeneous group of cell population during circulation and establish distant metastasis. The CTCs are rich source of liquid biopasy and "information center" for early diagnosis, monitoring, and treatment [3]. Several techniques have been implicating to increase the number and their sensitivity depending increase (5.0-10ml) the quantity of blood samples [4]. Clinically, the acute ALL cases were indistinguishable by cytologically, or immunology, but peripheral blood cells show blast cell with anemic, thrombocytopenia and sudden increase of fever. The finally the genetic basis of diagnosis becomes quite relevant to explore the etiopathology of the disease. In human cancer cytogenetics, the ALL patient shows poor or 'fugginess' morphology of chromosomes. The karyotypic variation shows non-random pattern of involving additional chromosome number 3, 6 and 21. These constitutional chromosomal aberrations (aneuploidy) warned the individual are at higher "risk" of developing leukemia in the patients of Down's syndrome (47+21) and Klinefelter syndrome (47, XXY or mosaic) patients. The genetic etiology of human cancer is highly complex due to involvement of multiple factors. In lymphocytic leukemia is commonly observed in Down's syndrome cases due non-disjunction in mitotic event during cell – division.

In the present case study, CTCs were isolated using two different procedures and check their sensitivity was evaluated using three different epithelial to mesenchymal transition (EMT) markers - SOX4, EpCAM and CK19. These markers are highly conserved in nature and extensively used for morphogenetic transformation during metastasis. A novel procedure was developed to increase the number and their sensitivity after collection of small amounts of blood (0.5ml) form the patient like ALL for CTCs using in - vitro techniques. Further, the same study was extended to assess the "risk factor" by using MTHFR C677T gene polymorphism in the same case of ALL, after isolation of genomic DNA form CTCs. The methylenetetrahydrofolate reductase (MTHFR) gene is a critical enzyme to regulate the folate metabolism for DNA methylation in pediatric cancer [5]. In the present rare cases of ALL, the authors try to explore the etiopathology between the genetic link and risk factor of the disease to determine genetic heterogenicity.

MATERIAL AND MTHODS

Clinically diagnosed, a female of six-year age old girl referred to cytogenetic and molecular genetic laboratory of the department of Pathology/Lab Medicine, All India Institute of Medical Sciences, Patna, Bihar for confirmation of diagnosis based on involvement of genetic

component (chromosome) risk factors and their correlation to the onset of disease. The family history was recorded to develop pedigree analysis to find out the mode of inheritance in the proband but failed due to lack of cooperation of family member belong to rural area of Bihar. Family history showing lack of environmental exposure either to radiation or prenatal exposure to drug involvement during antenatal development of disease except sudden increase of fever.

Development of Karyotypes

The peripheral blood sample (1.5ml) was collected under sterile condition to set up short term lymphocytes cultures in triplicates (n=3), each culture contain 0.5ml of ALL peripheral blood. The RPMI 1640 media containing phytohemagglutinin-M, FBS (5%) and antibiotics (streptomycin - penicillin) for 72 hours at 37 C was used for cytogenetic analysis as well as used for isolation of circulating tumor cell (CTCs). The karyotypes were developed from well spread metaphase after harvesting the cultures by incorporation of colchicine (2hrs) prior to arrest the dividing cells. The cells were fixed in 3:1 methanol: acetic acid solution after hypotonic treatment. At least twenty well spread metaphases were selected for karyotyping after GTG banding [6]. The karyotypes were prepared according to the recommendations of the International System for Chromosome Nomenclature (ISCN 2016) The second and third set of ALL cultures were used for the isolation and characterization of circulating tumor cells.

Isolation and Characterization of Circulating Tumor Cells (CTCs)

The cultured blood (second and third set) was collected and wash with 3.0 ml of media RPMI 1640 three times and second set of cultured blood used for the isolation of CTCs using Ficoll's gradient) methods. Blood was mixed (3.0 ml) of Ficoll-Paque Plus in conical glass test-tube and centrifuged at 400 x g for 30 minutes at 20°C. After centrifugation, a ring was obtained at the junction of plasma and Ficoll's layer. The upper layer was drawn off leaving the lymphocyte layer undisturbed which was then isolated carefully and kept in an Eppendorf tube for DNA extraction using kit (Promega, USA). In the third set of cultured cell were collected and follow the same procedure as mentioned above except failed to add Ficoll-Paque Plus. The genomic DNA was isolated using kit quantified by nanodrop spectrophotometer. The comparative analysis and sensitivity of CTCs was carried out by adapting two different procedures after using known markers of transition from epithelial to mesenchymal cells named SOX 4, EpCAM and CK19 [6-8]. Further, the confirmation and characterization of the CTCs makers were performed with specific forward & reverse primers using RT PCR and individual bands were characterize on (1.5%) agarose gel electrophoresis. The bands were visualized on GelDoc system (BioRad) after Et.br staining.

Characterization of CTCs markers-SOX 4, EpCAM and CK-19

Table-1, showing the specific set of primers of SOX4, EpCAM and CK-19 for the identification of CTCs in ALL case 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 Go Taq DNA polymerase (5U/µl). The genomic template of DNA (50ng) is mix with reaction mixture before using PCR. The reaction profile was different for each of the CTC's marker i.e. carried out for 35 cycles comprising, initially denaturation at 95°C for 5 minutes. There are three markers showing different PCR protocols 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, the denaturation at 95°C for 45 seconds, annealing at 58.7°C for 1 minute,

elongation at 72°C for 1 minute, followed by final elongation at 72°C for 7 minutes. and CK-19 (Denaturation at 95°C for 45 seconds, annealing at 60.2°C for 30 seconds, elongation at 72°C for 1 minute, followed by final extension at 72°C for 7 minutes) as shown. The amplified products were characterized on 1.5% agarose gel as illustrated as above.

MTHFR C677T Gene Polymorphism

MTHFR gene polymorphism analysis helps to assess the genetic heterogenicity and "risk factors" by using ARM-PCR in ALL case. This is highly sensitive, reliable technique used for SNP analysis to detect mutant alleles of MTHFR, based on Tm values to increase the specificity of specific primers (tetra plex) as details of primers are documented in table-1. The primers were designed for genotyping of MTHFR C677T (http://cedar.genetics.soton.ac.uk/public_html/primer1.html)

CAGTAGGGATAACCGTCCAATGGGGTTTCCCATAAACAGGAATGAAGT
ATGAACCCACGCTACCACCAGGATGAACCTGAGGACGTATTGCTGAGT
GAAAGAAGTCAGTCGCAGCAGGCCGCACATGGTGTGTTTCATTTATGT
GAACGTCCAGGCAAATCCACAGACACAGGTACAGCCACGTCGGAATTG
TTTCATGTCGGTGCATGCCTTCACAAAGGAAAGCGGGTGGGTGGTTGCC
TGGGGCCGGGGGCAGGGAGCATGAACTTCCTTCCACACAGGACCCCGC

A and further confirmed by BLAST (http:// www.ncbi.nlm.nih.gov/blast) to determine the specificity of the primers using RT-PCR technique.

Table 1: RT-PCR strategy showing the set of primers (forward/reverse) used for circulating tumor cells (CTCs) as specific markers and the four set of primers for MTHFR C677T genes polymorphism used in the present study.

S. No.	Oligo Type	Oligonucleotide Sequences (5'-3')	Annealing Temp. (°C)	Amplicon Size (bp)	References
1.	SOX4	Forward-GGTCTCTAGTTCTTGCACGCTC Reverse CGGAATCGGCACTAAGGAG	57.2	183	[7]
2.	EpCAM	Forward GCCAGTGTACTTCAGTTGGTGC Reverse CCCTTCAGGTTTTGCTCTTCTCC	58.7	359	[8]
3.	CK 19	Forward ATTCCGCTCCGGGCACCGATCT Reverse CGCTGATCAGCGCCTGGATATGCG	60.2	573	[9]
4.	*MTHFR C667T	Forward TGTCATCCCTATTGGCAGGTTACCCCAAA Reverse CCATGTCGGTGCATGCCTTCACAAAG	58.0	171	[10]
		Cpoly GGCGGGCGGCCGGGAAAAGCTGCGTGATGATGAAATAGG		150	
		Tallele GCACTTGAAGGAGAAGGTGTCTGCGGGCGT		105	

Note: *ARMS PCR is used using four different set of primers.

To increase the specificity of the reaction, the allele-specific primers were selected and confirmed by software to obtain maximum *Tm* values [9]. This tetra primer selected for ARMS - PCR of MTHFR C677T genotype i.e. CC (wild type) and CT (mutant) either in homozygous and heterozygous condition using SYBR green. The primers used in present study- MTHFR-T, 5' – GCACTTGAAGG AGAAGGTGTCTGCGGGCGT-3'; MT MTHFR-C-poly G, 5'-GGCGGGCGGCCGGGAAAAGCTGCGTG ATGA TGAAATAGG-3'; MTHFR-cf, 5'-TGTCATCCCTATTG GCAGGTTACCCCAAA-3'; MTHFR-cr, 5' - CCATGTC G GTGCATGCCTTCACAAAG-3'. The reaction mixture consist of a total volume of 20µl containing 10µl of SYBR Green PCR Master mix , 1 µl of each primer per reaction, 40ngm of genomic DNA, and distilled water was used for RT- PCR analysis. The PCR protocol initially consist of denaturation step (95 C for 7 min) was followed by amplification and quantification steps repeated for 30 cycles (950C for 10 s, 60 C for 10 s, 72s, with a single fluorescence measurement at the end of the elongation step at 72° curve analyzed the data and reaction was terminated

by cooling to 40°C. Melting curves (*T*m) 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, RT- PCR, a plot was developed between fluorescence versus temperature (dF/dT) for the amplification of candidate gene products and finally measured at 530nm. PCR products were further analyzed on agarose gel electrophoresis by evaluating the appearance of additional band of 105bp confirming heterozygosity (CT allele) in ALL case.

RESULTS

Cytogenetics analysis was carried out using short term lymphocyte cultures using RPMI1640 media and the GTG banded karyotypes were developed. More than 30% metaphase plates showing chromosomal complements 47+XX (trisomy 21) as shown in Figure-1 and ~70% cells showing normal chromosomal complements (46, XX). The karyotype was developed according to ISCN 2016 and analyzed by ASI software on Cytoscan system of Olympus (Japan). This showing the confirm the involvement of genetic factor in the proband of the ALL.

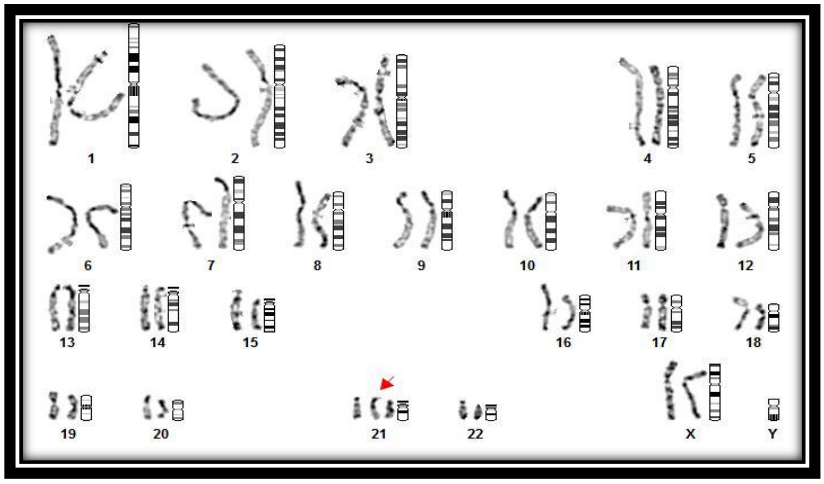


Figure 1: Karyotypic picture showing (arrow) 47+XX (Trisomy -21).

Circulating Tumor Cells (CTCs)

Circulating tumor cells are important and highly sensitive noninvasive technique for the diagnosis and management of early solid tumors. Figure 2A & 2B. The RT PCR analysis showing SOX4 (183bp) and CK19 (573bp) gene expression (Figure 2A) and EpCAM (359bp) as shown in Figure 2B in CTCs isolated form acute lymphoblastic anemia (ALL) with help of specific forward and reverse primers. The relevant finding in the present study of ALL case is SOX4 of amplicon 573bp expression showing down regulation as compared to EpCAM and CK19 as shown in Figure 2A, L1 & L2.

The appearance of specific bands using forward and reverse primers of epithelial to mesenchymal transition (EMT) markers on 1.5% agarose gel electrophoresis confirm the sensitivity of circulating tumor cells in ALL patient.

MTHFR C677T Gene Polymorphism

The folate is an essential component for DNA methylation during proliferation of cells division. MTHFR C677T gene play a key role to regulate folate metabolism either in homozygous (CC genotype) or in heterozygous (CT genotype) condition. ARMS RT - PCR is highly sensitive technique used to assess the genetic heterogeneity.

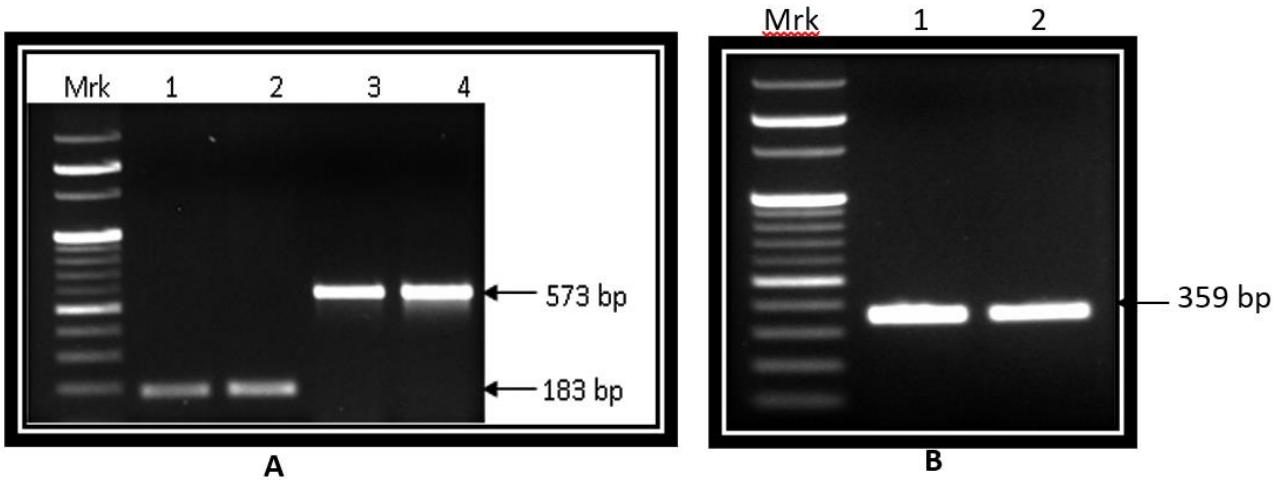


Figure 2A & 2B: RT- PCR analysis showing SOX4 and CK-19 gene expression in Acute Lymphoblastic Leukemia and its respective female control (Mrk-100bp, L1 case & L2 control showing SOX4 gene (183bp), respectively. The L3 case & L4 control showing CK-19 gene expression (573bp) as shown in Figure 2A. Similarly, the EpCAM gene (359bp) showing Mrk-100bp, L1(case) & L2 (controls).

Table 2: RT- PCR strategy showing amplification (Ct) cycle and melting peak (*Tm*) for MTHFR C677T gene analysis in circulating tumor cells.

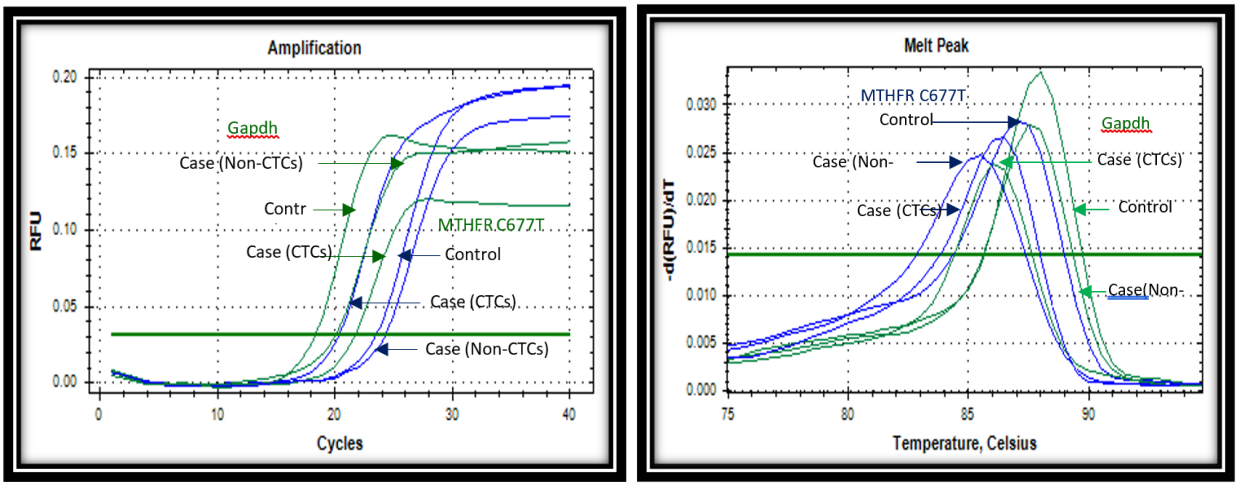
S. No	Target	Sample Types	Cycles (Ct value)	*Tm* values
1.	MTHFR	CTCs (Ficoll's method)	20.35	**86.50**
2.	GAPDH	CTCs	21.72	86.00
3.	MTHFR	Non-CTCs (without Ficoll's)	24.29	**85.50**
4.	GAPDH	Non-CTCs	20.10	87.50
5.	MTHFR	Native Control	23.51	**87.00**
6.	GAPDH	Native Control	18.33	88.00

**Notes: ***CTCs = Circulating Tumor Cells; NonCTCs = Blood samples; Native Controls= Normal female

Table 1 showing the details values of Ct and *Tm* of CTCs isolated from ALL case using two different procedures i.e. with Ficoll-Paque Plus or without Ficoll's (directly). The Ct vales in native female act as control is 23.51 and, in ALL case, (20.35) apparently showing significant correlation when corelate with *Tm* vales (87.0) and shift to ALL case (85.00) & (86.00) in CTCs in both the procedure used as shown in Figure 3A and 3B. The shifting of *Tm* values between 0.5-1.5 confirm the genetic heterogeneity of MTHFR C677T gene, where the nucleotide cytosine substitute in to thymidine (C→T) followed by change of amino acid alanine into valine and simultaneously also confirm the validity and sensitivity in the procedures used for isolation of CTCs in the cancer like ALL.

DISCUSSION

The acute lymphoblastic leukemia (ALL) is one of the major pediatrics cancers originates either from T or B lymphocytes and 80% of the children predisposed to genetic syndrome such as Down syndrome or Fanconi anemia [11]. Present case of ALL agree of the above statement and 30% metaphase cells shows trisomy-21, after GTG bandings as shown in Figure 1 which confirming the genetic association with other factors. The CTCs play an important role in tumor biology as non-invasive manner in different type of human cancer patients including solid tumor.



(A) (B)

Figure 3A & 3B: RT-PCR analysis of MTHFR C677T gene in acute lymphoblastic leukemia (ALL) case and normal female as native control (figure 3A). Amplification Curve showing Ct values of the case (CTCs) with 20.35cycles, Case (cultured blood) with 24.29 cycles and Control with 23.51 cycles. The melting peak analysis showing shifting of *Tm* values from 85 .50°C-86 .50°C (Case) to 87.0°C (Control) as shown in (Figure 3B).

The CTCs is essential component of liquid biopsy and rich source of information for cancer diagnosis and prognosis. The frequency and sensitivity CTCs varying in different age groups and types of tissues [3]. Earlier report on CTCs is highly confusing regarding numbers, sensitivity and large (4-10ml) of blood sample are required for diagnosis [4], seems to be unethical from cancer patients. Therefore, present study is a novel noninvasive approach based on *in - vitro* culture technology, required small amount of sample and the sensitivity was checked by using highly conserved metastatic epithelial to mesenchymal transition markers SOX4, EpCAM and CK19 using polymerase chain reaction with forward and reverse sets of primes as documented in Figure-2 A &B. The present study has been designed which is simple and based on *in-vitro* cell culture techniques after collection of small amounts of blood was obtained from the family of proband suffering from acute lymphoblastic leukemia (ALL). This is the first report of this kind which proofs the sensitivity by using specific EMT markers. These EMT markers are highly sensitive for CTCs and has been selected for ALL case. SOX 4 is a early transcription factor belong to SRY related high mobility homo box group, encoding proteins responsible for embryonic development, cell differentiation including stem cell activation and maintenance [7] The over expression of SOX4 has been reported during transition from epithelial to mesenchymal due to aberrant pathway induced by TGF-β (3,4,5T). CK19 is an epithelial marker of filamentous cytoskeleton protein and member of polygenetic family of cytokeratins (CK). The expression of CK19 mainly were observed in head & neck carcinoma, neuroendocrine disorders, renal cell carcinomas and used as prognostic marker for blood as well as bone marrow. The existence also been noticed in epithelial cancers such as lungs, breast, pancreas and gastric tissues [4]. The epithelial cell adhesive molecule (EpCAM) is a membranous glycoprotein consist of 314 amino acids and play an important role in cell signaling, cell proliferation, differentiation and maintenance of organ morphology. The over expression has been reported in breast cancer patient during metastasis process [4,12]. However, the down regulation of SOX4 gene as compared to CK 19 and EpCAM, suggesting either due to different heterogenous cell population or interference of new variants pre termination (stop) codons (PTC) of MTHFR C677T gene [5] as shown in Figure 2A L1 and L2. Genetics and

epigenetic factors play an important role to regulate folate metabolism during DNA methylation in cancer of pediatrics age group [5]. In cancer biology, dietary factor plays an important role for *de novo* synthesis of folates for the cell proliferation and highly sensitive towards environmental mutagens. MTHFR C677T gene polymorphisms increase risk factor due to missense mutation where, cytosine change into thymidine (C $\rightarrow$ T) leading to the substitution of amino acid alanine by valine resulting reduced the enzymatic activity of the enzyme resulting decrease the deficiency of folate and increase homocysteine level blood plasma. In the present cases of ALL Figure showing genetic heterogeneity by substitution of nucleotide cytosine change in to thymidine confirm to increase the risk factor of the disease in heterozygous (CT) condition due change of *Tm* values between case and controls as shown in Figure-2A &B. Earlier studies of the same author on MTHFR C677T gene polymorphism in different tumors suggests that genetic susceptibility increase due missense mutation in the proband and risk factor. Although, this is the first kind of the report on ALL case where CTCs establish positive correlation between two different procedures used and establish genetic link and risk factor of the disease. However, other predisposition cancer genes or environmental factors are required for further study in different labs to explore the etiopathology of cancer like ALL, otherwise the study will be remains in complete.

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