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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

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ABSTRACT

BACKGROUND

Circulating tumor cells (CTCs) are important genetic biomarker of early prognosis and diagnosis during tumorigenesis. The sensitivity of CTCs during transition was identified and characterized by EMT (epithelial mesenchymal transition) markers - Sox4, CK-19 and EpCAM. Presently study has been designed with the aims to identify the role of stem cells (Sox2 & Oct4) along with epithelial mesenchymal transition markers (EMT) Sox4, CK 19 and EpCAM from CTCs due to early signaling transducing factor to maintain pluripotency. The study was further extended to evaluate the genetic heterogeneity of MTHFR C677T gene polymorphism.

METHODS

The genomic DNA was isolated from clinically diagnosed cases of pancreatic tumors (n = 7) including one female case. Stem cell Sox2 and Oct 4 gene was characterized by RT-PCR techniques. In one female case, the curiosity has been developed to assess the role of CTCs in pancreatic tumor, after isolation from cultured cells using Ficoll's plaque gradient procedure. After isolation of CTCs, the sensitivity was characterized and compared with non-cultured CTCs using EMT (Sox4, CK 19 & Ep CAM) markers. The genetic heterogeneity of MTHFR C677T gene polymorphism was studied by ARMS-PCR to assess the "risk factor" of the disease.

RESULTS

Interestingly, the findings revels >16% cases of adenocarcinoma of pancreatic tumor shows Oct 4 gene mutation and Sox2 shows variation in the gene expression, suggesting failed to maintain the pluripotency and may also likely to increase the aggressiveness of tumor. The finding of the CTCs on the basis of EMT markers shows the loss (mutation) of CK 19 band consist of 573bp to confirm the sensitivity, followed by changes in architecture and physiological function of the tumor. Further, the MTHFR C677T gene polymorphism again showing significant variations in Tm values between GAPDH (88.00) and case

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(86.00) due to point mutation followed by substitution of nucleotide cytosine to thymidine (C→T), resulting change of amino acid alanine to valine in heterozygous (CT genotype) condition.

CONCLUSION

Over all our data support that mutation of stem cell Oct 4 gene failed to maintain the pluripotency and the loss of epithelial marker (CK19) during metastasis is responsible for poor diagnosis to the clinicians. Secondly, MTHFR C677T gene variant confirm the "risk factor" due to dysfunction of folate metabolism. Interestingly, present study shows disconcordance between folate metabolism, cytokeratin and stem cells in pancreatic tumors.

KEYWORDS

Pancreatic tumor; Sox2; Oct4; EMT markers; MTHFR C677T gene polymorphism

INTRODUCTION

Stem cells, are known as early transcription factors and play a crucial role during organogenesis since after the discovery in 2003, identified as pluripotency markers - Oct4, Nanaog3 and Sox2 to decide the fate of three germ layers i.e. ectoderm, mesoderm and endoderm [1]. The metastasis is main cause of mortality in tumor biology due to invasion of circulating tumor cells (CTCs) to the neighboring organs. Epigenetic factors modulate the activity of stem cells during therapeutic regime has been poorly under stood. Pancreatic tumor shows poor sensitivity in clinical diagnosis after local invasion to metastases. Because, the CTCs are rich source of "liquid biopasy" and act as an "information center" for the clinicians as a source of early diagnosis, monitoring and cancer management in variety of tumors. The identification and characterization of CTCs are difficult task due to involvement of several factors such as sensitivity, (~1%) small quantity (number), survival and heterogeneity in population [2-4]. The collection of blood samples (5 ml - 10 ml) from the cancer patients raises the question of ethics and still shows lack of consistency in their findings for the study of CTCs even after using EMT markers [5]. Several efforts have been developed to improve the number of CTCs with sensitivity for early diagnosis due to clinical interest and to assess the metastatic risk factor in tumor.

CTCs cells have a characteristic feature of developing adaptive niche after infiltration to distant organs like liver, kidney and nervous system, where they act as circulated "seeds" for metastasis as shown in Figure 1.

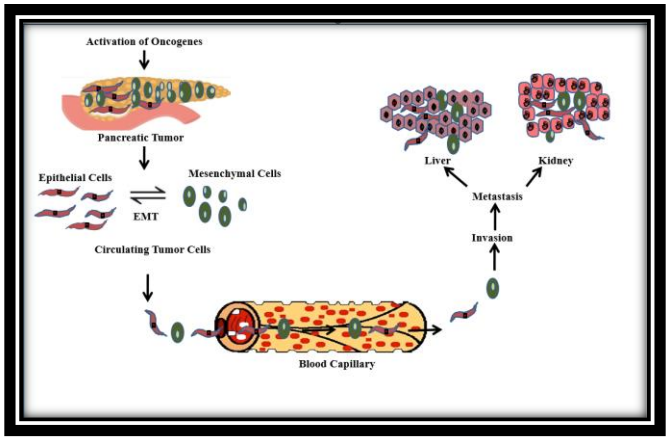

Figure1: The transformation model of circulating tumor cell (CTCs) between epithelial and mesenchymal cells by activation of oncogene through blood vessels reach in two different metastasis sites.

The study of CTCs is difficult phenomenon due to several reasons: 1) small cell number in blood, 2) heterogeneous population of cells, 3) variation in expression of EMT markers sensitivity during distant invasion (metastasis), 4) also shows tissue and site specificity, and 5) application of technique (s) used for the study such as flowcytometry or immunochemistry or RT-qPCR shows discordance in the findings. Figure 1, showing the transition model during migration of primary tumor cells activated by oncogene (KRAS) at epithelial site to distant metastatic stage of

tumor site during circulation and reach to the neighboring tissue, where they settle down due to favorable niche, forms colony for further differentiation, proliferation of after signaling by mutagenic products (truncated proteins) of oncogenes [6]. However, the exact mechanism of activation between oncogenic product leading to the target site is still confusing in tumor biology. The sensitivity of CTCs was characterized by epithelial to mesenchymal transition (EMT) markers such as SOX4, EpCAM and CK19. These markers are highly conserved in nature and extensively used during morphogenetic transformation at metastasis stage of the distant organs. SOX4, a member SRY- high mobility homeobox region is belong to early transcription factor, encodes proteins, responsible to decide fate of cell proliferation. The over expression of SOX4 associated to oncogenic activities of epithelial cells during transition of aberrant epithelial to mesenchymal pathway after activation of TGF-β [7]. The epithelial cell adhesion molecule (EpCAM) is a kind of glycoprotein and expressed on cell surface of stem or progenitor cells and play a significant role in cell adhesion, maintenance of cell morphology during cell-migration [8]. Similarly, cytokeratins (CK19), is another epithelial marker with high molecular weight proteins has been associated with the angiogenesis in variety of tumor [9]. Therefore, a novel approach been used based on in-vitro techniques with the aims to enhance the CTCs number and to sustain the sensitivity when compared with non-culture cells using Ficoll's gradient method. Folate metabolism is an essential component of DNA methylation and regulates by methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism and increase genetic susceptibility in variety of cancers using highly sensitive ARMS-PCR [10-13]. Therefore, the study of becomes imperative to assess the "risk" factor by evaluating genetic heterogeneity to explore the mechanism of etiopathology in pancreatic tumors.

MATERIALS AND METHOD

Clinically diagnosed, blood samples (3ml) of pancreatic tumor (n = 7) were collected form the OPD of the All India Institute of Medical Sciences, Patna, Bihar between 48 years - 58 years old age group. The clinical symptoms of long-term complaint of indigestion and vomiting tendency was recorded during collection of patients performa. All the cases belong to rural population and associated with farming, while collecting family history none of them showing lack of drug or radiation exposure. Another female case of 57-years-old belong rural region, poor socioeconomic conditions and family history again showing lack of exposure with drug or radiations as well as pedigree analysis also reveals lack of cancer in the family. In this female case, the CTCs was isolated from 0.5ml blood cultured (n = 3) and sensitivity was compared with non-cultured samples using Ficoll's gradient methods as detail shown in Figure 2. After collection of blood (3.0 ml) mix with Ficoll-Paque Plus in glass tube and centrifuged at 400 × g for 30 minutes at 20°C, centrifuge a ring was formed at the junction of plasma and Ficoll's layer.

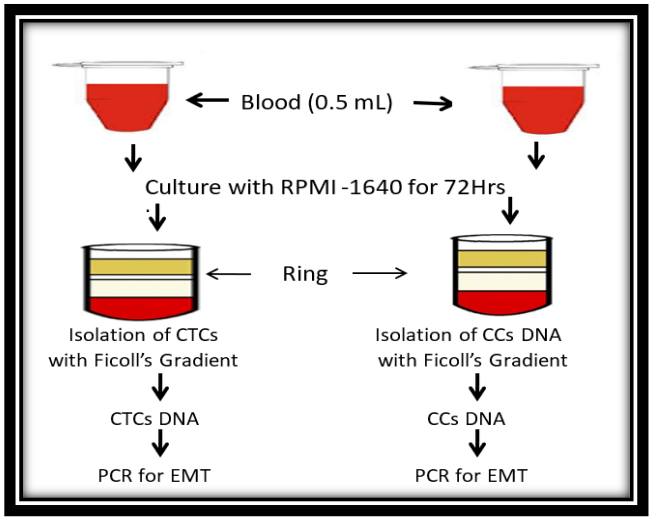

Figure 2: Diagrammatic representation of isolation of circulating tumor cells (CTCs) after short term culture and compared the same with non-culture cells and the sensitivity was characterize during transition of epithelial to mesenchymal markers - Sox4, EpCAM and CK19 in pancreatic tumor.

The upper layer was drawn off leaving the lymphocyte layer undisturbed which was then isolated gently for isolation of g DNA (Promega Kit), quantified by nanodrop spectrophotometer. The curiosity has been arisen with the aim to evaluate the genetic profiling of stem cells and EMT markers -Sox 2, Sox 4, Oct 4, EpCAM and CK19 using RT- qPCR technique. The 3D analysis of individual bands was characterized and visualize on agarose gel (1.5%) electrophoresis after staining with ethidium bromide on Gel Doc (Bio Red) system.

Molecular Characterization EMT markers in CTCs

Table 1, showing the details of specific set of forward and reverse primers of Sox4, EpCAM and CK-19 for the identification of CTCs in the female case after confirmation of sequences from NCBI (BLAST/http://blast.ncbi.nlm.nih.gov.).The PCR reaction was achieved in a 25 µl 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, 0.2 µl of Go Taq DNA polymerase (5 U/µl). The genomic template of DNA (50 ng) 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 in Table 1. The amplified products were characterized on 1.5% agarose gel and visualized by Gel Doc systems after ethidium bromide staining.

MTHFR C677T Gene Variants Using ARMS PCR for CTCs

MTHFR gene polymorphism analysis helps to assess the genetic heterogeneity and "risk factors" by using allele refractive mutational system. 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 specific primers were designed for genotyping of MTHFR C677T (http://cedar.genetics.soton.ac.uk/public_html/primer1.html) and further confirmed by BLAST (http://www.ncbi.nlm.nih.gov/blast) to determine the specificity of the primers using RT-PCR technique. To increase the specificity of the reaction, the allele-specific primers were selected and confirmed by software to obtain maximum Tm values [10]. The tetra primer selected for ARMS - PCR of MTHFR C677T genotype i.e., CC (wild type) and CT (mutant) in heterozygous condition using SYBR green.

The primers used in present study-
MTHFR-T, 5' – GCACTTGAAGGAGAAGGTGTCTGC GGGCGT-3'; MT MTHFR-C-poly G, 5'- GGCGGGCGG CCGGGAAAAGCTGCGTGATGATGAAATAGG-3';
MT HFR-cf, 5'-TGTCATCCCTATTGGCAGGTTACCC CAAA-3'; MTHFR-cr, 5' – CCATGTCGGTGCATGCCT TCACAAAG-3'. The reaction mixture consists of a total volume of 20 µl containing 10 µl of SYBR Green PCR Master mix, 1 µl of each primer per reaction, 40 ngm of genomic DNA, and distilled water was used for RT- PCR analysis. PCR protocol initially consist of denaturation step (95°C for 7 minutes) was followed by amplification and quantification steps repeated for 30 cycles (95°C for 10 seconds, 60°C for 10 seconds, 72 seconds, 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 (Tm) values 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 530 nm. PCR products were further analyzed on agarose gel (1.5%) electrophoresis by evaluating the appearance of additional band consist of 105 bp, confirming the heterozygosity (CT genotype) of the disease like pancreatic tumors.

Figure 3: PCR based analysis of Oct 4 and Sox2 stem cell in pancreatic tumor showing complete disappearance of Oct-4 band of amplicon 577 bp as shown in Lane-2 (arrow), while Lane-1&6 showing down regulation. Similarly, Sox4 (236 bp) gene showing lack of mutation but differential expression was observed in individual cases (lane 7-12).

S. N.	Types of Markers	Oligonucleotide Sequences (5'-3')	Anne. Temr (°C)	Ampl. (bp)	Ref.
1.	OCT 4	F [5]GACCATCTGCCGCTTTGAG[3]	60.0	577	[1]
		R [5]CCCCCTGCCCCCATTCCTA[3]			
2.	SOX 2	F[5]GGCAGCTAGAGCATGATGC[3]	60	236	[1]
		R [5]TCGGACTTGACCACCGAAC[3]			
3	SOX4	F [5]GGTCTCTAGTTCTTGCACGCTC[3]	57.2	183	[7]
		R [5]CGGAATCGGCACTAAGGAG[3]			
4.	EpCAM	F[5] GCCAGTGTACTTCAGTTGGTGC[3]	58.7	359	[8]
		R[5]CCCTTCAGGTTTTGCTCTTCTCC[3]			
5.	CK 19	F[5] ATTCCGCTCCGGGCACCGATCT[3]	60.2	573	[9]
		R[5]CGCTGATCAGCGCCTGGATATGCG[3]			
6.	*MTHFR C667T	F[5]TGTCATCCCTATTGGCAGGTTACCCCAAA[3]	58.0	171	[11]
		R[5]CCATGTCGGTGCATGCCTTCACAAAG[3]		171	
		Cpoly:[5]GGCGGGCGGCCGGGAAAAGCTGCGT GATGATGAAATAGG[3]		150	
		T-allele, [5]GCACTTGAAGGAGAAGGTGTCTGCGGGCG T[3]		105	

Table 1: RT-PCR showing the set of primers (forward/reverse) used for the identification and characterization of EMT markers from circulating tumor cells and specific primers used for MTHFR C677T genes polymorphism in the case of pancreatic tumor.
*ARMS PCR is used using four different set of primers.

RESULTS

Stem Cell Gene Marker Oct4 and Sox2 PCR based Study

Pancreatic tumors are highly aggressive in nature and our findings are based on the basis of mutation of gene (complete disappearance of band) or differential expression after 3D analysis of individual band using specific amplicons of stem cell markers using RT-PCR techniques. The differential expression of stem cells Sox2, and Oct-4 gene was observed as depicted in Figure 3, where, lane-6 showing very poor expression in Oct-4.The most interesting finding was observed that more than 16% cases of pancreatic tumor showing complete loss or disappearance of band (577 bp), confirming the mutation of Oct 4 gene as shown in lane-2 (arrow).Similarly, the Sox2 showing lack of disappearance of band (236 kd) except differential expression were observed after 3D analysis of the individual band intensity on 1.5% agarose gel.

Epithelial - Mesenchymal Transition Markers Analysis by RT-PCR

The findings of pancreatic tumors are quite interesting based on early transcription factors (stem cell-based gene regulation), epithelial- mesenchymal transition markers and folate metabolism with the help of RT PCR techniques after using highly sensitive forward/reverse set of amplicons. Another interesting finding shows differential expression of EMT markers with two different approaches i.e., with cultured and without cultured cell of the same patient were used for comparative analysis of sensitivity as shown in Figure 3. The most common procedure for the isolation of circulating tumor cells is with Ficoll's as shown in L1,3,5 and L2,4,6 after the cultured cells. Apparently, Sox4 (183 bp) showing lack of significant differences in the intensity of band (L1 non-culture) and L2 culture cells.

The most relevant findings were the disappearance of band of 573 bp (arrow) as shown in Lane 3-4, confirm the mutation of CK 19 and EpCAM gene showing up regulation (lane-5) in non-cultured cells, when compared with cultured cells (lane -6) in pancreatic tumor.

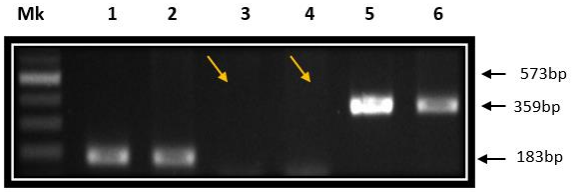

Figure 4: RT-qPCR analysis showing differential expression of EMT markers Sox4 (L1-2), and EpCAM L5-6) while Lane 3-4 showing complete disappearing of 573bp band of CK 19 confirm the mutation in both non-cultured and cultured Circulating Tumor Cells isolates from pancreatic tumor. (L1,3,5 from non-cultured and L2,4,6) from cultured lymphocytes.

MTHFR C677T Gene Polymorphism Analysis by ARMS PCR

Folate is an essential component of DNA synthesis for proliferating tumor cells. MTHFR C677T gene play a key role in folate metabolism either in homozygous or in heterozygous condition. Allele specific mutational spectra (ARMS) RT-qPCR is highly sensitive and reliable techniques for allele analysis due simultaneously use of four different primers in reaction. Figure 5A and Figure 5B showing the Ct values after obtaining amplification curve (Figure 5A) and melt curve (Tm) in cultured CTCs after isolation of genomic DNA from the same patient of pancreatic tumor. The melt peaks of ARMS PCR showing significantly shift of Tm values between control (88.00) and case (86.50) confirming the point mutation.

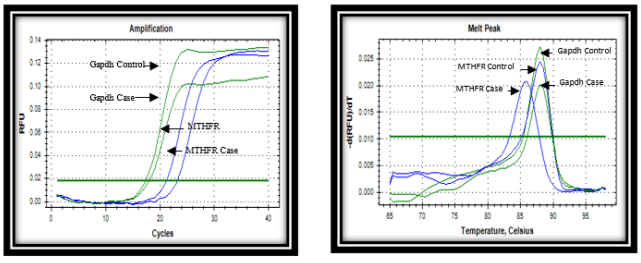

Figure 5A & 5B: showing the details finding of Ct values (Figure 5A) and Tm values (Figure 5B) of MTHFR C677T variants, after isolation of genomic DNA from female cases followed by allele refractive mutational system (ARM-PCR).

DISCUSSION

CTCs are highly sensitive biomarker for the study in tumor biology from liquid biopsy during metastasis. The majority of the adenocarcinoma of pancreas (>95%) dies due to poor prognosis, diagnosis and develop aggressive behavior [18]. Stem cells are early transcription factor and play a major role before or after chemotherapeutic regime during metastasis in cancer. The antineoplastic drugs changed the activity of progenitors or stem cells resulting fail to maintain pluripotency. Present study is the evidence to confirm that the mutation of Oct4 and variable expression of Sox2 might be the important “risk factor” to modified signaling, resulting failed to maintain pluripotency followed by onset of tumor development. Secondly, these changes in stem cells not only responsible for physiological activity, but similarly responsible for aggressive behavior resulting poor prognosis and diagnosis. Pedigree analysis also showing that none of the family member affecting such type of disease (adenocarcinoma of pancreas), suggesting such mutations and variable expression of stem cells in proband seems to be sporadic in nature.

The study of circulating tumor cells (CTCs) is quite interesting because of sensitivity for early diagnosis. In literature large number of techniques are available to identify and characterize CTCs using highly sensitive biomarkers for epithelial mesenchymal transition markers like S0x4, EpCAM and CK19, and showing disconcordance in numbers and sensitivity resulting poor prognosis and diagnosis of followed by high risk of mortality in cancer patients. There is also observed ambiguity during collection of blood samples varying from 4 ml to 20 ml from the cancer patient, which fail to justify ethically for the study of CTCs. Now the question arises how to increase the number and sensitivity of CTCs for early diagnosis of cancer patients, until use of cell and tissue culture-based technology. However, the present study showing the mutation of CK19 band (573 bp) might have change the physiological activity of higher molecular

weight proteins either of collagen (300 kd) or mucin (200 kd) followed by modification in the architecture during metastasis and make the aggressive behavior in the patients suffering from pancreatic tumor. Therefore, the present study becomes imperative to designed and developed using cell & tissue culture-based technology, after collection of small amounts of (0.5 ml) sample form such patients. The details of the technology have been documented earlier in the case acute lymphoblastic leukemia [10]. Recently, CTCs number determined by Cell search technology platform (Veridex, LLC, Rartian, NJ, USA) also showing limitations and fail to detect EpCAM negative cells from pancreatic tumors. In the controls (non-malignant) cases the study CTCs population remain >1% or even extremely rare [15-17]. However, the most conventional procedure for the isolation of CTCs is the Ficoll's gradient methods with short comings like poor yield (~1%) population, large sample (5 ml - 20 ml), hence, suggesting, the present procedure as depicted in Figure 2 becomes quite relevant with maintaining sensitivity including folate regulating enzyme MTHFR C677T after using EMTs markers again becomes evident to support our findings of relevance with justification for the study of CTCs in such cancer patients.

In cancer patient's folate metabolism play an important to determine genetic heterogeneity and to confirm "risk factor" in heterozygous condition. Because, CTCs, a non-invasive biomarker used for early diagnosis and prognosis of the patient it becomes necessary to assess the genetic susceptibility in such patients and corelate to the aggressive behavior of the disease during chemotherapy [18]. However, the differential expression of Sox4 gene as compared to EpCAM, suggesting either due to frequency (%) of heterogenous cell population or interference of new variants of MTHFR gene leading to pre termination or stop codons [5]. The most relevant findings are the disappearance of band of 573 bp of CK19 in CTCs increase risk of aggressiveness 577bp) while mutation of Oct4 confirm fail to maintain pluripotency during tumorigenesis the disease either alone or together with folate metabolism, although, folate is an important component of DNA methylation for cell-kinetics and cell proliferation during tumorigenesis. Similarly, in tumor biology, dietary factor plays an important role for de novo synthesis of folates and highly sensitive towards environmental mutagens. The C677T variants of MTHFR polymorphisms increase risk due to missense mutation, where, the nucleotide cytosine substitute into thymidine (C□T) followed by change in amino acids from alanine to valine resulting decrease of the enzymatic activity and increase of homocysteine level in blood plasma other than cancer patients [19]. Figure 5A & Figure 5B showing the genetic heterogeneity due change of Tm values between case and controls and confirm genetic susceptibility in heterozygous (CT genotype) condition. Earlier studies of the same author on MTHFR C677T gene polymorphism demonstrated in variety of tumors like carcinoma of cervix and ovary [12,13] including Wilm's tumors, suggests that the increasing "risk factor" due to involvement new variants of MTHFR C677T alleles including "stop codons or pretermination codons" followed by structural modifications owing truncated proteins after decoding of nucleotide substitutions [5]. Although, this is the first kind of the report on pancreatic tumors in India, where CTCs establish positive correlation between two different procedures used followed by established genetic link between stem cell biology and "risk factor" of the MTHFR C677T allele in cancer. The majority of the cases are males and belong to same profession i.e. farming, also concludes that they exposed constantly or consistently by strong mutagenetic agents like either insecticide or pesticide fumes interfere to the changes in the DNA profile leads to the development of cancer.

However, other factors like prediction of new predisposition of unknown genes and their expression exist on human genome associated interference to signaling due to oncogenic proteins (truncated) are required further

search in different laboratories with different population group to increase sample size, help to explore the mechanism of etiopathology of such tumors to make the study more interesting and authentic for the clinicians, otherwise the study will be remains obscure.

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