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MTHFR New Gene Variants Increase Risk Factor in Wilms' tumor and Prediction of 3D Structure Modulates Functional Activity During Drug - Protein Interaction

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Abstract

Wilms' tumor (WT) is an embryonic tumor of kidney that belongs to paediatric age group. The etiopathology is highly complex due to interaction between genetic and epigenetic factors. The genetic heterogeneity of methylene tetrahydrofolate reductase (MTHFR) gene polymorphism increase "risk factor" of the disease. The present study has been designed to identify new gene variants single nucleotide polymorphism (SNP) of MTHFR using Sanger's sequencing and decode the nucleotide sequences into corresponding amino acids to understand the translational events. Further, allele refractive mutation system with polymerase chain reaction (ARMS- PCR) was also used to confirm mutations (frequency) in the cases of WT and compare with age matched controls. Present findings reveal that genetic heterozygosity was observed in 20% cases of WT by substitution of nucleotide cytosine in to thymidine (C→T) followed by change of amino acid alanine is replaced by valine due to missense mutation. DNA sequencing data varies in different cases of WT that includes - first case shows four new SNPs -1) nucleotide cytosine is substitute by thymidine (C→T) followed by change in amino acid alanine is replaced by valine, 2) thymidine change into adenine (T→A) results in isoleucine→asparagine, 3) cytosine is substitute by adenine (C→A) results in isoleucine →asparagine, and 4) thymidine is substitute by cytosine (T→C), where phenylalanine →serine. Similarly, Second and third case of WT again showing the missense mutation, where the nucleotide cytosine is substitute by thymidine (C→T) followed by alanine→valine and thymidine into adenine (T→A) followed by change in isoleucine→asparagine, respectively. Based on bioinformatics analysis, the 3D structure predicted that the mutation in MTHFR gene modulate the functional activity of ligand binding sites either with protein or methotrexate. Collective findings of PCR and DNA sequencing suggests that these new gene variants which has not been reported earlier might have interfere in folate - metabolism during DNA methylation and increase genetic susceptibility and "risk factor" in WT cases.

Keywords: Wilms' tumor • Gene variants • MTHFR • Sanger sequencing

Introduction

Wilms' tumor (WT) is a nephroblastoma of early childhood, whose incidence is 1 in 10,000 live birth (1,2). Epidemiological study reveals that the majority (98%) of WT cases are sporadic and 1-2% cases (rare) are familial in nature [3,4].The pathogenecity is highly complex due involvement of WT1 and WT2 gene mutation including loss of heterozygosity (LOH) in WT cases [5,6] There is still lack of knowledge that how single nucleotide polymorphisms (SNP) of MTHFR gene interact with folate - metabolism and increase risk factor by modulating DNA synthesis in WT. Earlier study reveals that "stop codon" of transforming growth factor receptor (TGFβR1) and tumor suppressor gene (p53) play a significant role in tumorigenesis in different type of cancer. [7,8].

Methylenetetrahydrofolate reductase (MTHFR) gene is mapped on 1p36.3 chromosome and contains 2.0 kb coding region with eleven exons that encodes an enzyme concerned in folate metabolism pathway [9]. MTHFR is a crucial enzyme to regulate intracellular concentration of folate during de-novo synthesis of methionine and homocysteine after conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.

Folate is an essential component to transfer of one carbon unit in various biochemical pathways to regulate metabolic processes during DNA, RNA synthesis and repair mechanism. Thus, low folate concentration may alter DNA methylation results chromosomal breaks and disruption of DNA repair mechanism. MTHFR C677T allele is highly polymorphic in nature and the most common variant of nucleotide cytosine is substituted by thymidine (C→T) results alanine is replaced by valine due to point mutation. This mutations lead to affect enzymatic activity decrease up to 30 - 40% in differentiating cell results affecting DNA synthesis and increase "risk factor" in heterozygous condition in variety of diseases other than in cancer [9,10,11]. In literature, there is still lack of new predisposition gene variants modulate "risk factor" based on MTHFR gene polymorphism associated folate – metabolism in WT cases. Earlier study of the same group shows that spectrum of new gene variants including "stop codon" modulate functional activity of 3D protein structure between ligand (drug) and protein in WT cases [8]. Therefore, the present study has been designed with the aims to explore the mechanism of "risk factor" by incorporating new gene variants of MTHFR based on DNA sequencing (Sanger's) and also evaluate the (%) frequency of mutant "T" allele either in heterozygous or homozygous condition. Further, the 3D protein structure was predicted with help of bioinformatics tools to evaluate the functional activity after mutation between ligand (drug) and protein interaction. Hence, the present study become relevant to explore the mechanism of tumorigenesis by amalgamation of penetrance of new MTHFR variants causes dysfunction of folate - metabolism followed by increase of "risk factor" in cancer patients. Furthermore, the predicted helical structure of protein also validates the functional activity after mutation with protein- ligand (drug) interaction with folate - in WT cases.

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Materials and Methods

Blood samples (0.5ml) were collected in sterile EDTA vial from clinically diagnosed cases of WT with respect to age matched controls from the OPD of All India Institute of Medical Sciences and IGIMS Patna. The samples were stored at -20°C till further process for MTHFR C677T polymorphism and DNA sequencing.

Selection of Primers and RT PCR analysis of MTHFR C677T allele

MTHFR gene polymorphism analysis helps to assess the genetic heterogenecity and "risk factors" by using ARM- PCR in WT cases. This is highly sensitive, reliable method for SNP analysis and our interest to detect mutant alleles of MTHFR, based on Tm values to increase the specificity of specific primers (tetra plex). The primers were designed for genotyping of MTHFR C677T

(http://cedar.genetics.soton.ac.uk/public_html/primer1.html)

CAGTAGGGATAACCGTCCAATGGGGTTTCCCATAAACAGGAATGAAGTATGAACCCACGCTACCACCAGGATGAACCTGAGGACGTATTGCTGAGTGAAAGAAGTCAGTCGCAGCAGGCCGCACATGGTGTGTTTCATTTATGTGAACGTCCAGGCAAATCCACAGACACAGGTACAGCCACGTCGGAATTGTTTCATGTCGGTGCATGCCTTCACAAAGGAAAGCGGGTGGGTGGTTGCCTGGGGCCGGGGGCAGGGAGCATGAACTTCCTTCCACACAGGACCCCGCA 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 (12). These 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' – GCACTTGAAGGAGAAGGTGTCTGCGGGCGT-3'; MT MTHFR-C-polyG, 5'-GGCGGGCGGCCGGGAAAAGCTGCGTGATGATGAAATAGG-3'; MTHFR-cf, 5'-TGTCATCCCTATTGGCAGGTTACCCCAAA-3'; MTHFR-cr, 5' - CCATGTCGGTGCATGCCTTCACAAAG-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 (950C 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 analysed the data and reaction was terminated by cooling to 40°C. Melting curves (Tm) were constructed by lowering the temperature to 65°C and later increasing the temperature by 0.2oC/s to 98°C to measuring the change fluorescence consistently. After obtaining Tm values, RT- PCR, a plot was develop between fluorescence versus temperature (dF/dT) for the amplification of candidate gene products and finally measured at 530nm. PCR products were further analysed on agarose gel electrophoresis by evaluating the appearance of additional band of 105bp confirming heterozygosity (CT allele) in WT cases.

Analysis of 3D Structure by I-TASSER Server:

Bioinformatics techniques play an important role for the prediction of 3D helical structure of MTHFR and help to assess the functional activity between normal and mutated structure during ligand binding sites using I-TRASSER server (ITerative Threading Assembly Refinement).It identifies structural templates from the protein data bank by multiple threading approach LOMETS and atomic models were designed by interactive template-based fragment assembly. Recently, I-TASSER predict protein structure in community such as CASP7, CASP8, CASP9, CASP10, CASP11, CASP12, and CASP13 experiments [13]. Methotrexate, a common antineoplastic drug (antagonist of folate) is the reason to select during interaction between ligand (drug) and protein to evaluate the functional activity of the predicted protein structure. It act as an enzyme inhibitor during conversion of dihydrofolate to tetrahydrofolate in the presence of an enzyme reductase, resulting inhibit DNA synthesis. Molecular docking help to predict protein structure during ligand binding interaction based on free binding energy charges using auto dock tools software. AutoDock software was used for the calculation of Van der Waals and electrostatic forces for stable binding between non-polar hydrogen atoms and protein or drug (methotrexate) as model [14].

Statistical Analysis:

Chi square test (Two tailed) was used to calculate the level of significance ($p < 0.05$). The odd ratio (OR) was calculated with confidence interval (C.I) at 95% for MTHFR genotypes (CC, TT and CT) and individual frequency of allele (C/T) was calculated by Hardy Weinberg equilibrium to find relative "risk factor" between cases and control.

Results

In the present study, MTHFR C677T gene polymorphism analysis were carried out in WT Cases and aged match controls (n=40) using ARMS PCR with specific primers to evaluate the genetic heterogeneity.Figure-1A,B showing melt peak analysis of MTHFR C677T gene that shows shifting of Tm values 82.0 of wild type allele (CC) to 82.80 mutant allele (CT), suggesting (C→T) an increase of genetic heterozygosity in 20 % cases of WT . The same PCR product were further characterize to confirmed heterozygosity on 1.5% agarose gel (Lane - 4 & 7) that shows appearance of extra copy of band of 105 bp (arrow) of CT allele of MTHFR gene, while, lane- 1, 2, 3, 5, 6 & 8 showing 80% frequency of wild type (CC) allele in WT cases with respect to controls (lane-9, 10 & 11) as shown in figure-1C. However, there is lack of rare (TT) mutant allele in homozygous condition as observed in WT cases. Figure-1D showing significant variation in gene expression of MTHFR (C677) gene in all the cases of WT (bar 1-5) and controls (bar-6), GAPDH was used as housekeeping gene belong to homeobox region. The Hardy Weinberg Equilibrium was used to calculate individual frequency of MTHFR C677T allele and the frequency (0.9) of "C" allele was higher (0.1) than "T" allele. Statistical analysis showing significant (p=0.01730) association between 677 C→T allele variations and the value of confidence interval at 95% (0.0017- 0.5395) with odd ratio (0.030) between the cases and controls.

Figure-1 A B C & D: Showing MTHFR C677T gene polymorphism using ARMS PCR in Wilms tumors. Melt peak analysis of MTHFR gene showing shifting of Tm values from wild type (CC) allele (82.0) to 82.80 mutant allele (CT), suggesting (C→T) increase of heterozygosity (fig.1A, B).The findings were further confirmed by agarose gel electrophoresis where the same PCR product were used and Lane - 4 and 7 showing extra copy of band of 105 bp (arrow) of rare TT mutant allele in homozygous condition . The lane- 1, 2, 3, 5, 6 & 8 showing wild type (CC allele) in WT cases with respect to lane-9,10 & 11 act as controls (fig.1C). The findings of gene expression of MTHFR gene showing significant variations between cases of Wilms tumor (bar-1 to 8) with respect controls (fig.1D; bar-9).

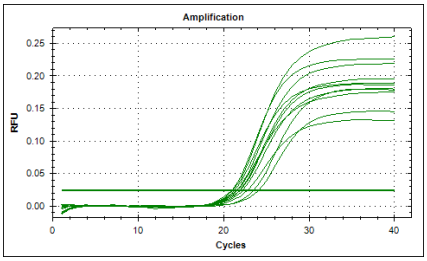

Figure-1A

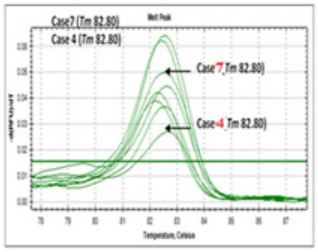

Figure-1B

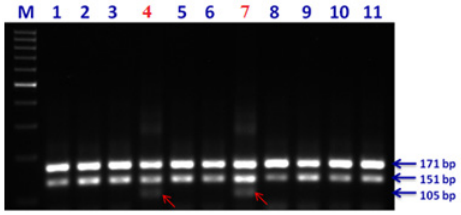

Figure-1C

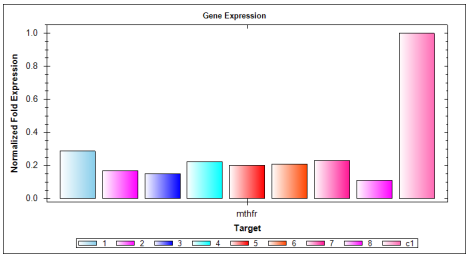

Figure-1D

Further, Sanger's sequencing was performed in selected cases (already shown WT1/WT2 gene mutation as well as loss heterozygosity) of WT along with controls. Figure-2A showing the details findings of the DNA sequencing which reveals large number of point mutation (substitution of nucleotide), deletion (loss) and further decode (http://blast.ncbi.nlm.nih) in to respective (translate) amino acids in three cases of WT. The first case of WT becomes quite interesting due to the appearance of large number of substitution of single nucleotide gene polymorphism (SNP) as well as deletion (loss) of nucleotides are as follows -1) cytosine is substitute by thymidine (C⟶T) followed by alanine is replaced by valine, 2) thymidine change into adenine (T⟶A) results isoleucine is replaced by asparagine, 3) cytosine is substitute by adenine (C⟶A) and isoleucine is replaced by asparagine, and 4) thymidine is substituted by cytosine (T⟶C), whereas the phenylalanine is replaced by serine. Beside, this the deletion (loss) of cytosine was also observed in the same case of WT. Second case showing the missence (point) mutation, where cytosine is substitute by thymidine (C⟶T) followed by change of amino acid alanine in to valine. Another case of WT showing substitution of thymidine into adenine (T⟶A) followed by change of amino acid isoleucine is replaced by asparagine. Further, the deletion (loss) of cytosine and thymidine nucleotide was also observed in mutated region after sequencing analysis and details are documented in table-1. The DNA sequencing data of the spectrum of MTHFR gene variants in the cases of WT after mutation decode their respective corresponding amino acids which shows that lysine is found to be the most common substitution of amino acid with respect to controls as depicted in supplementary table -1.

Figure-2 A,B & C: S(A) Details of DNA sequencing after alignment showing point mutation (SNPs) and deletion in different cases of Wilms tumor (https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_1370453188).(B-C) Showing normal MTHFR gene coded amino acids sequences (A) and mutated (B) using BLAST pair wise alignment.

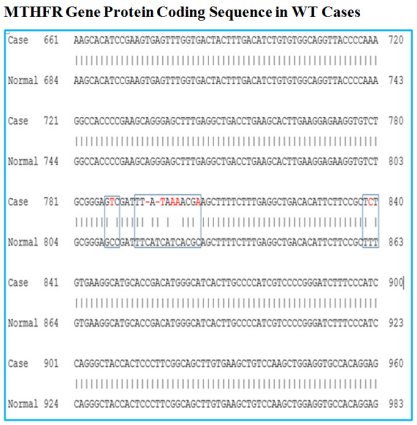

Figure-2A

Case No./Code	Transcriptional Event		Translational Event	
	Deletion	Substitution	Deletion	Substitution
352		C → T		Alanine → Valine
436	C	C → T T → A C → A C → A T → C	Phenylalanine	Isoleucine → Isoleucine Isoleucine → Lysine Glutamine → Lysine Phenylalanine → Serine
515	C and T	T → A	Phenylalanine and Isoleucine	Isoleucine → Asparagine

Table 1: Data of DNA sequencing of MTHFR gene variants showing the deletion and substitution after decoding (red text) with respective corresponding amino acids in the cases of Wilms' tumor and controls.

Phe	Ile	Ile	Gln	Phe	Ala	Amino acid
↓	↓	↓	↓	↓	↓	
TTC	ATC	ATC	CAG	TTT	GCC	Reference Sequence
↓	↓	↓	↓	↓	↓	
TT-	A-C/ATT	AAA/C	AAG	TCT	GTC	Mutated Sequence
	↓	↓	↓	↓	↓	
	Ile	Lys/Asn	Lys	Ser	Val	Mutated Amino acid

Supplementary Table-1 : Types of mutation of MTHFR gene and their corresponding amino acids between cases of Wilms tumor and controls.

Further protein sequence based on Sanger's sequencing data was predicted Uniprot (https://www.uniprot.org/blast/?about=Q13620[1-913]&key=Chain&id=PRO_0000393946), normal with mutated structure of MTHFR protein coded sequence was obtained from protein data bank (www.rcsb.org) after blast pairwise alignment. The changes of amino acid sequences are illustrated from normal MTHFR coded protein includes FII (phenylalanine, isoleucine and isoleucine) and FA (phenylalanine and alanine) is replaced by NKK (asparagine and lysine), whereas SV (serine and valine) (substitution) in mutated structure as shown in figure-2B and C , respectively. The 3D helical structure of MTHFR showing the substitution of amino acids after decoding from normal (white) to mutated (red) structure as shown in figure-3A Further, docking calculations were performed using Gasteiger charges method for the study of free binding energy between normal and truncated (mutated) protein to (ligand) amino acid residues using ball and stick model. After prediction of 3D normal protein structure showing the efficient binding to the respective amino acid with drug (methotrexate) between hydrophobic (polar) and van-dar wall force as shown in figure-3B. Similarly, after mutation and decode of respective amino acid changes the binding activity to the ligand (MTX) due to reduce the active sites of MTHFR protein (figure-3C). The findings were further confirmed by docking to calculate the free energy binding sites

which shows decreases from normal (-8.69kcal/mol) to mutated protein (-6.03 kcal/mol) structure (supplementary table 2A-B), suggesting free binding energies suggested a better interaction between drugs (MTX) and normal protein structure when compared to mutated (truncated) protein. The bindings between protein and ligand (drug) were further evaluated by construction of hydrogen bonding for parallel and ant paralleled protein sheaths. This approach is helpful for prediction of folding and flexibility with functional activity of the predicted structure as shown in figure-4 A-B. Thus on the basis of genomic data help to conclude good geometry for the prediction of 3D structure and function interaction binding energy and ligand (drug) .

Figure-2B

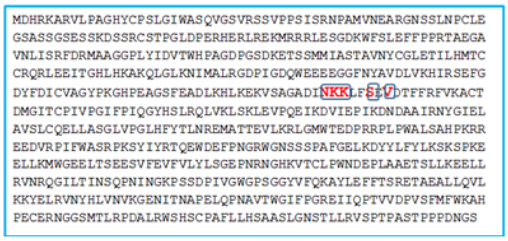

Figure-2C

Figure - 3 A,B & C:

(A) MTHFR 3D protein structure showing position of amino acid residues (white text) in normal while red text amino acid represents mutational residues in WT cases.

(B) Normal protein structure active site binding with ligand molecule in ball & stick model and interaction of amino acid residues of protein active site with MTX ligand binds with polar, hydrophobic and van-dal wall force.

(C) Mutated protein structure active site binding with ligand molecule in ball & stick model and interaction of amino acid residues of protein active site with MTX ligand binds with polar, hydrophobic and van-dal wall force.

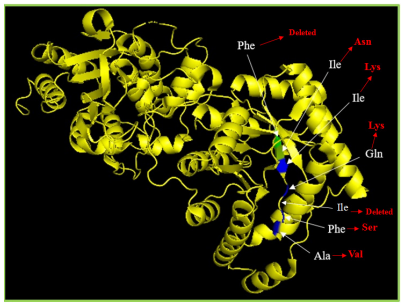

Figure-3A

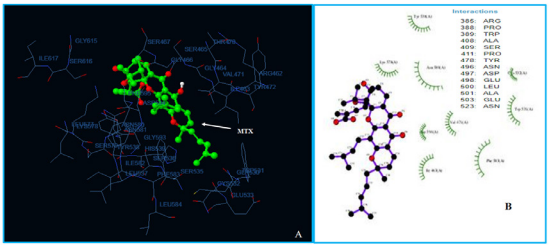

Figure-3B

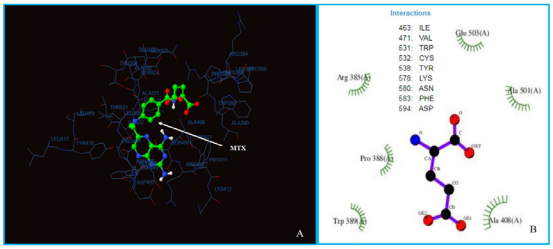

Figure-3C

Results Table

Rank	Est. Free Energy of Binding	Est. Inhibition Constant, Ki	vdW + Hbond + desolv Energy	Electrostatic Energy	Total Intermolec. Energy	Frequency	Interact. Surface
1.	-8.69 kcal/mol	423.68 nM	-9.80 kcal/mol	-0.30 kcal/mol	-10.10 kcal/mol	50%	1202.374
2.	-6.80 kcal/mol	10.43 uM	-8.36 kcal/mol	-0.40 kcal/mol	-8.76 kcal/mol	50%	1141.137

A

Results Table

Rank	Est. Free Energy of Binding	Est. Inhibition Constant, Ki	vdW + Hbond + desolv Energy	Electrostatic Energy	Total Intermolec. Energy	Frequency	Interact. Surface
1.	-6.03 kcal/mol	38.09 uM	-8.83 kcal/mol	+0.05 kcal/mol	-8.78 kcal/mol	20%	986.408
2.	-5.09 kcal/mol	185.16 uM	-8.74 kcal/mol	+0.68 kcal/mol	-8.06 kcal/mol	10%	1065.296
3.	-4.81 kcal/mol	297.86 uM	-7.89 kcal/mol	+0.26 kcal/mol	-7.64 kcal/mol	10%	1043.093
4.	-4.73 kcal/mol	340.40 uM	-7.27 kcal/mol	-0.29 kcal/mol	-7.56 kcal/mol	10%	908.091
5.	-3.86 kcal/mol	1.47 mM	-6.58 kcal/mol	+0.10 kcal/mol	-6.48 kcal/mol	10%	918.006
6.	-3.64 kcal/mol	2.14 mM	-6.17 kcal/mol	-0.02 kcal/mol	-6.19 kcal/mol	10%	872.572
7.	-3.38 kcal/mol	3.34 mM	-6.34 kcal/mol	+0.14 kcal/mol	-6.21 kcal/mol	10%	1050.884
8.	-3.10 kcal/mol	5.34 mM	-5.71 kcal/mol	+0.01 kcal/mol	-5.69 kcal/mol	20%	899.361

B

Supplementary Table 2: A & B : Illustrate the Mutated MTHFR protein binding sites with MTX ligand molecule and their decreasing energy where Rank 1 shown -6.03 kcal/mol is the best fit protein ligand structure (A), whereas in the case of normal protein interaction with MTX showing -6.80 kcal/mol (Rank 2) (B)

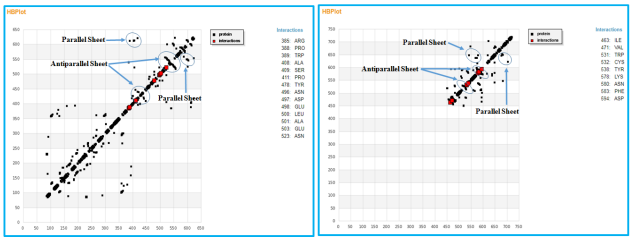

Figure 4 A&B: HB Plot representation of a protein containing parallel and antiparallel beta sheets in normal (A) and mutated protein (B).

Discussion

Genetics and epigenetic factors influencing folate-metabolism in cancer and the search of new predisposition factors (gene) becomes essential to explore tumorigenesis associated “risk factors” in WT. The etiopathology of WT is highly complex because of genetic heterogenicity in tumor cell population and interaction with unknown environmental factors [15]. Present study includes the involvement of new gene variants of MTHFR based on data obtained from Sanger’s DNA sequencing in WT cases and further prediction of 3D predicted protein structure after decoding with the help of bioinformatics techniques. Our recent study has shown that WT gene mutations and loss of heterozygosity (LOH) using microsatellite DNA markers (D11S935 and D11S904) increase genetic susceptibility in WT cases [16]. Further, the curiosity has been developed to find how MTHFR gene variations have been associated with increased

"risk factor" by modulating folate-metabolism in tumor. Folate, an important component for *de novo* synthesis of nucleotide during cellular proliferation and metabolism is highly sensitive towards environmental factors. MTHFR C677T allele is most common site of point mutation where nucleotide cytosine is substitute by thymidine (C→T) followed by change of amino acid alanine into valine in heterozygous condition. In tumor biology, the DNA synthesis of purine and pyrimidine are amino acid dependent process [17], and these amino acids play a significant role in folate metabolism. The gene expression is modulateted by arginine – derived polyamines during chromatin folding and glutamine act as an alternative source of energy in proliferating cancer cells during early tumor progression [18].

Earlier study of the same author reveals that MTHFR gene polymorphism play a significant role to increase "risk factor" in malignant [19, 20] and non-malignant conditions [10, 11, 21]. In the present study, the genetic heterogenecity were again observed due to substitution of nucleotide cytosine change into thymidine (C →T) followed by change of amino acid alanine in to valine up to 20% cases of WT in heterozygous condition. Interestingly, the same findings were observed during Sanger's sequencing, suggesting, that C→T substitution is one of the most common site of MTHFR gene mutation, beside this one case of WT shows the deletion or complete loss of nucleotide either "C" (cytosine) or "T" (thymidine) in the sequence confirming increase of genetic susceptibility and "risk factor" in heterozygous condition. However, our findings are similar to the findings of Gessler M et al. [22] who identified the similar frequency (20%) of WT gene mutation in sporadic cases of WT. Although, the mutation between WT and MTHFR genes are structurally different in nature, but the mode of action seems (functional) to be the same by inducing tumorigenesis and risk of the disease in WT. In the present study author fail to observe the rare mutation of MTHFR TT genotype in homozygous condition either due to small sample size or unknown environmental factors.

In tumor biology, dietary factors (amino acids) play a major role for the synthesis of macromolecules-proteins, lipids and nucleic acids. Amino acids provide both carbon and nitrogen for DNA synthesis, while purine biosynthesis requires aspartate, glycine and glutamine. Pyrimidine biosynthesis is simple than purine which requires only glutamine and aspartate. Single carbon unit derived from serine to glycine is essential component for thymidylate synthesis. The present study shows mutation of serine after decode of protein sequencing data of MTHFR gene, suggesting there might be changes in folate - cycle in WT . Thus it is not surprising that serine depletion results in glutathione reduction [23]. Although, folate plays an important protective role in the maintenance of genomic instability by providing methyl group during conversion of uracil to thymine for the synthesis of S-adenosylmethionine, required for the DNA methylation in mammals [24,25]. Serine and glycine are inter converted by serine hydroxymethyltranferase (SHMT1), is one of the key enzyme for folate mediated one carbon metabolism (26). Tetrahydrofolate (THF) serves as universal one carbon acceptor and may accept one carbon after conversion from serine to glycine or methionine to homocysteine. During oxidation of THF, exist in two different states- 5,10-methylene tetrahydrofolate is required for pyrimidine and 5-methyl tetrahydrofolate for purine biosynthesis [27]. Therefore, the discovery of predisposition of new MTHFR variants become essential to correlate with the 3D protein structure and also to assess the functional activity between protein and ligand (drug) binding sites. Authors also hypothesize that WT is an early embryonic tumor of kidney and believe that foetus might be exposed antenatally to the teratogen such as cyclophosphamide (antitumor drug) which interfere during normal organogenesis and induce tumor progression [28]. Thus, the functional genomics becomes an essential component of tumor biology to explore the mechanism of "risk factor" in WT cases. The exponential increase of "T" allele frequency followed by thermal instability of the enzyme might have altered folate-pool in the blood of the WT cases. During analysis of DNA sequencing data confirm the substitution of thymidine in to adenine (T →A) followed by change in corresponding amino acid isoleucine into lysine or asparagine, suggesting modulation of biosynthesis of non - essential amino acid such as asparagine, aspartic acid, cysteine, glutamic acid, glutamine and glycine [29]. However, authors hypothesize that these new variants of MTHFR gene modulate disequilibrium of intracellular folate pool, results hypomethylation and may activate oncogenes because of low folate level. Further, such alterations lead to an increase in the frequency of "T" allele with homozygous condition, resulting in higher "risk factor" of developing embryonic tumor due to penetrance of these gene variants translate truncated peptides which increase genetic susceptibility in the cases of WT [30].

Interestingly, the bioinformatics study after prediction of 3D protein helical structure of MTHFR shows significant interaction between ligand (methotrexate) binding site to protein as shown in figure 4 and 5.Furthermore,after mutation in MTHFR gene it functional activity might be change due to remodelling of the predicted 3D structure based on substitution of amino acid residues in the active sites, or by changing of free binding energy during interaction between polar and non-polar bonds to protein or drug (methotrexate). This might have altered the efficiency of drug, while use for the tumor patients during therapeutics regime. However, the present study has widened the scope of developing new strategies for incorporating amino acid as dietary supplements to prevent mutations by maintaining the folate pool in tumor cell.

Conclusion:

Present study identifies novel MTHFR variants based on Sanger's sequencing and high frequency (20%) of MTHFR CT genotypes that suggest increase of heterozygosity, followed by increase of "risk" of disease like WT. The prediction of 3D protein structure further suggests that significantly hampered folate metabolism either due to penetrance of mutant gene in the family of the proband or by modulating epigenetic unknown factor that induce tumorigenesis in the cases of WT. However, these new gene variants of MTHFR needs further validation in different group of population to correlate this finding in larger sample size inducing "risk factor".

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Authors Contribution:

AKS, for manuscript preparation, VK is for Clinical Diagnosis and provide sample of Wilms tumor, while AK and CKS for genetic analysis and bioinformatics analysis during research work

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