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Detection of Methylene Tetrahydrofolate Reductase Gene Polymorphism (C677T) in Sudanese Patients with Chronic Myeloid Leukemia

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ABSTRACT

Chronic myeloid leukaemia (CML) is a kind of cancer that affects the white blood cells and resort to progress slowly through many years. It's occur at any age, but is most common in older (60-65 years) of age. This is a cross sectional study aimed to detect MTHFR gene polymorphism (C677T) among Sudanese patients diagnosed with Chronic Myeloid Leukaemia and conducted at the research laboratory of the national center of neurological sciences (NCNS), Khartoum, Sudan.50 patients with Chronic Myeloid Leukemia (CML) diagnosed as BCR-ABL positive by RT-PCR used as a cases and 50 apparently healthy individuals as a control. A 5 ml of blood samples were collected in EDTA anticoagulant container for DNA Extraction and white blood cells count, hemoglobin level and platelets count. Genotyping of the MTHFR was carried out using PCR technique and the SNP (C677T) confirmed by sequencing a subset of samples. The results were analyzed using bioinformatics tools. The results showed; the most affected age group in the patients was 51-60 years followed by 41-50 years which constituted 32% and 30%, respectively. The hematological findings revealed that, the mean of TWBCs was 47.4, HB was 11.9 for patients, 7.2 and 14.1 respectively for control group (P = 0.000). PLT was 313.5 for patients and 287.5 for control group (P = 0.187). MTHFR gene was detected in the all patients (198pb) by the PCR, Sequence results were aligned with the reference sequence of MTHFR gene, the polymorphic C >T was found to be matched with the registered mutation in NCBI data base. This study provides the first evidence for associations of MTHFR gene polymorphism with the risk of chronic myeloid leukemia in Sudanese patients. The C >T genotype of the rs 677 polymorphism in MTHFR gene may have a promoting effect on chronic myeloid leukemia.

Keywords: Chronic myeloid leukaemia (CML), DNA, PCR, RT-PCR, MTHFR.

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INTRODUCTION

Chronic myeloid leukaemia (CML) is a kind of cancer that affects the white blood cells and resort to progress slowly through many years. It's occur at any age, but is most common in older (60-65 years) of age. It was first recognized as a clinical entity by John Hughes Bennett in the mid-1840s. It is also known as chronic myelocytic or chronic myelogenous leukemia [1].

Chronic myeloid leukaemia arises from a translocation between the BCR gene on chromosome 22 and the ABL gene on chromosome 9. This reciprocal translocation creates the Philadelphia chromosome (t 9;22) and the consequent formation of a unique BCR-ABL protein product. This protein has constitutive kinase activity that drives uncontrolled proliferation of hematopoietic stem cells, reduced apoptosis and generating genomic instability [2].

In the early stages CML usually have no symptoms and may only be picked up during tests done for other disease. No one is born with CML, it occurs when there is an injury to the DNA of a single bone marrow cell; scientists do not yet understand why the BCR-ABL gene that leads to CML is created in some people and not in others. In a small number of patients CML is occurred by exposure to very high doses of radiation, such as individuals treated for other cancers, like lymphoma.[3,4]

Incidence of overall leukemia in 2012 to be 351,965 cases (4.7 per 100,000). The incidence of overall leukemia in more developed regions in 2012 was estimated as 141,274 (7.2 per 100,000) versus an incidence of 210,691 (3.8 per 100,000) in less developed regions. Information on CML incidence and prevalence is scare, as CML is a rare disease.[5]

Methylene Tetrahydrofolate Reductase (MTHFR)

A key enzyme in homocysteine (Hcy) and folate metabolism, plays a role in DNA methylation and provision of nucleotides for DNA synthesis . MTHFR gene located on the short arm of chromosome 1 at 1p36.3. The cDNA sequence of this gene is 2.2kb long and is composed of 11 exons (103 to 432 bp) [6,7].

Mutations in the MTHFR gene may result in enzyme deficiency, low plasma folate levels and hyperhomocysteinemia, a risk factor for coronary and peripheral vascular obstructive events and neural tube defects. While, under situations of decreased MTHFR activity, more MTHFR substrate is available for purine and pyrimidine synthesis, which may prevent imbalances of nucleotide pools during DNA synthesis and potentially oncogenic alterations in DNA methylation [8].

There are two common genetic polymorphisms that have been shown to decrease the activity of MTHFR, which are the C677T mutation at codon 222 in exon 4 and the A1298C mutation at codon 429. Hyperhomocysteinemia is a consequence of single nucleotide polymorphisms (SNPs) in MTHFR 677 C>T that can cause homocysteine levels in the blood to increase, usually exceeding 15 µmol/L. MTHFR 677 C>T is the result of a missense mutation occurring at position 222, where alanine is substituted with valine. The mutation results in a thermolabile MTHFR that has lower enzyme activity at 37°C, leading to increased total plasma homocysteine [10]. Individuals with the MTHFR C677T TT genotype have been shown to have 30 percent in vitro MTHFR enzyme activity compared with the wild type, whereas those with the heterozygous (CT) genotype have been found to have 60 percent wild-type MTHFR enzyme activity [9].

The etiology of most types of leukemia remains unknown, leukemias commonly originate as a result of DNA translocations, inversions, or deletions in genes regulating blood cell development or homeostasis. Folate deficiency has been engaged in increasing the risk of chromosomal aberrations because it is associated with uracil miss incorporation into DNA. Indeed several studies reported that is the correlation between the presence of C677T polymorphism of the MTHFR gene and the risk of different leukemias. The aim of this study to investigate MTHFR gene polymorphism (C677T) might increase the risk for CML in Sudanese patients.

MATERIAL AND METHODS

This is study a cross sectional study, conducted at the research laboratory of the national center of neurological sciences (NCNS), Khartoum, Sudan during the period May 2019 to July 2019. Fifty patients diagnosed with CML were used as a case and fifty apparently healthy individuals were used as a control. Five ml of blood samples was collected in EDTA anticoagulant container for DNA Extraction and HB, platelets and white blood cells count.

The data was collected using pre-designed structural questionnaire, the demographic and clinical data concerning each participant was obtained from the registry data base office and the laboratory data included hematological results, Polymerase Chain Reaction findings and sequencing results. The study was approved by the ethical committee of the National Center for Neurological Sciences.

White blood cells Count

WBCs have been analyzed using electrical impedance method for cell counting Auto Hematology Analyzer (Mindray BC_3200).

Molecular Analysis

DNA extraction and purification:

Genomic DNA was isolated from peripheral blood leukocytes by the standard phenol chloroform extraction method; 10 ml of RCLB was added to 2.5 ml of blood then centrifuged for 5 minutes at 6000 rpm , this step was repeated until a clear pallet of white blood cell appeared, the supernatant was discarded and 2ml of WCLB, 1 ml of Guanidine Hydrochloride, 300 µl of ammonium acetate and 10 µl of proteinase K was added and incubated at 37ºC overnight. In the next day the mixture was cooled to room temperature and 2 ml of pre-chilled chloroform was added, the mixture vortex then centrifuges for 5 minutes at 6000 rpm after that, the upper layer containing DNA was collected to a new test tube and 10 ml of pre-chilled Absolute Ethanol was added and kept at -20ºC for 2 hours.

After incubation the precipitate of DNA was centrifuged for 10 minutes at 6000 rpm, after that the supernatant discharges then the pellet was washed in 4 ml of 70% ethanol then the pellet centrifuge for 10 minutes at 6000 rpm, after centrifugation the supernatant poure off and the pallet was left to dry overnight. The DNA is dissolve in 100 µl of ddH2O. Then vortex, incubates at 4ºC for 24 hours and store at -20ºC till use

MTHFR gene amplification

The MTHFR gene was amplified using PCR. The following primers sequences were used to obtain the fragment (198 bp);

Forwards: 5- TGAAGGAGAAGGTGTCTGCGGGA -3

Reverse: 5- AGGACGGTGCGGTGAGAGTG-3

In a PCR test tube, 14.5 µl ddH2O, 4 µl of master mix containing (1.5 buffer, nM MgCl2, 200µm of dNTPs and 0.5 units of Taq polymerase) were added, and then 0.75 µl from each primer and 2 µl of genomic DNA were added. The PCR was carried out using a commercial thermal cycler (SwiftTM MaxPro SWT-MXP-BLC-4). The amplification steps consisted of an initial 12 minutes of denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 seconds, the primers were annealed at 60 °C for 30 seconds, then the elongation period was 1 minute at 72 °C , the final elongation was adjusted for 5 minutes at 72 °C. The PCR amplification product was separated on a 3 % agarose gel and trans-illuminated with UV light with a 100-base-pair ladder.

Sequencing:

PCR products was sent for sequencing to Macrogen Europe Laboratory and BGI solutions Hong Kong Co. Ltd. Respectively.

SPSS13.0 statistical software (SPSS Inc., USA) was used for statistical analysis.

RESULTS

In the present study 50 of chronic myeloid leukemia patients were included. Among them, 35 were males, while 15 were females. In addition, 50 of apparently healthy individuals were selected as control group, 43 were males, while 7 were femal. (fig 1). The most affected age group in the patients was 51-60 years followed by 41-50 years which constituted 32% and 30%, respectively (Table 1).

The hematological findings revealed that, the mean of TWBCs was 47.4, HB was 11.9 for patients, 7.2 and 14.1 respectively for control group (P = 0.000)) (table 3). PLT

was 313.5 for patients and 287.5 for control group (P = 0.187) (table 3).

In the present study 198 bp of MTHFR gene was detected with gel electrophoresis after PCR (Figure 4). Thereafter, 198 bp of MTHFR gene was sequenced using Sanger sequencing methods.

Sequence results were aligned with the reference sequence of MTHFR gene (accession number NG 013351.1 in NCBI) (Figure 5). In this study the polymorphic C >T was found to be matched with the registered mutation in NCBI data base (Figure 6).

Table 1. Age groups of affected patients

Age groups	Frequency	Percent
Less than 30	3	6.0
30 – 40	11	22.0
41 – 50	15	30.0
51 – 60	16	32.0
More than 60	5	10.0
Total	50	100.0

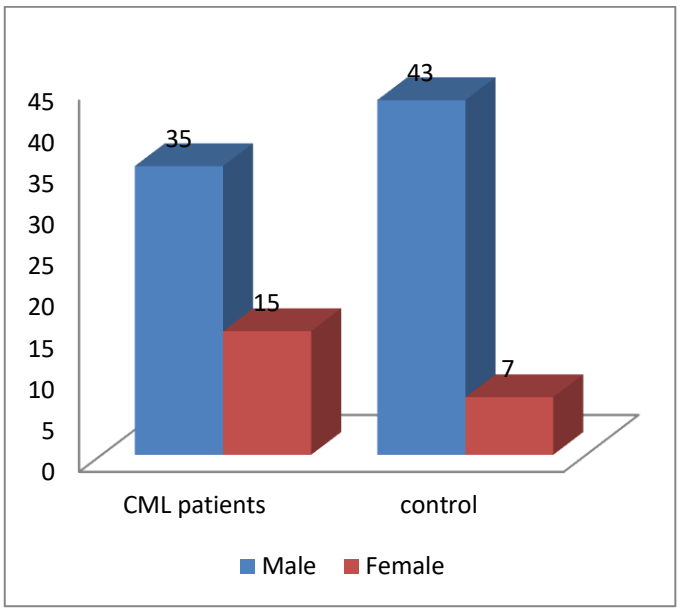


Fig (1): Frequency of gender among study population

Table (2): Descriptive Statistics of variables

Variables	Study population	N	Minimum	Maximum	Mean	Std. Deviation
Population	CML patients	50	22	70	47.4	10.3
	Control	50	18	58	37.9	12.9
TWBCs (×10⁹/l)	CML patients	50	5.4	237.0	71.1	62.1
	Control	4.2	10.9	7.2	2.2	4.2
Hemoglobin (g/dl)	CML patients	50	7.0	15.5	11.9	1.9
	Control	12.5	15.7	14.2	0.8	12.5
Platelet count (×10⁹/l)	CML patients	80	490	313.5	103.1	80
	Control	155	460	287.5	91.9	155

Table (3): Comparison white cells Count, hemoglobin and Platelet count between CML patients and controls.

Parameters	Population group (Mean ± SD)		P. value
	CML patients (n=50)	Normal individual (n=50)	
TWBCs (×10⁹/l)	71.1 ± 62.1	7.2 ± 2.1	0.000
Hemoglobin g\dl	11.9 ± 1.9	14.1 ± 0.9	0.000
Platelet count (×10⁹/l)	313.5 ± 103.1	287.5 ± 91.9	0.187

Table (4): Comparison of age, white cells Count, hemoglobin and Platelet count among gender of CML patients

Parameters	Gender (Mean ± SD)		P. value
	Male (n=35)	Female (n=15)	
TWBCs (×10⁹/l)	78.9 ± 66.7	52.7 ± 46.6	0.173
Hemoglobin (g\dl)	11.7 ± 2.0	12.2 ± 1.8	0.429
Platelet count (×10⁹/l)	299.5 ± 99.7	346.4 ± 106.9	0.142

Table (5): Correlation between white cells Count and Platelet count

Correlations			
		TWBCs	Platelet count
TWBCs	Pearson Correlation	1	-.089
	Sig. (2-tailed)		.540
	N	50	50
Platelet count	Pearson Correlation	-.089	1
	Sig. (2-tailed)	.540	
	N	50	50

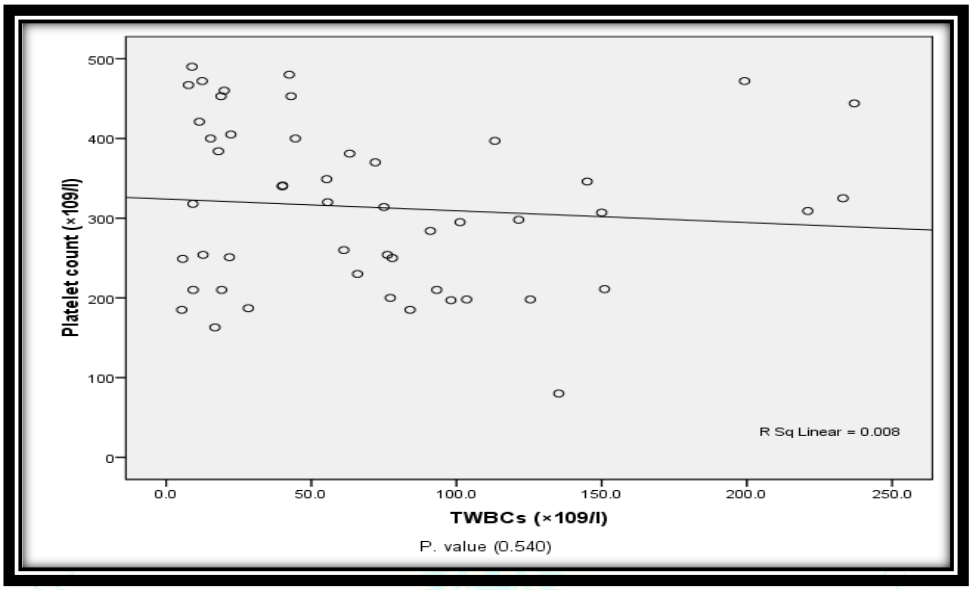


Fig (2): Correlation between white cells Count and Platelet count

Table (6): Correlation between white cells Count and hemoglobin

Correlations			
		TWBCs	Hemoglobin
TWBCs	Pearson Correlation	1	.076
	Sig. (2-tailed)		.602
	N	50	50
Hemoglobin	Pearson Correlation	.076	1
	Sig. (2-tailed)	.602	
	N	50	50

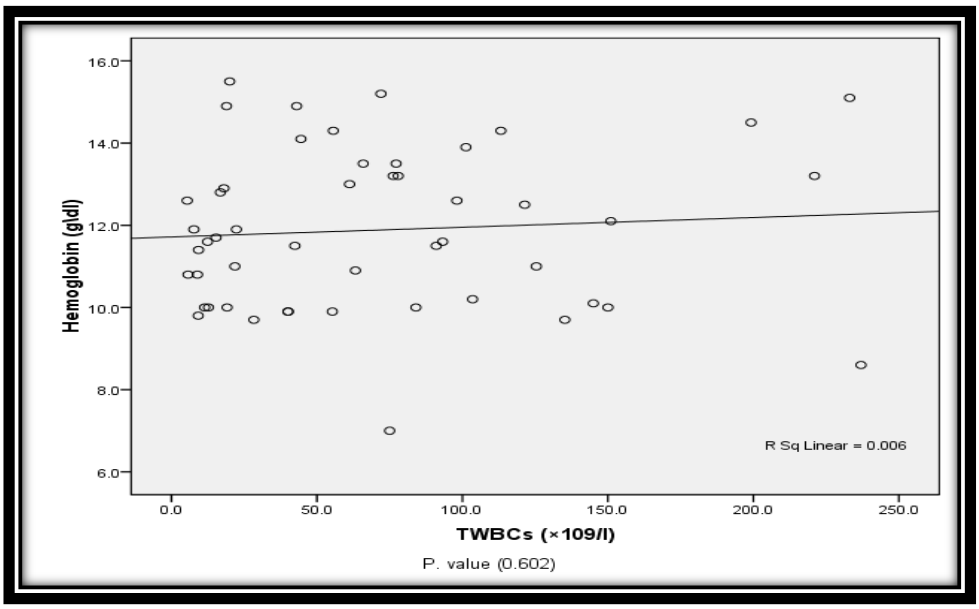
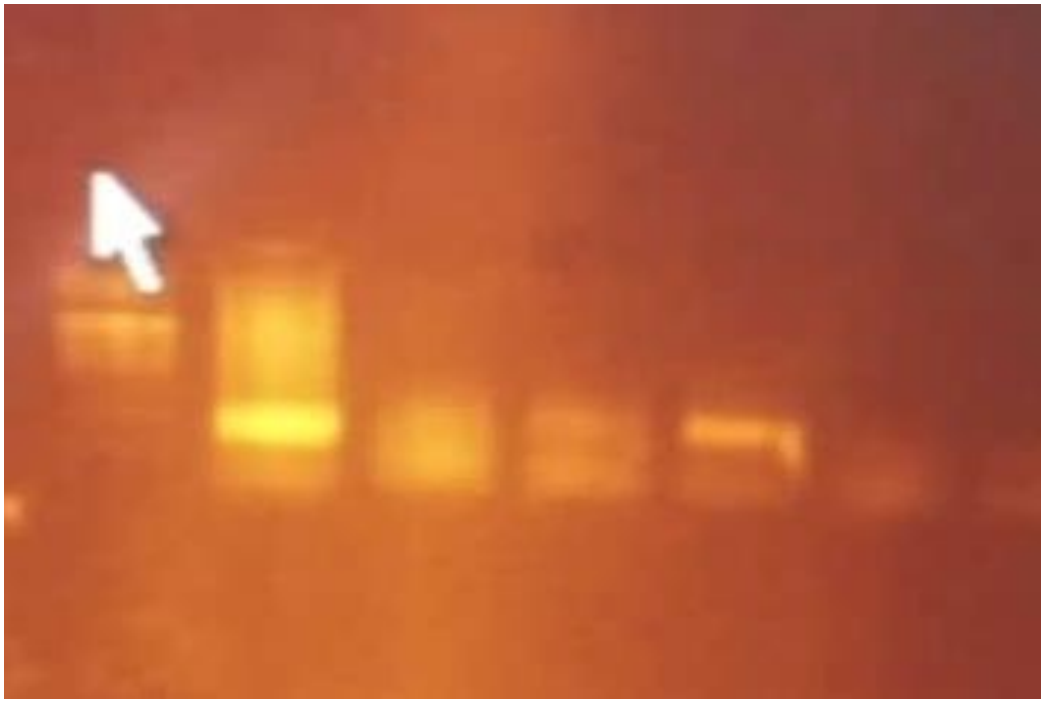


Fig (3) Correlation between white cells Count and hemoglobin



Fig(4): Gel electrophoresis for MTHFR gene

lane 1 (100 bp ladder), lane 2, 3 and 4 (198 bp product of IL-16 gene)

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Homo sapiens methylenetetrahydrofolate reductase (MTHFR), RefSeqGene (LRG_726) on chromosome 1	200	200	86%	1e-47	93.38%	NG_013351.1
Homo sapiens 5,10-methylenetetrahydrofolate reductase (NADPH) (MTHFR) gene, complete cds	200	200	86%	1e-47	93.38%	AY338232.1
Human DNA sequence from clone RP11-56N19 on chromosome 1, complete sequence	200	200	86%	1e-47	93.38%	AL953897.6
Homo sapiens SDS-stable vimentin-bound DNA fragment HEF42VIM21	200	200	86%	1e-47	93.38%	AJ297061.1
Rhesus Macaque BAC CH250-220G4 () complete sequence	156	156	86%	3e-34	87.50%	AC191444.6
Homo sapiens methylenetetrahydrofolate reductase (MTHFR) gene, intron 7	147	147	62%	2e-31	93.81%	AF080506.1
Homo sapiens methylenetetrahydrofolate reductase (MTHFR) gene, complete cds	135	135	53%	4e-28	95.35%	AH007464.3

Fig (5): MTHFR gene accession number

```
Query       19     CTTTACCTCTCAGGAG-A-CCAAACCGAAATGGTCACAAAGTGAGTGATGCTGG-AGTGGGGACCCTGGTTCATCCCCTGCCCCTGGACA  105
NG_013351.1 16708  ..........G....A.-........G.........................-.............................C.T  16795
AY338232.1  10672  ..........G....A.-........G.........................-.............................C.T  10759
AL953897.6  80420  ..........G....-.A........G.........................-.............................C.T  80333
AJ297061.1  684    ..........G....A.-........G.........................-.............................C.T  771
AC191444.6  37442  ..C........G....-.G........G..................C.C.....-GC.....T...................A...C.T  37529
AF080506.1  1                                  ..............-.............................C.T  49
AH007464.3  2558   ..........G....A.-........G.........................A...............................  2643

Query       106    GAcccccccTGCCCGCCAGGCTGCGGGGCTGTGACTTCCCCATCCTGT  153
NG_013351.1 16796  ......AG....AG.................................  16843
AY338232.1  10760  ......AG....AG.................................  10807
AL953897.6  80332  ......AG....AG.................................  80285
AJ297061.1  772    ......AG....AG.................................  819
AC191444.6  37530  ......AG....AG.............................C.....  37577
AF080506.1  50     ......AG....AG.................................  97
```

Fig 6: Multiple sequence alignment using Bio-Edit clustal W for samples with reference gene sequence of MTHFR gene

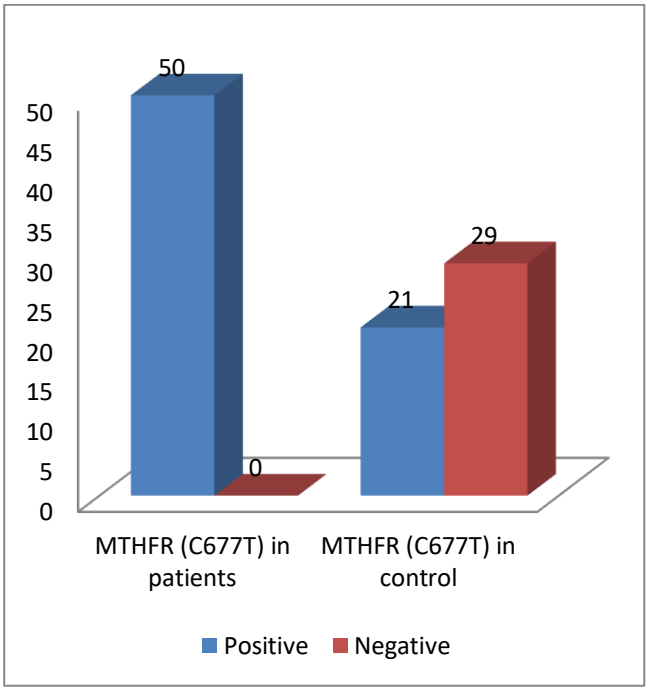


Fig (7): Results of MTHFR (C677T) among population

DISCUSSION

In the present study our results showed the most affected population were men and the most affected age group by CML was ranging between 51-60 years. One of the studies reported that; most cases of CML occur in adults. From 2007 to 2011, the median age at diagnosis for CML was 64 years a small number of children develop CML.[9]

The hematological findings of white blood cell count resulted that there was significant difference between patients and control group, the mean of WBCs was 47.4 for patients (P = 0.000).Also there was significant difference between patients and control group for the HB level the mean of HB was 11.7 for patients (P = 0.000). The same finding was reported by the other study; significantly related to high WBC counts at presentation: age<40 years, hemoglobin levels<120 g/L, (all P<0.05).[10] Regarding the platelets count our results reveled that; insignificant difference between patients and control group, the mean platelets was 313.5 for patients (P = 0.187). In contrast in the other studies; there was significant differences of platelet count with (P = 0.005). [10,11]

Methylenetetrahydrofolate Reductase (MTHFR) is the key enzyme in folate metabolism. It Irreversibly reduces 5, 10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyl-THF which is the main form of folate in serum that is involved in amino acid synthesis where it remethylates homocysteine into methionine at the expense of nucleotide synthesis. [12] There are two common genetic polymorphisms that have been shown to decrease the activity of MTHFR which are the C677T mutation at codon 222, and the A1298C mutation at codon 429.[13]

Since the MTHFR gene has an essential role in folate metabolism, genetic polymorphisms in this gene could alter the susceptibility to different cancers including hematological malignancies, as it was shown that an appropriate supply of folate is crucial specially for rapidly replicating cells such as hematopoietic cells. Indeed, several previous studies have noticed a change in cancer risk in individuals with mutated MTHFR genotypes. However, only a small number of reports have studied the effect of MTHFR gene alterations on the risk of developing leukemia. Those reports have focused on acute leukemias such as acute lymphoblastic leukemia (ALL), and acute myelogenous leukemia (AML), where they described a lower risk of ALL in individuals with mutated MTHFR alleles, but not in the case of AML.[14] Only very few studies addressed the association between MTHFR genotypes and the risk of CML.

In this study, the association between MTHFR polymorphism C677T and the risk of CML is thoroughly investigated in the Sudanese population for the first time. The results of this study showed that all the patients were having CT allele. One of the studies reported that; the difference in frequency of the heterozygous C677T CT genotype between CML patients and controls was not statistically significant [15]. Another study agreed that the C677T variants had no effect [16]. A study done in Jordanian population reported that; the present study demonstrates a clear association between mutated MTHFR genotypes and an increased risk of CML [17].

CONCLUSION

This study provides the first evidence for associations of *MTHFR* gene polymorphism with the risk of chronic myeloid leukemia in Sudanese patients. The CT genotype of the rs 677 polymorphism in MTHFR gene may have a promoting effect on chronic myeloid leukemia.

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