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GENETIC STUDY OF THE *METHYLENETETRAHYDROFOLATE REDUCTASE* POLYMORPHISMS IN IRAQI COLORECTAL PATIENTS

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ABSTRACT
Forty blood samples of CRC patients were collected, besides blood samples were taken from twenty apparently healthy persons and considered as control groups. Polymerase chain reaction restriction fragment length polymorphism (RFLP-PCR) technique was used to detect *MTHFR* C677T and A1298C polymorphisms, ELISA kit for homocysteine measurement was used. Genotype analysis of *MTHFR* A12987C revealed AA: 42.5%, AC: 30% and CC 27.5% among CRC cases and 25%, 40% and 35% among controls, while *MTHFR* C677T undetectable. Homocysteine measurement revealed significant increase ($p<0.05$) with CRC risk, 21.351±1.84 nmol/ml among patients and 6.97± 9.16 nmol/ml among controls. In conclusion homocysteine increase significantly among CRC patients. *MTHFR* 1298 A/C and C/C genotypes have no effect on CRC Iraqi patients, and this probably due to ethnic diversity in this study.

KEYWORDS: Genetic polymorphism - colorectal cancer - methylenetetrahydrofolate - genomic DNA, Iraq.

1- INTRODUCTION

Methylenetetrahydrofolate Reductase (MTHFR) is an important enzyme involved in folate metabolism, which effects DNA methylation and synthesis.[1,2,3] It converts 5, 10-methylenetetrahydrofolate irreversibly to 5-methyltetrahydrofolate which in turn donates its methyl group to homocysteine in the generation of Sadenosylmethionine (SAM). SAM is a major source of methyl groups used for DNA methylation. The MTHFR maintains circulating levels of folate and methionine, and prevents the accumulation of homocysteine.[1,2]

MTHFR C677T polymorphism results from C-to-T transition at nucleotide 677 in exon 4 resulting in an amino acid substitution of alanine to valine at amino acid 222.[4,5] The CC genotype of MTHFR gene at 677 is usually referred to as the wild type, CT as the heterozygous form, and TT as the homozygous variant. Subjects with the TT or CT genotype have lower levels of enzyme activity, 30% and 65%, respectively, relative to enzyme activity in subjects carrying the CC genotype.[4,6,7] Additionally, this single nucleotide polymorphism (SNP) decreases the thermal stability of this enzyme.[3] It has been reported that this substitution may lower levels of 5- methyltetrahydrofolate, and increase plasma homocysteine levels.[4,8,9] MTHFR A1298C polymorphism results from A-to-C transversion at nucleotide 1298 in exon 7 resulting in an amino acid substitution of alanine to glutamate at codon 429 of the protein.[10,11] The enzyme activity in vitro is decreased in homozygous variants (CC) and to a lesser extent, in heterozygotes compared with those without the variant.[10] Studies of A1298C and plasma folate and homocysteine are inconsistent.[11-15]

Enzyme activity in vitro for compound heterozygotes (i.e., heterozygotes for C677T and for A1298C) is unclear.[14]

Various studies discussed the effect of MTHFR gene SNPs (C677T and A1298C) on CRC patients at the areas surrounded Iraq[16,17,18,19], in addition, it was not noticed any research show the effect of C677T and A1298C mutations of the MTHFR gene on CRC Iraqi patients, so this study was carried out in order to determine the effects of Methylenetetrahydrofolate Reductase (MTHFR) polymorphisms and homocysteine levels on risk of colorectal cancer (CRC) among Iraqi patients.

2- MATERIALS AND METHODS

2.1 Specimens

This study was conducted at the department of Gastroenterology in Endoscopic Unit and Surgery at Baghdad and Al-yarmouk teaching hospitals for CRC diagnosis. The biochemical tests was performed at Alnadaer clinical laboratory (private laboratory) and the molecular work at AL-Nahrain Forensic DNA Research and Training Center from November 2013 to September 2014.

A sample of forty cases diagnosed CRC and hisopathologically approved with Dukes classification according to Astler and Coller, 1954.[20]

The sample included twenty two males and eighteen females with age ranged from 23 to 80 years, patients were excluded if they were suffering from: haemorrhoid, familial adenomatous polyposis, other tumors, chronic ulcerative colitis, diabetes mellitus, fatty liver, hepatic cirrhosis, metabolism syndrome, severe cardiovascular diseases and previous radiotherapy or chemotherapy.

A random sample of twenty apparently healthy persons were used as control groups and included eleven males and nine females with age ranged from 25 to 73 years.

In all sample cases and control group, a full history and complete physical examinations including body weight, height, smoking, tumor site, fertility, and body mass index were done by using a structured questionnaire.

Blood samples were collected from patients and control groups after an overnight fasting. Two and half milliliter of blood collected in EDTA anti-coagulant tubes, while another two and half milliliter of blood were collected in tubes without any anti-coagulant, these tubes were immediately placed on crushed ice, protected from light, then the blood samples were allowed to clot for 30 min and serum was collected.

The serum was again centrifuged at 4000 rpm at 4°C in cooling centrifuge and then the purified serum samples were used for evaluation of homocysteine and folic acid concentrations. EDTA tubes blood samples were kept at -20 then used for DNA extraction and molecular analysis.

Body Mass Index (BMI) was calculated according to (WHO, 2004).[21]

2.2 DNA Extraction

DNA was extracted from frozen EDTA blood tubes by using kit (Relia Prep™ Blood gDNA Miniprep System (A5081) according to the manufacturer's instructions.

2.3 MTHFR genotyping

Genotyping was performed for both polymorphisms, *C677T* and *A1298C* by using RFLP-PCR method, depending on Yousef *et al*. (2013)[19] and by using a thermal cycler (Veriti PCR- Applied Bio systems).

Primers, size of PCR product and annealing temperature shown in table (1).

Table 1: Primers, size of PCR product and annealing temperature (Yousef *et al*., 2013)[19]

polymorphism	Primers	PCR product size	Annealing temperature
MTHFR	F:5'TGAAGGAGAAGGTGTCTGCGGGA3'	198bp	65°C
C677T	R: 5'AGGACGGTGCGGTGAGAGTG3'		
MTHFR	F: 5' CAAGGAGGAGCTGCTGAAGA3'	128bp	58°C
A1298C	R: 5' CCACTCCAGCATCACTCACT3'		

The optimum PCR conditions were: 8 minutes of initial denaturation at 95C, followed by 40 cycles of 95C for 40 seconds, 63C for *C677T* while 58 C for 60 seconds, and 72C for 40 seconds, with a final extension at 72oC for 7 minutes.[22]

The PCR products of *C677T* and *A1298C* were separately digested with the *Hinf*I and *Mbo*II restriction enzymes (Promega, USA), respectively. Resulting fragments were visualized using ethidium bromide staining and 12% polyacrylamide (BIO BASIC, Canada) gel electrophoresis.

2.4 Measurement of Homocysteine concentration

Serum homocysteine concentration was measured by using ELISA Kit (CUSABIO, China) according to the manufacturer's instructions.

2.5 Statistical analysis

All data were analyzed using the Statistical Package for Social Science (SPSS), version 21 for windows.[23]

A two – way analysis of variance and paired-samples T – test was used to test the significance difference between the group means of patients and control group.

3. RESULTS AND DISCUSSION

A total forty CRC patients and twenty healthy controls were included in this study. The patients comprised 22 males (55%) and 18 females (45%) (M/F ratio=1.2) with age mean56.8±1.97. The controls consisted of 11 males (55%) and 9 females (45%) (M/F ratio=1.2) with age mean 52.9±2.97. Non-significant differences (p>0.05) have been observed within gender of CRC patients compared with control groups.

It has been noticed that the age of CRC patients have been distributed as follow, 4(10%) patients with age group ranged from 20 to 40 years, 16(40%) patients ranged from 40 to 60 years and 20 (50%) patients ranged from 60 to 80 years.

Distribution of age group and CRC in this study may explain that CRC increase with age group ranged from 40 to 80 years and this result were concordant with the finding of Iraqi cancer registery, (2010).[24]

BMI statistics analysis revealed, the patients slightly higher BMI mean 24.92±1.13 than controls 24.26±0.80, no significant difference between CRC patients and controls BMI (p>0.05). BMI values within normal range according to WHO, (2004).[21]

It has been noticed in many studies[25,26] that the obesity has been positively associated with the risk of CRC.

Classification of Duke's stage for CRC patients revealed, 1 (2.5%) A dukes stage, 4(10%) B dukes stage, 18(45%) C dukes stage and 17 (42.5%) D dukes stage.

Result of serum homocysteine measurement revealed significant increase (p<0.05) of CRC patients compared with control groups, where the mean of homocysteine level 21.35±1.85, 6.98±1.16 nmol/ml for CRC patients and control group respectively. It has been also noticed that there was interaction between the mean of serum homocystein level, age, sex and Duke's stage tend to be increased insignificantly (p>0.05) among CRC patients with age group ranged from (40 to 60 years) 20.35 ±2.42 nmol/ml and from (60 to 80 years) 19.373±1.01nmol/ml, and among males 20.5747±1.508 nmol/ml than females 18.65±1.66nmol/ml within CRC patients while increase among Duke's stage C 20.65±2.45 nmol/ml compared with Duke's stages A,B and D in figure (1).

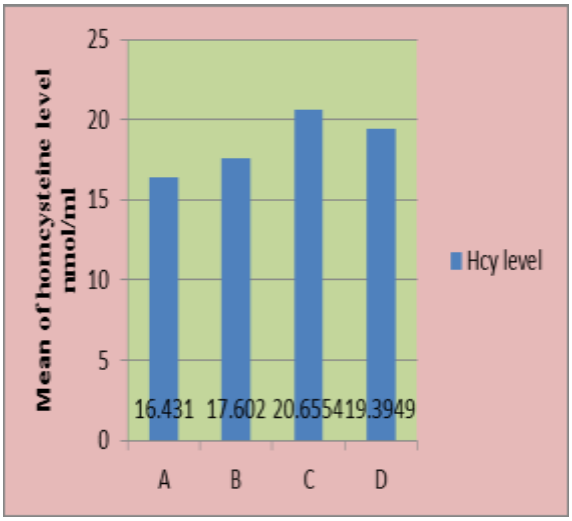

Figure 1: Distribution of homocysteine mean in serum and Dukes stages A, B, C and D for CRC patients.

The increased level of tHcy in serum of CRC patients is probably due to deficiency in folate, B12, B6, B2 and betaine which are considered as important factors in Hcy metabolism.

Furthermore, there are some common mutations in enzymes, transporters and receptors involved in Hcy metabolism that affect tHcy levels such as: cystathionine –synthase *(CBS);* methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (MTR*),* methionine synthase reductase *(MTRR)* and thymidylate synthase (*TS)*.[27,28]

It has been found that serum tHcy concentration increases with age (about 90% of CRC patients with age group ranged from 40 to 80 years) and this may be due to decrease in kidney functions, general slowdown of metabolism, increased intestinal malabsorption or insufficient nutritional supply.[29,30]

It was also noticed that men have higher serum tHcy level than women, and this may be due to the men have relatively more muscle mass and therefore produce more creatinine and when creatinine is formed Hcy is also formed.[29,30] The level of serum tHcy among Dukes stage revealed that C and D have higher than A and B Dukes stages and this probably due to C and D stages are advanced cancer, particularly with metastasis, can cause cancer cachexia, characterized by a symptomatic tetrad of involuntary weight loss, anorexia, muscle weakness, bleeding, body function and a feeling of poor health.[31]

Also uncontrolled growth and spread of abnormal cells of CRC[32], which probably due to nutrient depletion such as: folate, B12, B2 and B6, all these factor will effect on expression of the genes responsible of Hcy metabolism.

Many studies show agreement with result of this study which revealed the significant association of Hcy with CRC[33,34,35], while other studies revealed no significant association.[36,26]

PCR amplification the region containing the C677T mutation resulted single specific product of 198bp without primer dimers but with non-specific PCR products. *MTHFR* C677T SNP undetectable due to nonspecific PCR products that effect on *Hinf*I digestion.

PCR amplification the fragment containing the A1298C SNP (single nucleotide polymorphism) resulted single specific product of 128bp without primers dimers and non-specific products.

The 128 bp PCR products digested for 4hrs with *Mbo*ll and the digestion products were separated by polyacrylamide 12% gel electrophoresis.

Staining with EtBr and UV visualization resulted in the identification of the different genotypes. The digestion product of the *A1298C* revealed that there were 72 bp and 28 bp (2 fragments) for homozygous AA wild genotype, 28 bp, 72 bp, and 100 bp for heterozygous AC mutant genotype, and 100 bp and 28 bp for homozygous CC mutant genotype (Figure-2).

Results of analyzing *MTHFR* gene SNP among CRC patients and controls summarized in table (1).

The frequency of 1298A/A genotype *MTHFR* gene has 42.5% in CRC patients and 25% in controls while the frequency of 1298A/C genotype has 30% in CRC patients and 40% in controls and frequency of 1298 C/C

genotype has 27.5% in CRC patients and 35% in controls.

The frequency of 1298A allele has 57.5% in CRC patients and 45% in controls, while 1298 C allele has 42.5% in CRC patients and 55% in controls.

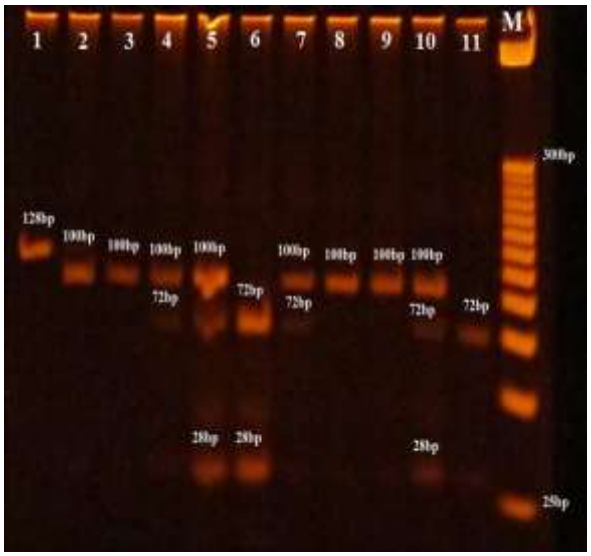

Figure 2: Ten samples were analyzed for *MTHFR* A1298C SNP using *Mbo*II based RFLP-PCR by electrophoresis on a 12% polyacrylamide gel (3V/cm) for 2hrs. (A/A: homozygous for wild genotype; C/A: heterozygous for mutant genotype; C/C, homozygous for mutant genotype). (Lane M: Marker, 25 bp DNA ladder; Lanes 6, 11 A/A genotype- (72bp-28bp two fragment); Lanes: 2,3,8,9 C/C genotype- (100bp-28bp); Lanes: 4, 5, 7, 10 A/C genotype-(100bp-72bp-28bp); Lane 1: negative control-(128bp).

Table 2: MTHFR A1298C genotypes and alleles frequencies among Iraqi patients with CRC compared with healthy controls.

Genotype/allele MTHFR A1298C	CRC n=40	Control n= 20
A/A	17(42.5%)	5(25%)
A/C	12(30%)	8(40%)
C/C	11(27.5%)	7(35%)
A	46(57.5%)	18(45%)
C	34(42.5%)	22(55%)

N: number of Sample, SNP: single nucleotide polymorphism.

It has not been specified the genotype of C677T in MTHFR gene and allele frequency in CRC patients group and control groups this has probably due to use primer which cause technical error responsible for appearance of non-specific PCR products.

The 128 bp PCR product contain two restriction site for Mboll, if no mutation lead to Mboll cut in both restriction site resulted the fragments have 72 bp and 28 bp (2 fragments) for homozygous A/A wild genotypes, while if mutation occurred lead to delete one of the restriction site and resulted the fragments have28 bp, 72 bp, and 100 bp for heterozygous A/C mutant genotypes and 100 bp and 28 bp for homozygous CC mutant genotypes that shown in figure (2).

Many studies discussed the *MTHFR* gene SNPs might be a risk factor for CRC in the surrounded area of Iraq[16,17,18,19], hence, in this investigated the prevalence of A1298C, one of two common *MTHFR* gene SNP in Iraqi population especially at Baghdad region to determine the relation of this SNP related with CRC.

Homozygous *MTHFR* 1298 A/A wild genotype more frequently occur among CRC patients (42.5%) higher than controls group (25%) this result concordant with Yousef *et al*. (2013)[19] result while disagree with (El-baz *et al*., 2010)[17] and Naghibalhossaini et al., (2010)[18] result. Heterozygous *MTHFR* 1298 A/C mutant genotype has frequency (30%) in CRC patients less than controls group (40%) this agreement with Yousef *et al*. (2013)[19] and disagreement with[17,18] results.

Homozygous *MTHFR* 1298 C/C mutant genotype has frequency (27.5%) in CRC patients less than controls group (35%) this results dissimilar with.[17,18,19]

4. CONCLUSION

In general, one can conclude that *MTHFR* 1298 A/C and C/C genotypes have no effect on CRC Iraqi patients, and this is probably due to ethnic diversity and regarding with the total serum homocysteine level, it has been noticed a significantly increased (p>0.05) among CRC patients compared with control group.

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