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Study of Association of MTHFR C677T Gene Polymorphism and Folate Level in Colorectal Cancer Patients

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ABSTRACT

Background: Genomic DNA replication, DNA repair, and folate metabolism are all impacted by methyltransferase (MTHFR), an important enzyme in folate metabolism. Among the many diseases associated with common functional variants of MTHFR, cancer is one.

Objective: to study the link between the C677T mutation of the MTHFR gene and the risk of colorectal cancer (CRC).

Patients and Methods: Included in this case-control study were thirty patients with a confirmed colorectal cancer (CRC) diagnosis by histopathology and twenty healthy controls who were matched for gender and age. In a laboratory setting, PCR-RFLP was used to determine the MTHFR C677T genotype and blood folic acid levels.

Results: In comparison to the healthy controls, people with colorectal cancer had far lower mean folic acid levels. A much lower percentage of CC genotype was found in people with colorectal cancer compared to healthy controls. No statistically significant trend was observed in the increasing frequency of CT and TT genotypes among CRC patients. A statistically significant increase in CRC relative to controls was indicated by a 2.5-fold higher T allele frequency in CRC patients compared to controls. The CC genotype had a greater folic acid level than the TT genotype, according to an analysis of MTHFR C677T genotypes in healthy controls and colorectal cancer patients.

Conclusion: colorectal carcinoma is associated with low folate status. MTHFR 677 T allele is associated with colorectal carcinoma in cases of low folate concentration. Adequate folate supplementation is required for people who carry the 677TT genotype to lower the risk of colorectal cancer occurrence. Further study with large number of studied groups is recommended to confirm this result.

Keywords: MTHFR C677T gene, Polymorphism, Folate level, Colorectal cancer.

INTRODUCTION

One in ten new cases of cancer is colorectal cancer, making it one of the most prevalent malignancies globally [1]. Generally speaking, colorectal cancer incidence is low in African and Asian countries compared to Western countries. The incidence of colorectal cancer, particularly colon cancer, has risen sharply in Japan, which has been named to the list of nations with the highest rates globally [1].

Epidemiological studies on colorectal cancer have shown a great deal of interest in folate and enzyme genetic variants in folate metabolism within the last decade. An important enzyme in folate metabolism, methyltetrahydrofolate reductase (MTHFR) is believed to have an effect on DNA methylation and synthesis [1].

Recent genetic research lends credence to the hypothesis that low folate levels in the body increase the risk of developing several malignancies, the most common of which is colon cancer. In addition, it appears that a high folate intake protects against cancer. A case in point of the interplay between environmental and genetic variables in the development of colorectal tumours is the association between MTHFR mutations and dietary habits [2].

The irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate is carried out by MTHFR in order to produce S-adenosylmethionine (SAM). When it comes to methylation, SAM is the methyl-group donor of choice for a wide variety of biological substrates. The promoters of tumour suppressor and DNA repair genes may experience aberrant methylation of CpG clusters if folate/methyl deficit causes an increase in DNA methyltransferase. Hypomethylation of DNA on a global scale is another potential consequence. The MTHFR substrate, 5,10-methylenetetrahydrofolate, is necessary for the conversion of deoxyuridylate to thymidylate. When there isn't enough thymidylate in the pool, DNA synthesis mutations such single- and double-strand breaks happen [2].

There are two functional variations known to exist in the MTHFR gene. The C677T and A1298C polymorphisms are two instances of such variations; the former alters the amino acid sequence at codon 222 to valine, and the latter alters the amino acid sequence at codon 429 from glutamate to alanine. Heterozygotes (CT) have 65% enzyme activity compared to 30% in those with the 677TT genotype (variant homozygotes). Those who carry the variant homozygote form of the MTHFR A1298C polymorphism—specifically, the 1298CC genotype—show a 40% decrease in enzyme activity when contrasted with those who carry the 1298AA genotype [2].

This study aimed to examine the relationship between folate levels and the MTHFR C677T gene variant in individuals with colorectal cancer.

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PATIENTS AND METHODS

Thirty patients undergoing treatment for colon cancer at Zagazig University Hospitals' Oncology Department were a part of this study. Biopsy or excised tumor histopathology was used to diagnose colon cancer. The patients group included 21 males and 9 females; their age ranged between 35 to 85 years. Twenty age and sex matched healthy persons were studied as a control group. Patients with other diseases that were known to be affected by MTHFR gene polymorphism were excluded from study e.g. cardiovascular disease, thromboembolism and other types of cancers.

All individuals under study were subjected to the following:

1- Full history taking including family history of cancer, smoking, vitamins supplementation and alcohol intake.
2- Serum folic acid determination using "Cobas e 411 analyzer" (Roche diagnostics GmbH, Mannheim, Germany).
3- MTHFR genotyping (C677T) of peripheral blood mononuclear cells by PCR-RFLP technique.

Samples:

Four ml of venous blood samples were withdrawn from each subject participating in this study. Blood was collected from patients before starting of chemotherapy. Samples were divided into: - 2.0 ml on plain vacutainer for folic acid determination, 2.0 ml on sterile EDTA vacutainer for MTHFR genotyping.

Folic acid determination:

Principle:

The folic acid assay makes use of a folate-specific natural folate binding protein (FBP) in a competitive test. There is a competition for binding sites on FBP (labelled with ruthenium complex) between the folate in the sample and the additional folate, which is labelled with biotin.

Reagents:

- The first pretreatment reagent for PT1 is 40 g/L of sodium 2-mercaptothanesulfonate (MESNA) with a pH of 5.5.
- Reagent 2 for PT2 pretreatment: 25 g/L of sodium hydroxide.
- The microparticles are streptavidin-coated and have a concentration of 0.72 mg/ml. They are also used as a preservative.
- Rh1 folate binding protein-R: 75 µg/L of ruthenium-labeled folate binding protein, 70 mmol/L of borate/phosphate/citrate buffer, pH 5.5, a preservative, and human serum albumin (as a stabilizer).
- Biotinylated folate (17µg/L), biotin 120µg/L, human serum albumin (stabilizer), 100 mmol/L of borate buffer, pH 9.0, and a preservative are all components of R2 folate-biotin.
- A calibration curve, created by the instrument itself using 2-point calibration, and a master curve, supplied by the reagent barcode, were used to calculate the results.

MTHFR genotyping:

Mononuclear cells were used for genomic DNA extraction. PCR was used to amplify the MTHFR gene from the isolated DNA. After that, the MTHFR C677T genotype was ascertained by digesting the amplified gene with a restriction enzyme. Lastly, the amplified products were exposed to agarose gel electrophoresis for visualization.

1-DNA extraction:

Axygen Bioscience's AxyPrep Blood Genomic DNA Miniprep kit was used for DNA extraction. The kit was located in Union City, CA, USA.

2- PCR amplification:

MTHFR amplification was performed according to **Frosst *et al.*** [3] after modification.

Principle:

Two primers carrying the target gene's complementary sequence are utilised to direct the amplification to a particular section of DNA. Because of their polarity and the fact that they hybridize with the target DNA's two strands, these two primers allow DNA polymerase to expand the sequences between them.

Reagents:

- Primers

Forward primer 5'-TGA AGG AGA AGG TGT CTG CGG GA-3'

Reverse primer 5'-AGG ACG GTG CGG TGA GAG TG-3'

The primers (Metabion international, Germany) were supplied in a lyophilized form and dissolved in 200 µl distilled water to prepare working primers with concentration of 0.5 µM.

After a 2-minute denaturation phase at 94°C, the thermal cycling process began with 40 cycles of the following: 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds. A 7-minute extension at 72°C followed. From Perkin-Elmer, USA, came the Gene Amp PCR system 2400, which was used for amplification.

3- Digestion of the amplified DNA by restriction enzymes:

Amplified DNA was digested with Hinf1 restriction enzyme (Jena Bioscince GmbH, Jena, Germany).

Restriction enzyme (Hinf1):

Cleaving sequence 5'…G ▼ANTC…3'

5'…CTNA ▲G…3'

Concentration: 10 u/µl.

Buffer supplied: 10 x B3 (including 10 x BSA).

4- Visualization of the PCR product:

An ethidium bromide 5% agarose gel was used for electrophoresis in order to see the digested DNA products. The electrophoresis was run for one hour at 90 volts. The filter portion of the UV transilluminator was covered with the gel. A single band at 198 bp is produced by homozygous wild type (677CC). Two bands, one at 175 bp and one at 23 bp, are produced by

the homozygous mutant (677TT). The 198-, 175-, and 23-base pair bands are all produced by the heterozygote (677CT) [3].

Ethical approval:

The Ethics Committee of Zagazig University Hospitals has given its approval to this study. Each participant completed a permission form when all information was received. Throughout its implementation, the study complied with the Helsinki Declaration.

Statistical analysis

The data were analysed using SPSS version 10. For quantitative data, the most common summary statistics were range, and mean± standard deviation. We utilised t-test to determine the difference in means between the two groups and analysis of variance (F-test) with least significant difference (LSD) test as a post-hoc test to compare multiple means. Frequency and relative percentage were used to display the qualitative data.

To assess the association between the variables, we computed a X^2-test or Fisher's exact result, the latter of which was advised when the expected cell size was less than 5. To assess the risk among patients compared to controls, Odd's Ratio and 95% CI were computed. Any p-value below 0.05 is deemed statistically significant.

RESULTS

Table (1) shows that there was no discernible difference between the control group and colorectal cancer patients when it comes to age, sex, smoking, or family history of the disease.

Table (1): Mean value of age, frequency of sex, family history of cancer, smoking and alcohol consumption in controls and CRC patients.

Parameter	**Controls n= 20** X ± SD No. (%)	**CRC patients n=30** X ± SD No. (%)	t. test	**P**
Age (years) Range	51.5 ± 9.38 (36-72)	57.13 ± 13.41 (36-82)	1.629	0.11
Sex Male Female	12 (60%) 8 (40%)	21 (70%) 9 (30%)	$\chi 2$=0.535	0.465
Family history -ve +ve	20 (100%) 0 (0%)	27 (90%) 3 (10%)	Fisher's exact test	0.265
Smoking -ve +ve	11 (55%) 9 (45%)	13 (43.3%) 17 (56.7%)	0.654	0.419
Alcohol -ve +ve	20 (100%) 0 (0%)	30 (100%) 0 (0%)	Can't be computed	
Multivitamins use -ve +ve	18 (90%) 2 (10%)	30 (100%) 0 (0%)	Fisher's exact test	0.155

Table (2) shows that the mean folic acid levels in colorectal cancer patients were much lower than those in controls.

Table (2): Mean folic acid levels in controls and CRC patients.

Parameter	**Controls (n=20) X ± SD (range)**	**CRC patients (n=30) X ± SD (range)**	**t. test**	**P**
Folic acid (ng/ml)	10.93 ± 2.58	6.44 ± 1.54	3.574	0.001

Table 3 shows that compared to healthy controls, patients with colorectal cancer had a substantially lower percentage of the CC genotype. While the frequency of CT and TT genotypes does rise in CRC patients, it was not yet statistically significant. The CC genotype has a 0.29 odds ratio for CRC formation, the CT genotype has a 2.43 odds ratio, and the TT genotype has a 2.92 odds ratio.

Table (3): Frequency of MTHFR genotypes in controls and CRC patients

Genotype	Controls N= 20 No. %	CRC Patients N= 30 No. %	OR (95% CI)	$\chi 2$	P
CC	12 60.0	9 30.0	0.29 (0.07-1.00)	4.43	0.03
CT	7 35.0	17 56.7	2.43 (0.65-9.28)	2.26	0.13
TT	1 5.0	4 13.3	2.92 (0.27-7.50)	Fisher's exact test	0.64

Table (4) shows that there was a significant increase in T allele frequency in CRC patients compared to controls, T allele occurs 2.5 folds more in CRC patients than in controls.

Table (4): Frequencies of MTHFR allele in controls and CRC patients.

Allele	Controls (n= 40) No. %	CRC Patients (n= 60) No. %	$\chi 2$	P	OR (95% CI)
T allele	9 22.5	35 58.3	12.507	<0.001	2.5 (1.0-6.0)
C allele	31 77.5	25 41.7			

Among controls there was one case of control subjects carrying TT genotype, CT and TT genotypes was considered as one group. Table (5) and figure (1) show that there was a significant decrease in folic acids levels in subjects carrying CT+TT genotypes compared to CC genotype. Among CRC cases the highest level of folic acid was in CC genotype followed by CT genotype and the lowest folic acid level was in TT genotype with significant difference between TT genotype and both CC, CT genotypes.

Table (5): Mean folic acid levels in different MTHFR genotypes in controls and CRC patients

Parameter	CC (n=12) X ± SD (range)	CT+TT (n=8) X ± SD (range)	t. test	P
Folic acid (ng/ml)	13.72 ± 3.31	6.73 ± 1.54	5.55	<0.001

CRC patients					
Parameter	**CC (n=9) X ± SD**	**CT (n=17) X ± SD**	**TT (n=4) X ± SD**	**F test**	**P**
Folic acid (ng/ml)	7.55 ± 1.74	6.53 ± 1.48	3.55 ±0.83	9.82	<0.001

	CC	CT
CT	> 0.05	
TT	< 0.001	< 0.01

Table (6) shows that there was a significant decrease in folic acid levels in T allele compared to C allele among controls.

Table (6): Mean folic acid levels in different MTHFR alleles among controls.

Parameter	T (n=9) X ± SD	C (n=31) X ± SD	t. test	P
Folic acid (ng/ml)	6.46 ± 1.51	12.22 ± 2.99	5.54	<0.001

Table (7) shows that there was a significant decrease in folic acid levels in T allele compared to C allele among CRC patients.

Table (7): Mean folic acid levels in different MTHFR alleles among CRC patients.

Parameter	T (n=35) X ± SD	C (n=25) X ± SD	t. test	P
Folic acid (ng/ml)	5.58 ± 1.27	7.06 ± 1.69	3.88	<0.001

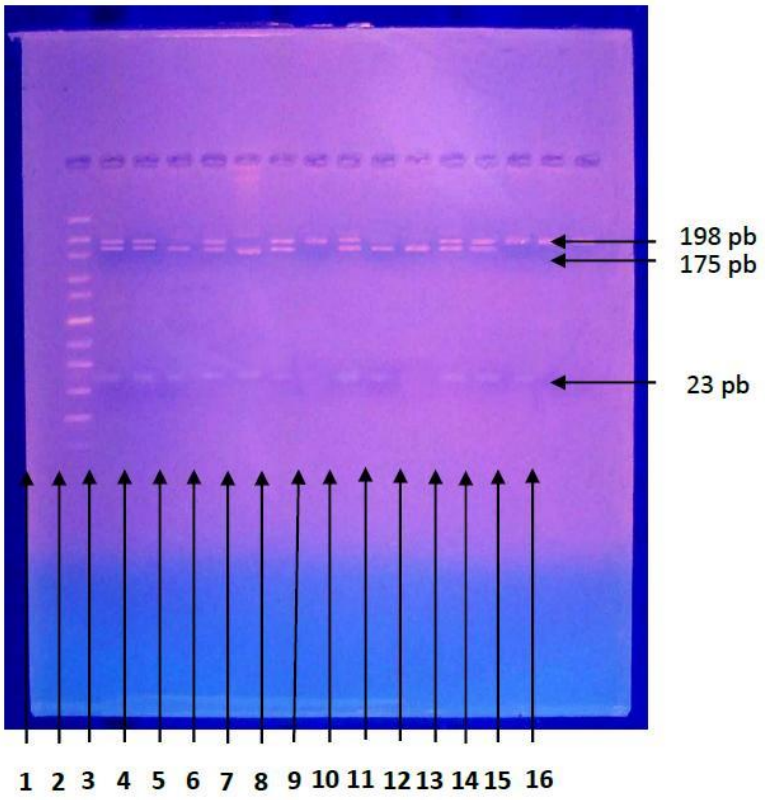

Figure (1): Gel electrophoresis of MTHFR gene polymorphism PCR products in CRC patients. [Lane (1) represents 10 bp DNA ladder; lane (2, 3, 5, 7, 9, 12, 13) represent CT genotype; Lane (8,14,15,16) represent CC genotype; Lane (4, 6, 10, 11) represent TT genotype].

DISCUSSION

Researchers have been interested in folate's possible preventive influence on cancer risk for the past ten years [4]. Folate is a methyl group provider during human de novo deoxynucleoside synthesis and intracellular methylation processes. Symptoms of low folate levels include DNA hypomethylation, chromosomal damage, DNA strand breakage, poor DNA repair, and uracil misincorporation during DNA synthesis [5]. Theoretically, variations in the sequence of genes that code for key enzymes in folate metabolism, such as MTHFR, may increase the likelihood of colorectal cancer [6].

At a pivotal point in the process of DNA synthesis, the enzyme MTHFR is involved in the methylation of proteins, lipids, and DNA [7]. C677T in exon 4 (Ala222Val) and A1298C in exon 7 (Glu429Ala) are two prevalent variant genotypes of the MTHFR gene that are linked to decreased enzyme activity [8]. Some research has linked mutations in the MTHFR gene, which affects DNA methylation and nucleotide synthesis, to an increased risk of colorectal cancer [9].

In this study, we aimed to investigate the association of MTHFR C677T gene polymorphism and folate level in colorectal cancer patients. In order to achieve this aim, 30 colorectal cancer patients and 20 healthy age and sex matched persons served as control were studied. The peripheral blood samples from all subjects were taken and subjected to folic acid determination and MTHFR C677T polymorphism detection using RFLP-PCR.

As people get older, their chances of having colorectal cancer also rise. Conditions such as inflammatory bowel illness, specific genetic disorders, and a personal or family history of cancer or colon polyps are additional risk factors. A person's risk for colon cancer increases when they don't exercise regularly, eat a diet high in fat and low in fibre, are overweight, drink alcohol, and smoke cigarettes [10].

The result of this study showed no significant difference regarding age, sex, family history of cancer, smoking, alcohol consumption and multivitamins use between CRC patients and controls (P> 0.05). Small sample size may attenuate our results regarding risk factors of cancer colon. There is another limitation in the study as we could not gather correct data from participants regarding alcohol consumption and multivitamins use since alcohol consumption is forbidden in our religion and due to insufficient education of most of the study participants.

Promthet *et al.* [11] and Wang *et al.* [12] showed that colon cancer risk was associated with alcohol consumption. **Hermann *et al.* [13]** and **Omata *et al.* [14]** also provide credence to the idea that white, Asian, and multiethnic people who drink moderately or not at all have a reduced risk of colon cancer. By contrast, **Wang *et al.* [15], Kim *et al.* [16] and Keku *et al.* [17]** showed in white, black, and Asian populations, there is no correlation between alcohol use and colon cancer.

Kennedy *et al.* [18] suggest that folate consumption is inversely related to the incidence of colorectal cancer.

In this study, there was a significant decrease in the mean folic acid levels in CRC patients compared to controls (F= 3.574 and P= 0.001).

This result goes with **Fujimori *et al.*** [19] who showed that to reduce the risk of colorectal adenomas, a serum folate level of approximately 8.0 ng/ml was required. In patients with serum folate levels above 8.0 ng/ml, there was no statistically significant difference in the risk of colorectal adenomas. Patients whose serum folate levels were lower than 8.0 ng/ml had a substantially higher risk of developing colorectal adenomas. A 50% greater risk was observed for males and a 23% increase for women. Those with the lowest folate and vitamin B6 consumption levels were the only ones in another study who had an increased risk [20].

The process of DNA methylation requires the active form of S-adenosyl-methionine (SAM), which in turn requires folic acid metabolites to convert homocysteine to methionine. Genetic instability and tumour growth are linked to decreased global DNA methylation, which can be caused by folic acid deficiency [21].

Our study revealed a significant decrease in MTHFR CC genotype in CRC patients compared to controls. Although there was increase in CT and TT genotypes among CRC patients; it does not reach the statistically significant level. The odds ratio of development of CRC was 0.29 in CC genotype, 2.43 in CT genotype and 2.92 in TT genotype. There was a significant increase in T allele frequency in CRC patients compared to controls. The risk of CRC was 2.5 folds higher in individuals carrying T allele.

A substantial association between CRC and the MTHFR 677T variant was discovered [22]. **Haghighi *et al.*** [23] and **Cao *et al.*** [24] also showed the MTHFR 677TT genotype has been linked to colorectal cancer. Some have hypothesised that this impact only manifests in patients with advanced colon cancer [24].

Alternatively, **Fernández-Peralta *et al.*** [25] found that controls were more likely to have the TT and CT genotypes of the MTHFR C677T than cases were, indicating that the 677T variant allele protects against CRC. The MTHFR 677T allele was associated with a reduced risk of colorectal cancer in another investigation that found similar results [26].

We found a significant difference between folic acid concentrations in different MTHFR genotypes in the control group, as the lowest folic acid concentrations were in CT and TT genotypes. Folic acid level was lower in healthy controls that carry the T allele.

These results go with **Nishio *et al.*** [27] who validated the finding that TT genotyped healthy subjects exhibited reduced serum folate levels, even after controlling for folate consumption in the diet. A decrease of almost 20% was observed on average.

One of the critical enzymes in the folate metabolism pathway is MTHFR. The principal form of folate that circulates in the blood is 5-methyltetrahydrofolate, which is processed by MTHFR. The substitution of valine for alanine occurs due to the C to T polymorphism at location 677 of the MTHFR gene. In homozygotes, the enzyme activity drops by 60% while in heterozygotes, it drops by 30% as a result of this substitution [28].

We found in this study that the highest level of folic acid among CRC patients was in CC genotype followed by CT genotype and the lowest folic acid level is in TT genotype with significant difference between TT genotype and both CC, CT genotypes. Folic acid concentration was significantly low in those patients who carry T allele. Under low folate conditions, these findings were linked to an elevated risk of colorectal cancer.

These results go with **Levine *et al.*** [29]**, Guerreiro *et al.*** [30] **and Kono and Chen** [31] who showed that individuals carrying the 677TT genotype were found to have a higher risk of colorectal adenomas when their dietary intake was low in folate, vitamin B12, vitamin B6, and methionine, and a decreased risk when their dietary intake was rich in these nutrients. **Le Marchand *et al.*** [32] showed that individuals with one or two MTHFR 677T alleles, especially those with folate deficiencies, have an increased risk of colorectal cancer (CRC) when they have microsatellite instability (MSI).

Methionine production relies on flavin-adenosine-dinucleotide (FAD), a cofactor that TT homozygotes are particularly susceptible to when their folate status is low, since the mutant enzyme requires a much higher quantity of folate than the healthy level to stabilize the binding of FAD [33]**.**

CONCLUSION

Colorectal carcinoma is associated with low folate status. MTHFR 677 T allele is associated with colorectal carcinoma in cases of low folate concentration. Adequate folate supplementation is required for people who carry the 677TT genotype to lower the risk of colorectal cancer occurrence. Further study with large number of participants is recommended to confirm this result.

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