Original Article

Association between Methylenetetrahydrofolate Reductase and Homocysteine Levels in Patients with Ovarian Cancer in Mosul City, Iraq

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Abstract

Introduction: The enzyme methylene tetrahydrofolate reductase (MTHFR) is the major regulator of homocysteine (HCY) levels in humans. Three missense mutations have a significant impact on the activity of this enzyme. **Aim:** The present study aimed to evaluate the association between HCY and MTHFR mutations in ovarian cancer. **Materials and Methods:** A cohort of 64 females suffering from ovarian cancer was investigated for resistin, malondialdehyde (MDA), glutathione (GSH), vitamin C (Vit. C) and HCY. MTHFR mutations were included in the study. **Results:** A significant increase in resistin and MDA while a significant decrease in GSH, Vit. C and HCY were detected in ovarian cancer patients. Also, a significant association between homocysteine and MTHFR was also observed in these patients. Interestingly, the results showed a correlation between ovarian cancer risk and MTHFR. **Conclusion:** MTHFR mutations were found to be associated with an increased risk of ovarian cancer in Mosul, Iraq.

Keywords: Ovarian cancer, MTHFR, resistin, malondialdehyde, vitamin C, glutathione, homocysteine.

Introduction

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme that regulates folate and homocysteine metabolism. Transformation of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate by reduction reaction produces the active form of folate that is required for remethylation of homocysteine to methionine.[1] Polymorphisms in the MTHFR gene are quite frequent.[2] MTHFR is a folate metabolic enzyme that controls methionine and homocysteine levels.[3] Folate is required for DNA methylation, DNA repair, and DNA and RNA synthesis in intracellular metabolic processes.[4] Folate may have a role in regulating the transfer of one carbon unit to a variety of metabolic events, which is complex in diseases including cancers of breast, ovary, gastric, colorectum and lung.[5] Despite several studies on the relationship between ovarian cancer and MTHFR BRCA1and BRCA2 polymorphisms, it is still not clear if MTHFR is its frequent risk factor or not. This might be due to several factors including race, origin and control group size. However, dependence found between MTHFR and unfavourable obstetrical/perinatal results has piqued people's curiosity.[6] Similarly, several studies have shown that A1298C,C677T and BRCA1 and 2 mutations in MTHFR have also been linked to recurrent miscarriage, retardation of intrauterine growth (IUGR), premature birth, hypertension, congenital abnormalities, fetal aneuploidies, and placenta elimination.[7]

Methionine synthase reductase (MTRR) and MTHFR, both are the primary enzymes which contribute to folic acid and methionine metabolism and affect the level of homocysteine in humans.[8,9] Three missense mutations in MTHFR i.e. A1298C, C677T and A66G have been found to have a significant impact on the actions of these enzymes.[10] The C677T mutation substitutes T for Cat position 677 and causes a substitution of valine for alanine, resulting in a thermolabile form with reduced activity. The replacement of base A for C at position 1298 causes glutamic acid to be replaced with alanine, decreasing enzyme activity. The MTRR A66G mutation disrupts the methionine/homocysteine cycle by converting isoleucine

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to methionine in the protein chain. Increased homocysteine levels have been linked to preeclampsia (high blood pressure during pregnancy), placental abruption (when the placenta separates from the uterus), and recurrent pregnancy loss. Homocysteine levels are highly significant in females who have a child with a defect of the neural tube (a problem with the brain or fetal spine).[11]

We hypothesized that MTHFR is a risk variable that needs other pathological circumstances to activate the chained biological processes which result in metabolic placental inflammation and intrauterine hypoxia. Thus, the present case-control study has been conducted to investigate the association of the *MTHFR* gene mutations with homocysteine in ovarian cancer patients. Exon 4 has been chosen in this study because of its highly efficiency in the detection of the strain.[12] Biochemical parameters has been included in this study as malondialdehyde concentrations (MDA) and GSH levels in serum because the association between this type of cancer and the state of free radicals and antioxidant in the body Sabuncu et al.[13] on the other hand vitamin C was measured since its role as reactive oxygen scavenger to prevent damage effect in the DNA.[14] Finally, resistin levels was determined since it promote the cell proliferation of the cancer as well as homocysteine levels which is considered as a tumor marker.

Materials and Methods

Study design

In this study, 64 patients with ovarian cancer and 40 healthy controls who visited the Nuclear Oncology and Medicine Hospital in Mosul, Iraq, between November 2019 and January 2020, were recruited. The patients were diagnosed by the physicians and the diagnosis was confirmed by specific tests. Among the patients, 33 had ages ranged from 30-40 years while the remaining patients were 40-50 years old.

Sampling

A total of 10 ml of venous blood was taken from all study participants. Then, the blood was transferred to plain tubes and centrifuged for 15 min at 3000 rpm to separate the sera which were later stored at -20°C.

Biochemical tests

The MDA concentrations were measured through thiobarbituric acid reactions while the thiobarbituric acid (TBARS) concentration and GSH in blood sera were estimated with Halliwell's[15] technique and the Mohammed and Kakey[16] method, respectively. The total concentration of vitamin C was determined by the 2,4-dinitrophenylhydrazine (DNPH) method,[17] following the sample deproteinization (60 g/L m-phosphoric acid; 2 mM EDTA). In this method, copper (II) sulfate oxidizes the Asc in the sample to DHA. Then, in an acidic environment, DHA starts the reaction with the DNPH reagent (1.88mmol/L of copper (II) sulfate, sulfuric acid, 4.05mol/L, 32.85mmol/L; of 2,4-dinitrophenylhydrazine, 90mmol/L and 1.88mmol/L of thiourea) to produce hydrazone complex with red color. Commonly available ELISA kits (MyBiosource, USA) were used to detect the concentrations of HCY and resistin in the serum of each participant. All of the reagents, samples, working standards and methods were produced according to the manufacturer's instructions. Thereafter, the optical density (OD) was measured at 450 nm using an ELISA reader (Bio-Tek). By comparing the OD of the samples with the standard curve, the concentrations of HCY and resistin in each sample were determined.

DNA extraction

Genomic DNA was extracted from whole blood according to the manufacturer's instructions using the DNA Blood Kit (Roche, Germany). The elute containing the DNA was incubated at room temperature for 5 minutes, and then centrifuged at 4,500 g for 2 minutes to extract highly concentrated DNA. The DNA was then dissolved in 200 microlitres of distilled water and stored at $^{-}$20°C until analysis.

Assessment of the quantity and quality of DNA

The extracted DNA was analyzed by using a spectrophotometer of NanoDrop at 260 nm. Then, the absorbance ratio was calculated at 260/280. No evidence of substantial band shearing or contamination was found.[18]

Primers of MTHFR gene

Table 1 lists the primers used in conventional PCR investigation for the MTHFR gene exon 4 region. Primer3 software version 0.4.0 was used to these primers. The chosen primer pair was verified against the BLAST database to ensure primer specificity and to check out the possibility of repeated sequences.

Table 1. Primers for MTHFR Exon 4

Primer name	Primer sequence	Primer length (bp)	Tm	GC%	Product length (bp)	Exon length (bp)
MTHFR (Exon 4\F)	5'-GTGACCAGTGGGAAGAGGAG-3'	20	59	60	102	111
MTHFR (Exon 4\R)	5'-CTGCCACACAGATGTCAAAG-3'	20	57	50	102	111

The Polymerase Chain Reaction (PCR) Conditions

Reaction mixture of100µl total volume was prepared with 10µl buffer, 5µl of 1U DNA polymerase, 5µl of .10pmol/l primer, 10µl of 200µmol/l dNTP and 10µl of 10ng genomic DNA. Finally, deionized distilled water was added to make the volume up to 100µl. The PCR was carried out on a Thermo cycler (^{3}Prime Thermal Cycler, UK), with following conditions i.e. denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec (30 cycles), annealing at 59°C for thirty seconds and elongation at 72°C for 30 minutes. The final elongation step took place at 72°C for 10 minutes

followed by cooling at 4°C. After the reaction, the PCR products were visualized on a 2% (w/v) agarose gel.

Statistical Analysis

Statistical analysis was done by using SPSS version 26.0 (SPSS Ltd., Surrey, UK). One-Way ANOVA was used to analyze the data and to resolve the discordance means, Duncan's novel multiple range test was applied.

Results

The comparison of biochemical parameters i.e. resistin, MDA, GSH, Vit. C and HCY among ovarian cancer patients and healthy controls are illustrated in Figure 1. The results revealed significant elevation of resistin and MDA concentrations in the patient groups (7.395±1.055 ng/l, 14.105± 1.76 µmol/L) respectively, than in the healthy control group (5.34±1.30 ng/l, 4.67±0.31 µmol/L) respectively. In contrast, levels of GSH, Vit. C and HCY were found lower in the ovarian cancer groups (5.32±0.96 µmol/L, 20.13±1.21 mmol/L, and 10.565±0.55 ng/ml), respectively than in the healthy control group (8.48±0.62 µmol/L, 33.61±6.036 mmol/L, and 17.62±2.10 ng/ml) respectively.

The patients' groups with respect to the age (30-39 years and 40-50 years) were compared and it was found that the resistin concentration in the prior group was higher (7.87±0.88 ng/l) than in the latter group (6.92±1.23 ng/l). Likewise, GSH, Vit. C and HCY ng/ml were also found in higher concentrations in 30-39 years old ovarian cancer group (5.89±0.90µmol/L, 20.45±1.33mmol/L, and 11.45±0.21ng/ml) respectively than in the other group (4.75±1.02µmol/L, 19.81±1.09mmol/L, and 9.68±0.89ng/ml) respectively (Figure 1). Moreover, the MDA level was found lower in patients 30-39 years old (11.43±2.71µmol/L) than in patients with age 40-50 years (16.78±0.81µmol/L). Among the 10 patient samples that were amplified, only 2 showed the presence of the MTHFR Exon 4 gene (Figure 2).

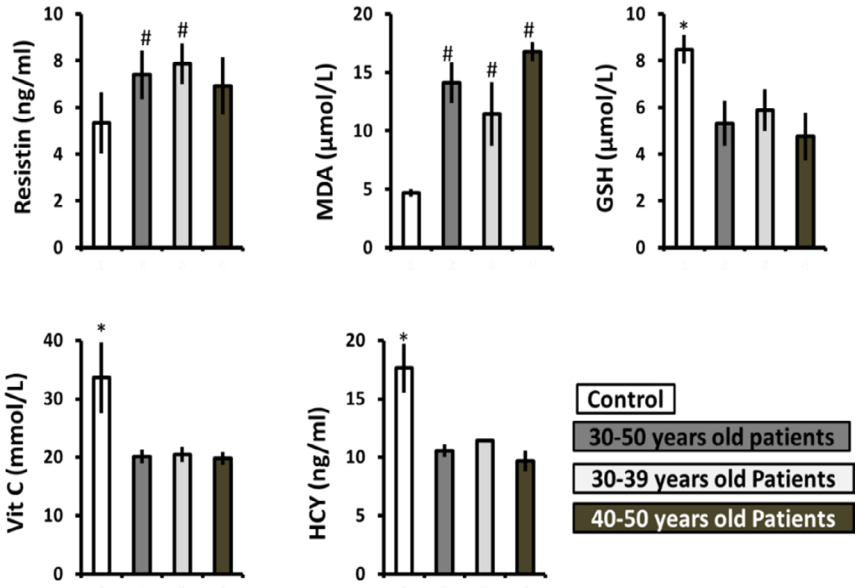

Figure 1. Cancer-related biochemical parameters detected in the serum of study participants. Data is expressed as mean±SD, * denotes the values were significantly higher (P<0.05) in the control group versus patient groups, and # denotes the values were significantly higher (P<0.05) in the patient groups versus control group. Data were analyzed with one-way ANOVA followed by Duncan's novel multiple-range test.

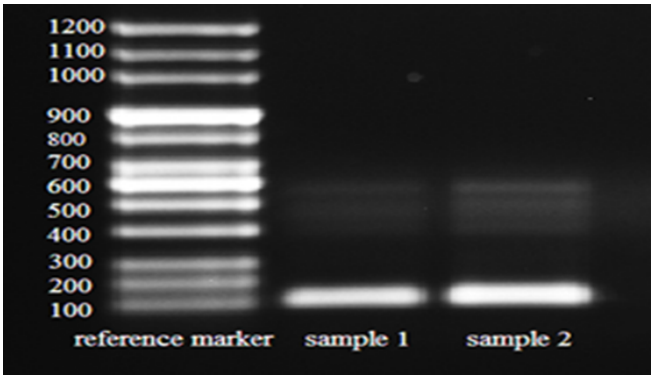

Figure 2. Amplified PCR products of MTHFR exon 4 gene at 102 bp size shown on a safe stained 2% agarose gel. Lan 1: 100bp DNA ladder 100bp, Lan 2-3: PCR products of 102bp band size.

DNA sequencing analysis was done with Geopiza software V1.4.0 to detect variations in the sequencing data of 2 samples for exon 4. The sequences of 2 samples (Query sequence) were compared with reference sequence (NM005957) in UCSC genome browser. After that, finch1.4.0 software (www.geospia.com) was used to double-check the results. Two mutations were found in exon 4 i.e. Glu (784) deletion (A) and Pro (777) deletion (C) in patient 1 (30-39 years) (Figure 3) and Asp (10877) and Lys (10844) deletion (G) in patient 2 (40-50 years) (Figure 4).

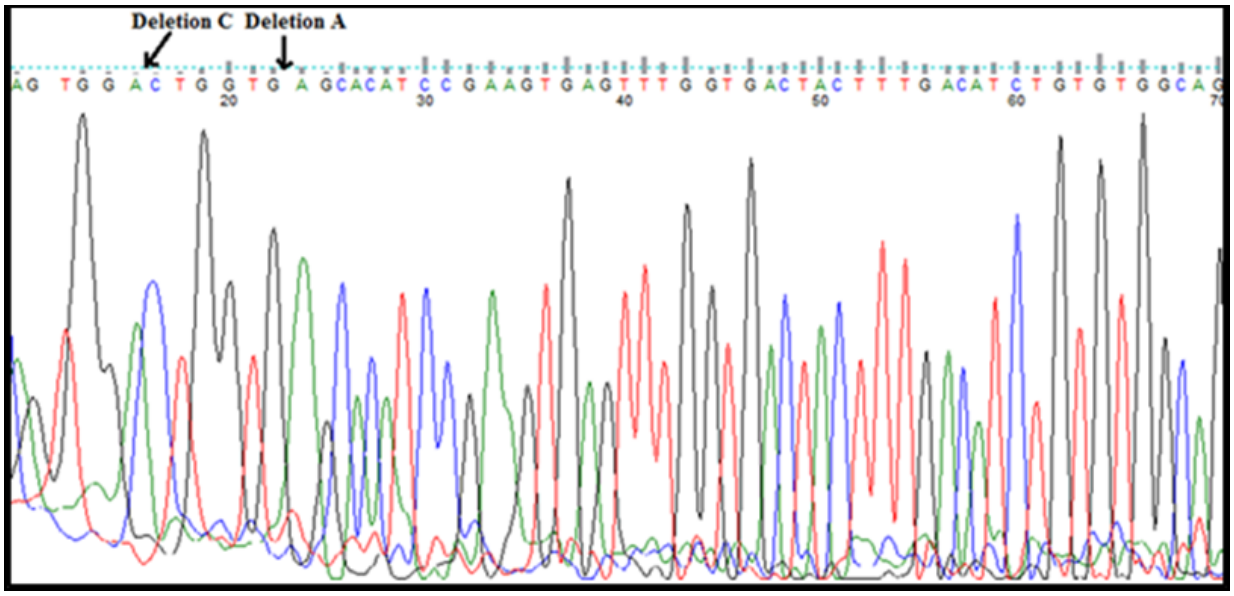

Figure 3. Sequence result of MTHFR/gene exon 4 in patient 1 (30-39 years)

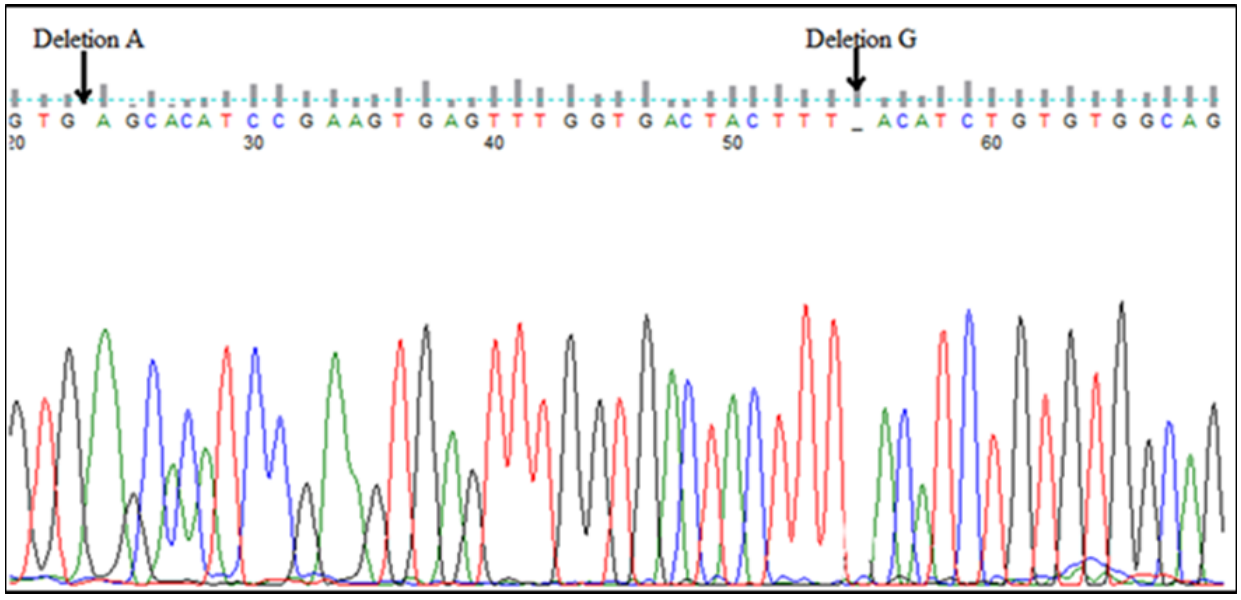

Figure 4. Sequence result of MTHFR/gene exon 4 in patient 2 (40-50 years)

Discussion

MTHFR is an enzyme that converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is necessary for the production of methionine from homocysteine remethylation. S-adenosylmethionine is formed after methionine is digested. As a result, any disruption in the process, whether due to a lack of cobalamin/folate or an MTHFR deficit, can lead to an accumulation of homocysteine and altered methylation patterns.[9] In the present study, we identified that two homocysteine-related gene mutations were associated with an increased risk of ovarian cancer and that the relationships were mediated by homocysteine levels. Homocysteine is a protein produced by the body that should be found in low amounts in the blood.[19] Homocysteine levels in humans are primarily influenced by folic acid and methionine metabolism,[8] however,

MTHFR and MTRR are the most important enzymes which deal with this condition.[9] Homocysteine elevation is associated with a variety of illnesses including ovarian cancer and cardiovascular disease.[20] In accordance with other studies, the current study revealed that there was a correlation between homocysteine-related mutations and ovarian cancer.[21]

Several clinical and experimental investigations have established the association between obesity and cancer, demonstrating that obese individuals are more susceptible to developing certain cancers.[22] Resistin may act as a metabolic regulation mediator, especially glucose homeostasis. It can also act as an adipogenesis regulator and a modulator in the inflammatory response.[23] There are a limited number of studies on the correlation between resistin and cancer.[24] In this study, the level of resistin was found to be higher in females with ovarian cancer than in healthy controls.

The present study found lower GSH levels in women with ovarian cancer than in healthy women. Given the critical function of GSH in ovarian cancer chemoresistance, attempts have already been made to counteract its effects on cancer cells. Different methods have been described thus far, such as blocking GSH production, utilizing GSH mimics, or medicines that target protein S-glutathionylation. However, this result is incompatible with results of a previous study which found that women with ovarian cancer had considerably elevated lipid peroxidation.[13] Owing to its function as an electron donor, ascorbic acid supports a variety of critical biological processes.[14] However, in clinical investigations and clinical practice, the status of vitamin in cancer patients is commonly neglected. The mean plasma vitamin C levels in these patients are consistently lower than in healthy controls, according to several studies.[25]

The spectrophotometric assessment of samples revealed that ovarian cancer patients had greater MDA levels than healthy people and similar findings have also been reported in other types of cancers.[26,27] High levels of MDA are associated with an elevation in oxidative stress experienced by cancer patients. The increasing levels of reactive oxygen species (ROS) and free radicals destroy the cellular components and more elevation in antioxidant concentrations occur.[28,29] MDA is one of the most well-studied lipid peroxidation products and is considered to be a tumor promoter and co-carcinogen due to its strong cytotoxicity and inhibitory impact on antioxidant enzymes.[29] The MDA level in the blood is a reflection of the cell damage caused by free radicals. It has a stronger cytotoxic and inhibitory impact on several defensive enzymes, causing carcinogenicity and tumour formation.[30,31] MDA levels are significantly elevated in a variety of cancers, including lung cancer, breast cancer, oral cavity cancer, oropharyngeal cancer, liver cancer, gastric cancer, colorectal cancer, ovarian cancer, cervix cancer, renal cancer, and prostate cancer.[32]

Conclusion

The MTHFR gene mutations and anomalies in homocysteine/methionine metabolism have been shown to correlate to a serious risk of ovarian cancer. Moreover, impaired nucleotide and DNA synthesis, which can increase the incidence of ovarian cancer, are also caused by the same mechanisms.

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Adherence to Ethical Standards

The study was approved by the College of Education for Pure Sciences-University of Mosul (Approval Letter 4S/226 on 10.11.2021).

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Conflict of Interest

The authors declare no conflict of interest concerned in the present study.

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