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5, 10-methylenetetrahydrofolate reductase C677T missense mutation is involved in recurrent pregnancy losses in Egyptian women.

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ABSTRACT

Objectives: Thrombosis usually associated with many mutations, the condition which predisposed for recurrent pregnancy losses in women. In the present study we aimed to test the link between recurrent pregnancy losses and C677T missense mutation of 5, 10-methylenetetrahydrofolate reductase in Egyptian women. **Methods:** In the present study we tested the methylenetetrahydrofolate reductase (MTHFR) C677T gene mutation, MTHFR activities in placental tissues and serum homocysteine level in Egyptian women with a history of recurrent pregnancy losses .The study included 150 subjects of a history of more than one recurrent pregnancy loss matched with 100 normal subjects with a history of normal delivery with no complications. **Results:** MTHFR C677T mutation was detected in (45.4%) of patients and in (4%) of control. Homozygous with mutant alleles were detected only among patients. The lowest placental MTHFR activities were detected among patients with heterozygous mutations, a significant increase in the plasma homocysteine is observed in all patients with MTHFR mutation compared with control. **Conclusion:** MTHFR C677T mutation is significantly involved in RPLs in Egyptian women.

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INTRODUCTION

Methylenetetrahydrofolate reductase (MTHFR) [5-methylTHF]: (acceptor) oxidoreductase, [EC 1.7.99.5]. MTHFR is considered a key enzyme in the one-carbon

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metabolism, because it is responsible for the irreversible conversion of 5, 10-methylenetetrahydrofolate to 5-methylTHF, which serves as methyl donor for methionine, precursor of S-adenosylmethionine (SAM). Not only is this pathway important for methyl group synthesis but the lack of methyl group production results in an intracellular accumulation of S-adenosylhomocysteine (SAH), as well as an increase in plasma of total homocysteine levels (Hcy) [(1)]. A common missense mutation was identified in the MTHFR gene, the C to T substitution at position 677; that encodes for a thermo labile variant with reduced enzymatic function [(2)]. The mutant T allele has been shown to be associated with higher levels of Hcy. The relationship between the MTHFR polymorphism and plasma folate values has been implicated as the likely link between the C677T polymorphism and cardiovascular disease [(3, 4)], as well as neural tube defects [(5, 6)]. Consistent with the concept that (TT) homozygosity reduces the in vivo availability of 5-methylTHF also the distribution of different co enzymatic forms of folate is altered in (TT) homozygotes [(7)]. The red blood cells (RBCs) of the (TT) homozygous mutants have diminished proportions of methylated tetrahydrofolates, as well as variable amounts of formylated folate. In contrast, cells from the wild-type individuals contain exclusively methylated folate derivatives [(7)]. Usually Subjects with hyperhomocysteinemia have a twofold to threefold increase in risk of developing cardiovascular disease or venous thrombosis [(8)]. Venous thrombosis, including deep-vein thrombosis and pulmonary embolism, is an important cause of morbidity and mortality, particularly in older people [(9)]. Most cases of venous thrombosis arise due to prolonged immobilization, major surgery, trauma or cancer, but genetic or acquired hemostatic abnormalities, including elevated plasma homocysteine levels, have also been implicated [(10)]. The initial epidemiological evidence that examined the association between homocysteine and venous thrombosis was derived from retrospective case–control studies (in which blood for homocysteine measurements was collected after onset of thrombotic events in cases) [(11-15)], but it was not possible to ascertain whether the higher homocysteine levels caused the thrombotic event or was a consequence of it. Subsequently, prospective studies (in which blood for Hcy measurements was collected before the onset of the thrombotic events) appeared to confirm these findings, but the weaker results raised questions about causality [(16-18)]. To the best of our knowledge there are little data explaining the role of MTHFR mutation in the incidence of RPLs. The aim of the present study is to examine the involvement MTHFR C677T missense mutation in the incidence of venous thrombosis and RPLs in Egyptian women.

Materials and Methods

Patients: - A case control study was used to evaluate the involvement of MTHFR C677T gene mutation in incidence of RPLs in Egyptian women. The patients group include One hundred and fifty (N= 150) women with a history of more than one adverse recurrent pregnancy losses who were admitted in Obstetrics and gynecology department in Kasr El Aini hospital, Cairo University during the period march 2010 – April 2012. Subjects were selected in consultation to include only women who are apparent healthy and did not suffer from any complications leading to adverse pregnancy outcome. The Control group include one hundred women (N=100) with no history of recurrent pregnancy losses, any pregnancy complications, venous thrombosis or other hematological disorders. Matches between patients and control groups were taken in mind; their

average age (32±3) years old, marriage age (22±3) years, with no smoking, no obesity and similar BMI. **Sampling protocol:** - Blood samples were collected in EDTA tubes. Separate the plasma from the blood cells by centrifugation at 2000 x g for 5 min at 0-2° C [19]. This was done as soon as possible after collection because erythrocytes continue to export HCy in collected whole blood [20]. Addition of sodium fluoride to a final concentration of 4 g/L of blood has been proposed to inhibit this release. Plasma was stored at -70 °C if it will not be analyzed immediately. Repeated freeze/thaw cycles may increase HCy levels and should be avoided. Samples for genotyping were processed to DNA extraction step. Placenta was collected immediately after the birth of the baby, then it was placed in ice cold, sterile 0.9% saline and washed thoroughly to remove any maternal blood from the surface. The sections were obtained from areas free of infarctions. These sections were washed again in ice cold 0.9% saline until they were pale and free from blood. They were then combined and minced. Biopsies were stored frozen at -20°C until assay. Placental tissues were prepared for enzyme assay by homogenization in 3 volumes of 0.01 mmol/L of potassium phosphate buffer pH 7.4, followed by centrifugation at 20,000 g for 80 min at 4°C. The supernant was collected for the enzyme activity.

DNA extraction and mutations detection: - DNA extraction, amplifications with biotinylated primers and reverse hybridization detection method were performed according to the kit protocol (Vienna Lab, GmbH, Asturia). 100 ul of whole EDTA blood was incubated with the lysis buffer for 15 minutes, then GenEXTRACT resin was added (it is important that the DNA concentration should not exceed than 20-200 ng/reaction). The amplification was consisted of 15 ul of amplification mix solution, 5 ul of Taq polymerase (Fermentas, Canada Inc., Canada) diluted 0.2 U/ml by dilution buffer supplied with the kit and 5 ul extracted DNA (40ng/ul). The reaction mix added in 0.2 ml eppendorf tube and incubated in thermal cycler (Applied Biosystem,USA) and denturated at 95 ^{o}C for 10 minutes followed by 30 cycles (94 ^{o}C denaturation, 60 ^{o}C annealing and 72 ^{o}C for extension) finally at 72 ^{o}C for 10 minutes. Amplicons were checked via gel electrophoresis. A strip assay protocol was started by incubation of 10 ul of amplicons with 10 ul of denaturation solution for 15 minutes, followed by hybridization with strip probes in tracks with hybridization buffer and adjusted hybridization temperature must be 45±5o C for 45 minutes in shaking water bath profiBlot device (Tecan, Switzerland) for hybridization. Results were easily interpreted visually; there is no need for any documentation system. A positive reaction of the control indicates the correct function of the Conjugate Solution and Color Developer.

Biochemical placental assay for MTHFR activity: - The physiologic assay for MTHFR was based on the spectrophotometric assay described by Matthews and Baugh [21]. The standard assay mixture contained, in a final volume of 100 µL: 0.05µmol/L potassium phosphate buffer, pH 6.6, 100 µmol/L (6 R)-5,10-methylenetetrahy-drofolate (Eprova AG; specified purity, 91%); 200 µmol/L-NADPH (Sigma), and enzyme extract containing 10–70 g of protein. The assay was performed in duplicate with and without 75 µmol/L FAD in the

assay mixture. Tenfold concentrated stock solutions of FAD and NADPH in doubly distilled water (stable for at least 2 months), and 5,10-methylenetetrahydrofolate in 0.5 mmol/L dithiothreitol (stable for at least 10 months) were stored at 20C. Unless otherwise stated, the assay time was 20 min at 37°C. The reaction was terminated by the addition of 50µL of 50 mL/L HClO4 in 10 g/L ascorbic acid, and the samples were mixed and placed on ice. For blanks, the enzyme extract was added after addition of the stop solution. The Km for 5,10-methylenetetrahydrofolate was determined by varying its concentration between 2.5 and 200 µmol/L in the presence of 200 µmol/L NADPH. Blanks were prepared for each 5,10-methylenetetrahydro-folate
concentration by replacing the enzyme extract with 50g of bovine serum albumin in each assay. The Km for NADPH was determined by varying its concentration between 10 and 250 µmol/L in the presence of 100 µmol/L 5, 10-methylenetetrahydrofolate. NADPH was omitted in the blanks. Although 5-methyltetrahydrofolate has to be protected from light during long-term storage, this was not necessary during the assay as well as during preparation of samples for HPLC separation in the dark. When samples were not processed immediately, they were stored at-20°C in the dark; they remained stable under these conditions for at least 2 weeks. **Plasma Homocysteine level:-** Plasma Hcy level was determined in plasma according to the method described by Solomon & Duda [(22)]. Aliquots of plasma (200 µL) are combined with two other reagents in 1.8 ml micro centrifuge tubes and allowed to react at room temperature for ten minutes. A protein- precipitating reagent is then added and the tubes are centrifuged for five minutes. A 10 µL aliquot of the supernatant is injected into the chromatograph, resulting in a chromatogram similar to that in Figure 1. Calibration either external aqueous calibrators or endogenous (using pooled plasma) calibrators may be used. We recommend the endogenous method because of greater stability of these samples (up to 24 hours at 4° C).

Statistical analysis

The data was processed using the statistical package for social science (SPSS Inc., Version 18, Chicago, Illinois, USA). Chi-square test was used for comparison of qualitative parameters. Statistical test results were evaluated by using an overall significance level of $p<0.05$ at 95% confidence interval.

Results

Methylenetetrahydrofolate reductase C677T mutation: the mutation is detected in 68 of patients (45.3%) with 11, 57 (7.3%& 38%) of patients are with homogenous and heterogeneous mutation respectively; while heterogeneous mutation is detected in (4%) of control with no detection of homogonous mutation in control at ($P\leq$ 0.05) figure 2.

Placental Methylenetetrahydrofolate reductase activity (µU/mg protein): subjects with homogenous mutation show the lowest enzyme activity; while the enzyme activity is lower in subjects with heterogeneous mutations than the negative subjects. Highest enzymatic activity is detected in the control negative at ($P\leq 0.05$) table 1, 2.

Plasma Homocysteine (µmol/L): A significant increase in the plasma homocysteine is observed in all patients with MTHFR mutation compared with

control. Highest levels are observed in patients with heterogeneous mutation. The lowest level is observed in control negative for the mutation at ($P \leq 0.05$) table 1, 2.

Discussion

In the present study we evaluated the MTHFR gene C677T missense mutation and its relation to placental MTHFR activity and plasma homocysteine (Hcy) levels in Egyptian women with a history of recurrent pregnancy losses. MTHFR C677T gene missense mutation encodes for a thermolabile variant with reduced enzymatic activity [(1)]; the condition which resulted in accumulation and elevation of plasma homocysteine [(23)]. High plasma homocysteine is implicated in venous thrombosis48. But it was not ascertain whether the high homocysteine level cause thrombosis or was a consequence of it [(23)]. Studies of genetic variants of MTHFR associated with high homocysteine level might prove the link of high homocysteine level and the incidence of venous thrombosis [(24)]. Previously; there is strong evidence on the implication of MTHFR mutation in the occurrence of venous thrombosis [(23, 25, 26)]. As it implicated in thrombosis for this reason; there is an evident involved MTHFR C677T mutation as a causal agent of RPLs due to thrombosis of utroplacental vasculature [(27)]. Our results prove this association as homozygous and heterozygous C677T mutation was detected in 11.7 % and 38% in subjects with RPLs respectively with total detection of the mutation by 54.6 % in patients if compared with control subjects (4%) only. On the other side; elevation of the plasma levels of Hcy has been associated with increased risk for cardiovascular and cerebrovascular disease in two studies [(28, 29)]. Strong evidence previously mentioned by Ma et al. [(30)] that a genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR), has been associated with elevated plasma homocysteine. This come in agreement of our data as the lowest enzyme activities (112±7.3) and highest HCy levels (23.12±2.7) were detected in patients with heterozygous mutation (TT); while the highest enzyme activity (447±15.6) and lowest Hcy levels (9.12±1.8) were detected in negative homozygous (CC) patients this confirm the observation of Van Dunné et al. [(23)]. Here the link between low enzyme activity and high Hcy levels and previous data that involved them in thrombosis explain how the thermo labile enzyme resulted from mutated gene is involved to induce RPLs in women. The possible explanation is due to vasculature disorders in the placental circulation. Great evidence about the association between the Hcy and venous thrombosis was presented; high plasma homocysteine is positively correlated with venous thrombosis [(31-33)]. In the same line, the link between thrombosis and recurrent pregnancy losses was previously studied [(34-37)]. Eldibany and Caprin [(38)] tried to demonstrate the mechanism; as HCy is formed from methionine by transmethylation reaction. Key enzymes in the rather complex homocysteine metabolic pathway are the cystathionine β synthase (CbS), the methionine synthase (MTR) and the methelenetetrahydrofolate reductase (MTFHR). The role of increased homocysteine as an independent risk factor for arterial and venous thrombosis has become approved. The proposed pathogenetic mechanisms are oxidative damage of the endothelium through suppression of the vasodilator nitric oxide, increasing the levels of

asymmetric dimethylarginine, and impaired methylation, vascular smooth muscle proliferation, promotion of platelet activation and aggregation and disruption of the normal procoagulant-anticoagulant balance favoring thrombosis. In conclusion our study proved the link between the MTHFR C677T mutation and the reduced enzyme activity with high Hcy levels to be the cause of RPLs in women with history of many pregnancy losses. This by the way confirms the association between the genetic vascular disorders and RPLs in Egyptian women.

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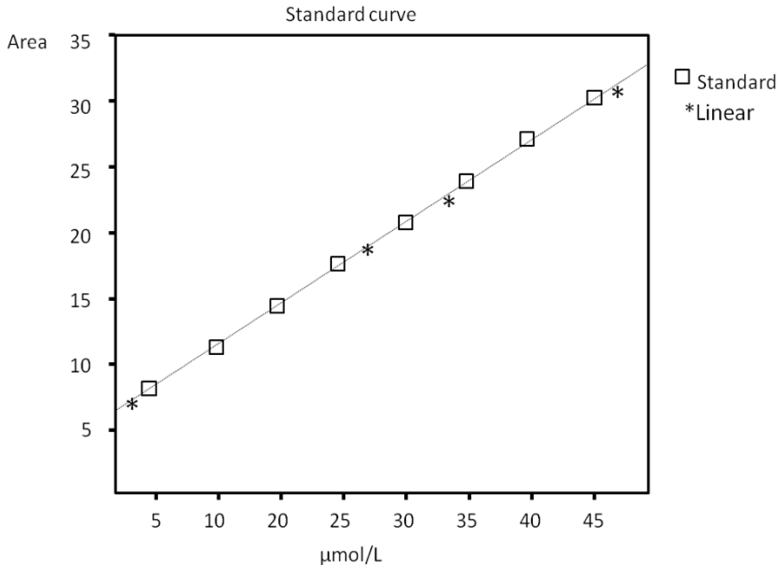

Figure (1): Standard curve for chromatographic separation

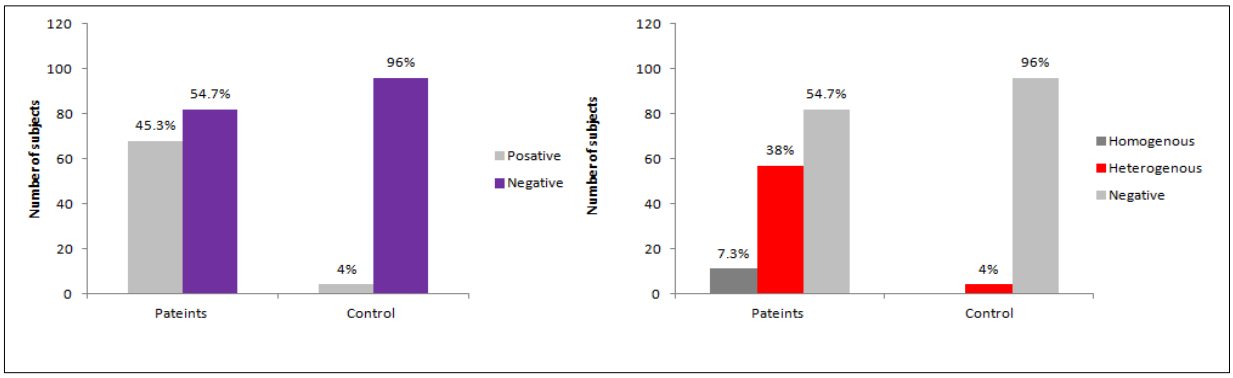

Figure (2): the incidence of methylenetetrahydrofolate reductase C677T mutation in both patients and control.

Table (1): Placental methylenetetrahydrofolate reductase activity and plasma homocysteine level of patients.

Genotypes	N	MTHFR activity (µU/mg protein)	Homocysteine level (µmol/L)
Homozygous (TT)	7	112±7.3[d]	23.12±2.7[a]
Heterozygous (CT)	11	415.4±8.2[c]	11.37±1.99[b]
Negative (CC)	82	447.5±15.6[b]	9.12±1.8[c]

Table (2): Placental methylenetetrahydrofolate reductase activity and plasma homocysteine level of controls.

Genotypes	N	MTHFR activity (µU/mg protein)	Homocysteine level (µmol/L)
Heterozygous (CT)	4	420±2.3^{c}	10.6±1.3^{bc}
Negative (CC)	96	474.6±46.9^{a}	8.8±1.3^{c}

Means of Methylenetetrahydrofolate reductase activity & homocysteine level that carrying different subscripts a, b, c& d are significant at ($P \leq 0.05$).
Data given as mean ± SD
µU= pmol/min.
N= number of subjects.