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Methylene tetra hydrofolate Reductase C677T Gene Polymorphism in Heroin Dependence

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Introduction

Dependence to opiates is a chronic relapsing brain disease that can cause major health, social and economic problems [1]. Dependence vulnerability is affected by environmental factors, drug-induced factors and hereditary factors, which represent approximately 40–60% of the risk of developing dependence [2]. Illicit drug use could lead to epigenetic changes in DNA methylation which may play an important role in the development of chronic substance abuse by enhancement of drug reward, craving and relapse use [3,4]. Epigenetic factors could be also responsible for vulnerability to drug response, dependence and addiction treatment response [5].

Methylene tetra hydrofolate Reductase (*MTHFR*) gene and enzyme are involved in complex biochemical pathways and folate metabolism [6]. Normal MTHFR activity may help to maintain the pool of circulating folate and methionine and possibly to prevent a build-up of homocysteine [7].

MTHFR gene has two single nucleotide polymorphisms C677T (rs1801133) and A1298C (rs1801131). Two common single nucleotide polymorphisms in MTHFR have been reported, a cytosine-to thymine (C→T) transition at nucleotide 677 in exon 4 and an A→C transversion in exon 7 at position 1298. Both of these polymorphisms are functional and result in diminished enzyme activity. For the C677T polymorphism TT genotype has 30% enzyme activity in comparison with CC genotype, while CT genotype retains 65% of CC MTHFR enzyme activity [8]. Healthy persons with the 677 TT genotype have lower levels of folate in plasma and red blood cell than persons with other genotypes [9]. Gilbody and his coworker's meta-analysis [10] demonstrated an association between the *MTHFR* C677T variant and depression, schizophrenia and bipolar disorder. In addition, association between the *MTHFR* C677T polymorphism and some of substance use was noted including nicotine smoking [11], alcohol [12] and cocaine dependence [13]. Although Yuferov et al., [1] found that there is a strong association between Heroin and cocaine addiction and polymorphism in genes; to the best of the authors' knowledge little is known about the association between MTHFR Genetic Polymorphisms and Heroin use.

We hypothesized that *MTHFR* C677T polymorphism and hyperhomocysteinemia are more common in Heroin dependence patients and are associated with severity of dependence. Therefore, the objectives of the current study are to examine the association of *MTHFR* C677T polymorphism and homocysteine level with Heroin dependence and its severity.

Methods

Study design

Due to the lack of specialized inpatient unit for drug dependence and treatment in the only two psychiatric public hospitals present in Mansoura city (Demira Mental Hospital and Mansoura University, Faculty of Medicine, Psychiatry department) the authors decide to choice two private hospitals to conduct this study. The two chosen private hospitals are considered the largest hospitals treating addiction in Mansoura.

The study was designed to be a case–control study conducted during the period from the 1st February 2012 until 31st August 2013 (18 months duration). During the study period all Heroin dependence patients (178) admitted to the two hospitals were examined.

Controls (192) were apparently healthy and free from any psychiatric disorders, chronic diseases or substance abuse. They were chosen from workers of both hospitals and blood donors attending Mansoura University Hospital Blood Bank. All subjects were examined by Arabic version of Mini International Neuropsychiatric Interview [14,15] to exclude any psychiatric disorders.

The study was approved by the Mansoura Faculty of medicine ethics committee and then it has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. A written informed consent was obtained from all participants before inclusion in the study.

Inclusion criteria for participation in the study include both sexes, age range from15 to 50 years old and solitary Heroin dependence (no other drug dependence, i.e. alcoholism). Exclusion criteria include any medical illness or other psychiatric disorders, mental retardation and signs or symptoms of vitamin B6, B12 or folic acid deficiencies. 98% of the population in Egypt consists of "Egyptians" ethnic who are native speakers of modern Egyptian Arabic. The ethnic minorities (2%) in Egypt include Berber (Siwa Oasis), Nubian (Nubian people clustered along the Nile in the southernmost part of Egypt), Bedouin (Arab tribes of the Sinai Peninsula and the eastern desert), and Beja 1%, Greek, Armenian, other European (primarily Italian and French) 1% [16]. Ethnicity was assessed in all subjects using a direct question to identify their ethnicity and to choice if he or his family roots come from one of previously mentioned groups. In our study, we excluded any subjects from any minority ethnicity to limit the ethnic factor in genetic study, which may be present even in very small effect.

Data collected from patients' sheets included socio-demo-graphics, e.g. age, sex, residence, education, marital status and work. Drug use data were collected as duration of dependence, dose of Heroin use in the last year (more than one gram or less than one gram) and number of previous relapses.

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All patients and controls were examined by a psychiatrist using an Arabic version of Mini International Neuropsychiatric Interview non-alcohol drug dependence and abuse [14,15]. All cases were diagnosed for Heroin dependence, according to Diagnostic and Statistical Manual of Mental Disorders, fourth Edition, Text Revision (DSM-IV-TR) [17].

Severity of opioids dependence was measured using 5-item Severity of Dependence Scale (SDS) questionnaire. Each of the five items was scored on a 4-point scale (0-3). By summing the scores of 5-item the total score is obtained. The higher the score, the higher is the level of dependence. The original SDS scale is in English language originally developed for assessing psychological dependence on Heroin with very good validity and reliability [18]. Because an Arabic version of the SDS is not available, the scale was first translated into Arabic language by three bilingual (native English and Arabic) persons resulting in three different Arabic form of SDS. Then one form of SDS was created by a board consisting of the three translators (and their translated form) and three senior professional psychiatrists who not involved in the translation process. After that, the Arabic version of the SDS was back translated by a native English speaker living in Mansoura for five years ago in British Council for translation and language training; this person was unaware of the original English document. Once the back translation finished, the board was asked to review and determine the difference between back translation and the original scale. Finally, a pretest was conducted with a group of lay native Arabic speakers (25 subjects). The internal consistency level (Chronbach's alpha coefficient) of Arabic version of the SDS was 0.87.

Blood sampling

A fasting five ml venous blood sample was collected and delivered to a centrifuge tube containing K_2 EDTA; one ml EDTA anti-coagulated whole blood was used immediately for DNA extraction and other 4 ml was centrifuged at 7000 rpm to produce plasma.

DNA extraction

Genomic DNA was extracted from EDTA–anticoagulated peripheral blood leukocytes using G-spin™ Total DNA Extraction Mini Kit (Cat, No. 17045, Intron Biotechnology, Sungnam-si, Kyeonggi-do, Korea) [19]. The average DNA concentration (0.127±0.005μg/μl) was determined from the absorbance at 260 nm (Jenway, Genova Model, UK). All samples had a 260/280 nm absorbance ratio between 1.6 and 1.79. The integrity of the DNA was checked by electrophoresis on 0.8 % agarose gel stained with ethidium bromide.

Genotyping of *MTHFR* C677T Polymorphism (rs# 1801133):

Polymerase Chain Reaction (PCR): Conventional method of PCR amplification was used for genotyping of C677T polymorphism in *MTHFR* by the method described by Han et al. [20]. The primer sequences used for DNA amplification are as follows: 5'-GCA CTT GAA GAG AAG GTG TC- 3' (forward) and 5'-AGG ACG GTG CGG TGA GAG TG- 3' (reverse). PCR was carried out in 50 microliters final reaction volume using PCR Master Mix (i-Taq™ Mix, Cat No. 25028) (purchased from Intron Biotechnology, Sungnam-si, Kyeonggi-do, Korea). The following mixture was prepared for each sample: 25 μl i-Taq™ Mix PCR reaction Mix (1X), 2μl (20 pmole) of forward primer, 2μl (20 pmole) of reverse primer, 2μl (200 ng) of genomic DNA and 19 μl of deionized water. This mix was put in a thin wall PCR micro-centrifuge tube and gently centrifuged to collect all components to the bottom of the tube. Amplification was carried out in a Thermal Cycler (TECHEN TC-312, Model FTC3102D, Barloworld Scientific Ltd. Stone, Stafford Shire, st 150 SA, UK). Initial denaturation at 94 °C for 5 min followed by 35cycles of denaturation at 94 °C for 30 sec, alignment at 55 °C for 30 sec, and an extension step at 72 °C for 45 sec, followed by a final extension step at 72 °C for 7min. The PCR amplification product was 203 bp.

PCR products were digested with HinfI (Promega Corporation, cat. number Part# TM367) using the following protocol: 16.3μl deionized water, 2μl Restriction Enzyme 10X Buffer, 0.2μl Acetylated BSA (10μg/μl), 1.0μl of DNA were mixed by pipeting, then 0.5μl of Restriction Enzyme 10u/μl was added to a final volume of 20 μl. The mix was centrifuged for a few seconds in a Micro-centrifuge and incubate at the enzyme's optimum temperature [37 °C for 2 hours] [21].

PCR and restriction enzyme digestion of all samples have been performed in duplicates and negative control samples were used for testing of contamination.

For the nucleotide 677, an undigested PCR product (203 bp) indicated a homozygous wild type (CC), three bands of 203, 173, and 30 bp indicated the heterozygous genotype (CT), and two bands of 173 and 30 bp indicated the homozygous genotype (TT). The DNA fragments were separated by electrophoresis in 3.0 % agarose gel and visualized using ethidium bromide via Light UV Trans-illuminator (Model TUV-20, OWI Scientific, Inc. 800 242-5560, France) and photographed.

Determination of plasma total homocysteine level using Homocysteine ELISA Kit from IBL International GmbH ELISA kit (Catalog Number: AX51301) is designed for detection of total homocysteine in human plasma [22]. This assay employs the solid-phase enzyme immunoassay technique, which was performed according to the manufacturer's instructions using a plate ELISA reader (Sunrise Rimote/Touch Screen-Tecan Austria GmbH, 5082 Grodig, Austria) for reading the absorbance of each sample at 450 nm wavelength.

Statistical method

Data was analyzed using SPSS 16. Qualitative variables were presented as number and percent. Chi square test was used for comparison between groups. Gene and allele frequencies and their 95% confidence interval were calculated. In addition, ORs and their 95% CI were calculated. Hardy-Weinberg equation was calculated. Quantitative variables were tested for normality distribution using Kolomogrov-Smironov test. Normally distributed variables were presented as mean and SD. The unpaired t - test was used for comparison between 2 groups and ANOVA (F) test was used for more than two groups with Bonferroni's multiple comparisons. Non-parametric variables were presented as median (minimum- maximum) and Mann-Whitney test was used for the two group comparisons and Kruskal-Wallis test. $P \leq 0.05$ was considered statistically significant.

Results

The results shown in (Table 1) reveal that cases and control were matched regarding their age, gender, religion, marital status, education and residence. However, they differ significantly in their working status. The majority of cases were students (54.5%) while the majority of controls were non-working (49%).

(Table 2) demonstrates that the duration of dependence varies from 12-39 months with a mean and median of 23.4 and 24; respectively. The dependence severity score varies from 3 to 15 with a mean and median of 7 and 6; respectively. The number of relapses ranged from zero to 10 with a mean and median of 4.12 and 4; respectively. About 60% of cases consume more than 1 g Heroin per day. A positive family history of drug dependence was reported by more than half of the patients (51.7%).

The genotypes CT and TT are 3.7 and 13.9 times more likely to be linked to drug dependence than genotype CC; respectively. In addition, the presence of allele T is associated with increased likelihood of dependence compared to allele C (OR=4.1; CI=2.8-5.96) (Table 3).

(Table 4) shows that the CT and TT genotypes have a significantly longer duration of dependence, higher severity score, more relapse, and larger dose of daily Heroin intake and higher percent of positive family history compared to genotype CC. The same pattern was observed in allele T compared to allele C. These data are not deviated from Hardy-Weinberg equilibrium.

(Table 5) shows that homocysteine level is significantly higher in cases than control. Among control homocysteine level does no significant variation according to genotype and allele type. On the other hand, in cases the homocysteine level is significantly higher in genotype CT and TT compared to CC and with allele type T compared to C.

	Control (192) N (%)	Cases (178) N (%)	Significance
Age: <30	49(25.5)	48(27.0)	χ^2=0.23, P=0.88
30-	97(50.5)	91(51.1)	
40 & more	46(24.0)	39(21.9)	
Mean ± SD	34.4 ± 9.6	35.5 ± 8.7	t=1.1,P=0.3
Gender: Female	60(31.2)	55(30.0)	^{2}=0.01,
Male	132(68.8)	123(69.1)	P=0.9
Religion: Muslim	185(96.4)	171(96.1)	^{2}=0.02,
Christian	7(3.6)	7(3.9)	P≤0.89
Marital status:			
Married	54(28.1)	48(27.0)	
Divorced	9(4.7)	7(3.9)	χ^2=0.5,
Single	117(60.9)	114(64.0)	P=0.9
Widow	12(6.2)	9(5.1)	
Education *:			
literate	64(33.3)	50(28.1)	
Primary-preparatory	46(24.0)	40(22.5)	χ^2=4.2,
Secondary school	68(35.4)	80(44.9)	P=0.24
Faculty education	14(7.3)	8(4.5)	
Work *:			
Not working	94(49.0)	66(37.1)	
Student	71(37.0)	97(54.5)	χ^2=14.96,
Semi-skilled	16(8.3)	13(7.3)	P=0.002
Skilled worker	11(5.7)	2(1.1)	
Residence: Rural	93(48.4)	89(50.0)	^{2}=0.1,
Urban	99(51.6)	89(50.0)	P=0.76

* This classification is according to the Office of Population Censuses and Surveys Social Trends (1988)

Table 1: Socio-demographic features of control vs. cases.

	Mean ± SD	Median (min-max)
Duration (months)	23.4 ± 8.6	24(12-39)
Severity scores	7.5 ± 3.2	6(3-15)
Number of relapses	4.12 ± 2.7	4(0-10)
	N (%)	
Dose: <1 g	72(40.4)	
≥1 g	106(59.6)	
Positive family history:	92(51.7)	

Table 2: Clinical data of addicts.

Discussion

Present study revealed that cases and control were matched regarding their age, gender, religion, marital status, education and residence. However, they differ significantly in their working status which could be explained by the effect of substance use and its hazard on social and occupational function.

In the current study, *MTHFR* C677T genotype CC was more prevalent in the control group (75%) with low prevalence of CT and TT (21% and 3%, respectively) while CT represented the most common genotype in the cases group (42%) then CC (38%) and lastly TT (19%). (P=0. 19 for the control group and 0.10 for cases group). There are statistically significant increases in CT & TT genotypes as well as T allele in the cases group when compared to the control group. These results confirm the idea recently emerged that opioid abuse is closely related to genetic polymorphism of some opioid receptor genes apart from *MTHFR* gene, [23]. For our knowledge, it is the first study to investigate the association of *MTHFR* C677T gene polymorphism and Heroin dependence. Previous studies showed that T allele and T carrier genotypes (TT & CT) were more prevalent in nicotine smoker [11] alcohol [12] and cocaine dependent patients [13] than in the control group. Yuferov et al., [1] found that there is a strong association between Heroin and cocaine addiction and polymorphism in genes. The similarity of the present study results and other drug dependence (cocaine, alcohol, and nicotine) could return back to the fact that these drugs share common final neurotransmitter release (dopamine) in the same brain area (brain reward system) and many other genotype polymorphism [1,23].

The current study shows statistically significant increase in plasma homocysteine level in the cases group than the control group. Also, there is increase in the TT genotype of the *MTHFR* C677T gene and T allele frequency in comparison to other genotypes (CT and CC) and C allele frequency in the cases group but not in the control group. These results are in agreement with Tomedi et al. [24]. Their study showed that the plasma homocysteine level is statistically higher in the opiate drug addict group even the treated subjects of them than that of their control group. The hyperhomocysteinemia associated with T allele and T

	Control		Cases		Significance	OR (95% CI)
	N (Frequency)	95%CI	N (Frequency)	95%CI		
Gene						
CC	144(0.750)	0.684-0.806	69(0.388)	0.318-0.461	χ^2=54.9, P≤0.001	1(r)
CT	42(0.219)	0.166-0.282	75(0.421)	0.351-0.495		3.7(2.3-6.2)
TT	6(0.031)	0.014-0.066	34(0.191)	0.140-0.255		13.9 (5.3-38.5)
H.W.	χ^2=1.73, P=0.19		χ^2=2.71, P=0.10			
Allele						
C	330(0.859)	0.821-0.891	213(0.598)	0.547-0.668	χ^2=64.5, P≤0.001	1(r)
T	54(0.141)	0.109-0.179	143(0.402)	0.352-0.453		4.1(2.8-5.96)

CI=Confidence Interval, r=reference category
H.W. = Hardy-Weinberg equation

Table 3: Gene & allele frequencies in control vs. cases.

	Genes			Alleles	
	CC Median (min-max)	**CT Median (min-max)**	**TT Median (min-max)**	**C Median (min-max)**	**T Median (min-max)**
Duration	14(12-26)^{AB}	28(12-39)^{A}	27.5(12-39)^{B}	18(12-39)	28(12-39)
Significance	K W. χ^2=104.2,P≤0.001			Z=8.95,P≤0.001	
Severity score	5(3-8)^{AB}	10(4-15)^{A}	10(4-14)^{B}	5(3-15)	10(4-15)
Significance	K W. χ^2=78.9,P≤0.001			Z=8.0,P≤0.001	
Relapse number	1(0-5)^{AB}	5(2-10)^{A}	5(1-10)^{B}	2(0-10)	5(1-10)
Significance	K W. χ^2=109.3,P≤0.001			Z=9.4,P≤0.001	
Dose (N& %): <1 g ≥1 g	 45(65.2)^{AB} 24(34.8)	 19(25.3)^{A} 56(74.7)	 8(23.0)^{B} 26(76.5)	 109(51.2) 104(48.8)	 35(24.5) 108(75.5)
Significance	χ^2=47.6,P≤0.001			χ^2=25.3,P≤0.001	
Family history (N&%): **negative** **positive**	 54(78.3)^{AB} 15(21.7)	 24(32.0)^{A} 51(68.0)	 8(23.5)^{B} 26(76.5)	 132(62.0) 81(38.0)	 40(28.0) 103(72.0)
Significance	χ^2=41.5,P≤0.001			χ^2=39.6,P≤0.001	

A & B significant difference between the corresponding groups by multiple comparisons (χ^2 for qualitative variables & Mann-Whitney for quantitative variables). KW=Krusskal-Wallis test, Z of Mann-Whitney test

Table 4: Variation of severity, duration, relapse, dose and family history according to genes & alleles.

Group	Genotype	Allele	Homocysteine Mean ± SD	Significance
Control **Cases**			13.7 ± 1.9 17.7 ± 2.9	t=15.5, P≤0.001
Control	CC CT TT		13.6 ± 1.98 13.98 ± 1.6 15.5 ± 1.9	F=0.6, P=0.6
Cases	CC CT TT		15.4 ± 1.5^{AB} 17.5 ± 1.2^{AC} 22.6 ± 1.1^{BC}	F=343.7, P≤0.001
Control		C T	13.7 ± 1.9 13.7 ± 1.8	t=0.74, P=0.46
Cases		C T	16.1 ± 1.7 19.96 ± 2.8	t=15.8, P≤0.001

A, B & C significant difference between the corresponding groups by Bonferroni multiple comparisons

Table 5: Homocysteine in control vs. cases & its variation with genotype & allele in each group.

carrier genotypes of the *MTHFR* C677T gene could be explained by the following mechanism; MTHFR synthesizes 5-methyltetrahydrofolate, the major carbon donor in the remethylation of homocysteine to methionine. C to T mutation causes the substitution of valine for alanine at amino acid 223 and renders the enzyme less efficient (raised levels of homocysteine especially in TT genotype) and thermolabile [23,25].

Explanation for how hyperhomocysteinemia (associated with T carrier genotype of the *MTHFR* C677T gene in the current study) could harm brain and influence Heroin dependence can be extracted from finding of previous studies. *MTHFR* C677T gene is linked with low folate levels and is associated with increased incidence of neuronal tube defects [26]. Moreover, in patients with severe MTHFR deficiency cerebral demyelination was detected [25]. In addition, MTHFR is involved in remethylation of homocysteine to methionine which is central for dopamine methylation and the synthesis of neurotransmitters [11]. In addition, hyperhomocysteinemia is associated with global DNA hypomethylation [27]. Major life events occurring before the onset of Heroin dependence and/or chronic Heroin use were found to modify DNA methylation [28]. The polymorphisms within the *MTHFR* gene also influence homocysteine related neurotransmitters like taurine (play important in oxidative process). Homocysteine increase superoxide and hydrogen peroxidase by auto-oxidation leading to oxidative stress and reduce the expression of glutathione peroxidase (an antioxidant enzyme) thereby causing the accumulation of its neurotoxic oxidative products [29]. Moreover, homocysteine was found to provoke neuronal cell damage and brain atrophy by stimulating N-methyl-D-aspartate (NMDA) receptors [30]. Therefore, it is not strange to find that elevated homocysteine (Hcy) levels associated with neuro-degeneration, vascular diseases and brain atrophy [31].

In this study, the dependence severity was more associated with the presence of a positive family history. This was consistent with previous studies by Pickens et al., [32] found that family history of substance dependence is a good predictor for more opioid dependence symptoms and more likely to be classified as severely dependent patient. Moreover, the current study revealed an association between the severity of dependence as measured by duration of illness, number of relapses, severity of dependence scale and the dose of the substance with genotype and alleles. All these measures are higher in CT & TT genotypes than CC genotype as well as T allele than C allele. A similar increase in severity of dependence with an increase in gene polymorphism was noted in alcohol dependence by Lutz et al., [12]. They found that the T - allele frequency increased from 0.28 in healthy control subjects to 0.33 in alcohol dependent patients suffering from mild withdrawal symptoms up to 0.40 in alcohol dependent men with a history of withdrawal seizure [12]. De Bree et al. [33] found that the frequency of substance abuse was associated with increase duration of dependence, average Heroin daily dose, score on the severity of dependence scale, and more relapse. This may be explained by the results that put a link between MTHFR enzymatic activity and the polymorphism of the responsible gene. A recent cross sectional study based on Dutch, found a significant lower level of serum folate with 677CT and 677TT genotypes among individuals with self-reported low folate intake [33]. The 677TT genotype even conferred lower serum folate in individuals with high folate intake compared to other genotypes. Individuals with 677TT genotype had elevated homocysteine levels when folate status is low [33]. Finnell and associates [34]. Studies of the folic acid biosynthetic pathway led to the discovery of an association between elevated levels of homocysteine (Hcy). The thermo-labile form of the enzyme led to elevated (Hcy) which may be the mediator of the dependence case severity.

Limitation of the study

Although we tried hardly to exclude ethnicity in this study using through history taking but this may not surly true. Moreover, the present study population was accessed at two private hospitals this may excluded a large portion of the population who could not be treated in private hospital. In our locality no public hospital treating addiction is present.

Conclusion

MTHFR polymorphism may contribute to the incidence and severity of heroin dependence, but that numerous other factors may play an equal or more significant role. In addition, plasma homocysteine may have a role in pathogenesis of Heroin related disorders. Multicenter studies and larger samples are recommended in further research to confirm the result of this study.

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