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ASSOCIATION OF 5, 10- METHYLENETETRAHYDROFOLATE REDUCTASE C677T POLYMORPHISM IN SUSCEPTIBILITY TO TROPICAL CHRONIC PANCREATITIS IN NORTH INDIAN POPULATION

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Abstract

MTHFR is a key enzyme in folate metabolism that catalyzes the conversion of 5, 10-methlenetetrahydrofolate (5, 10- methylene THF) to 5-methyltetrahydrofolate (5-methyl THF), a predominant circulatory form of folate and methyl donor for the remethylation of homocysteine to methionine. Some studies have shown that C667T polymorphism increases the risk of pancreatic cancer. Since MTHFR is involved in methylation, inflammation and protection against oxidative stress, the processes especially important for pancreatic homeostasis. The altered enzyme activity could play a role in pancreatic injury. The role of MTHFR C677T polymorphism in chronic pancreatitis has been explored by conducting a hospital based; case-control study involving 100 patients radiologically confirmed chronic pancreatitis and 329 healthy controls. All samples were analyzed for MTHFR C677T polymorphism using PCR-RFLP method. Restriction enzyme Hinf I was used to digest the 198 bp amplified product. The frequency of the MTHFR was 57.3%, 34.1% and 8.5% among cases compared with 87.2%,11.2% and 1.5% of controls for CC, CT and TT genotypes, respectively. The T Allele frequency was found significantly higher in patients than in controls. A significant association with T allele was observed with p-value (< 0.0001) odds ratio 4.475 and (95% CI=2.961-7.046). It could be predisposing to the traditional risk factors such as diabetes, dietary, alcohal and smoking habit that are known to be associated with chronic pancreatitis. Additionally it was observed that smoking increases the risk of chronic pancreatitis by 4.1 times. The T allele frequency of MTHFR (C667T) was found to be a significant risk factor for chronic pancreatitis playing a crucial role in altered folate metabolsim.

Key words: Chronic pancreatitis, MTHFR, C677T, smoking, alcoholic, diabetes.

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INTRODUCTION

Pancreatitis is a persistent inflammation of the pancreas characterized by repeated attacks of abdominal pain and impairment of both exocrine and endocrine functions (14). Tropical Chronic pancreatitis (TCP, OMIM 608189) is a juvenile form of chronic calcific non alcoholic pancreatitis with classical traid of symptoms include diabetes, steatorrhea, pancreatic calculi and finally clinical manifestation of end stage disorder i.e. pancreatic cancer (31, 9). The disease is reported from all parts of the Indian subcontinent. Balaghi and coworkers in 1993 reported the prevalence of calcific pancreatitis 98/ 100,000, which is far higher than that being reported for CP in the west 10-15/ 100,000 (1). Tropical CP has a strong genetic basis and increasing knowledge of gene- environment interactions has provided new insight into the pathophysiology of the disease. It is thought as a multifactorial disorder in which multiple risk factors are involved in the disease progression (16). TCP is considered to be a pre-cancerous condition that produces alteration in the microenvironment of pancreatic cells. It mainly targets the exocrine compartments and transformation by increasing genomic damage and cellular proliferation (20, 35).

The pancreas contains high level of folate, second only to the liver as evidenced from experimental studies which proved an association between folate levels and pancreatic disease (1). Some studies have suggested that reduced exocrine function was a result of disturbed methyl metabolism secondary to folate deficiency (19). It is possible that the disturbed metabolism may cause abnormal cellular differentiation, reduced exocrine function of the pancreas, increased toxic injury and carcinogenesis as a consequence of autolytic destruction of the pancreas in chronic pancreatitis, which has been associated with pancreatic cancer (3). Chronic inflammation in pancreatitis induces DNA damage and mutations and thereby facilitates the development of pancreatic cancer. The processes like DNA synthesis during replication and repair are largely dependent on the availability of one carbon metabolism pathway. Therefore, one-carbon metabolism polymorphisms may have the potential to modify or influence chronic pancreatitis (32).

5, 10-methylenetetrahydrofolate reductase (MTHFR) is the most extensively studied gene. It acts at a critical juncture in the folate metabolism. The MTHFR gene contains a 2.0-kbp coding region with 11 exons and is located on chromosome 1p 36.3. It is a key enzyme in folate metabolism that catalyzes the conversion of 5, 10- methlenetetrahydrofolate (5, 10- methylene THF) to 5-methyltetrahydrofolate (5-methyl THF,) a predominant circulatory form of folate and methyl donor for the remethylation of homocysteine to methionine (5). Methionine is the principal methyl donor for the methylation process. A reduction in MTHFR activity such as that caused by the C to T transition at position 677 of the MTHFR cDNA (C667T) or change in the alanine to valine at position 222 of the MTHFR aminoacid sequence, produces a thermolabile enzyme which results in increased plasma homocysteine level (8,13). Homocysteinuria is primary metabolic cause of chronic pancreatitis (28). DNA methylation is an epigenetic feature of DNA that influences cellular development

and function. The aberration(s) of DNA methylation may serve as a plausible candidate mechanism for the development of cancer. There were many reports of MTHFR with developmental disorders like neural tube defects (such as spina bifida, nonsyndromic cleft lip and Down's syndrome) and neurological impairment (36, 10, 27, 15). The genetic variation in this gene was susceptible to occlusive vascular defects (25), breast cancer (23), colon cancer (29) and leukemia (26).

Though there is a worldwide distribution of a common MTHFR mutation, previous studies of C667T mutation mainly concentrated on European population (24). The MTHFR polymorphism was found tested in every population. Unlike other mutation of trypsinogen activation and inactivation pathways like PRSS1, PRSS2 and Factor5, which are common in western countries, the C667T mutation exhibits relatively high frequency throughout the world (11, 4, 17). Some studies have highlighted the association of MTHFR gene with coronary artery disease in population of North India (22). We hypothesized that polymorphism of the MTHFR gene that results in reduced enzyme activity may modify chronic pancreatitis. To the best of our knowledge, no study has evaluated the role of MTHFR in chronic pancreatitis in Indian population. We therefore tested this hypothesis in a case-control study. Each eligible candidate was interviewed to collect demographic data and information on smoking history, alcohol (ethanol consumption) and other risk factors.

MATERIALS AND METHODS

Study Subjects

The study encompassed clinically diagnosed 100 patients of Tropical chronic pancreatitis. All these patients were recruited from the OPD of Prof Gourdas Choudhuri Department of Gastroenterology, Sanjay Gandhi Post Graduate Institute, Lucknow, India between 2009 to 2011. The biochemical tests like serum amylase, serum lipase, liver function test, lipid profile, fasting and PP glucose levels, HbA1c and serum calcium were done in all the cases as routine investigations. Sudan stain test was carried out to investigate the presence of fat in stool sample.

The recruited controls were 329 healthy, genetically unrelated family members, who had no history of pancreatitis. A prior written informed consent was obtained from both patients and controls. Diagnosis of chronic pancreatitis was based on the presence of exocrine and endocrine pancreatic insufficiency and the findings of morphological examinations such as calcification in pancreas detected by ultrasound, computed tomography or nuclear magnetic resonance of the abdomen, morphological changes of the pancreatic canalicular system detected by endoscopic retrograde cholangiopancreatography, and morphological changes of the pancreas detected by endoscopic ultrasound.

Genotyping

Genomic DNA was extracted from the whole blood collected in EDTA vials using Phenol-Chloroform method described by Ponez et al. (21). Polymerase chain reaction (PCR) was performed in a total volume of 50µl containing genomic DNA and 20 pmol of each primer. The primer sequence and PCR conditions were similar as described by Frosst et al. (6). The PCR product (198bp) of the MTHFR polymorphism was digested with H*inf* I enzyme as per manufacturer's instructions. The restriction digestion product was separated on a 3% agarose gel and visualized by ethidium bromide staining. The wild type MTHFR C677C was characterized with absence of restriction site (single band of 198bp); heterozygote's C677T showed 198bp, 175bp, and 23bp fragments, while homozygote's for T allele yielded 175bp and 23bp fragments.

Statistical analysis

The clinical data were analyzed using SPSS 15 software. The chi-square test was used to compare the incidence of variables in the two groups. Genotype and allele frequency were analyzed using the GraphPadInStat3 (demo version) software package. Allele frequencies were deduced from genotype distribution. The observed genotype frequencies were compared with those calculated from the Hardy-Weinberg disequilibrium theory ($p2 + 2pq + q2 = 1$), where p is the frequency of the variant allele and $q = 1-p$. Multiple logistic regression models were used to evaluate the combined effect of genotypes and exposure (cigarette smoking, alcohol consumption). Heavy smokers were defined as those who smoked >20 pack-years and heavy drinkers were defined as those who consumed >86 g ethanol/wk. Variables for combined effects was coded using a common reference group (MTHFR CC and CT genotypes).

RESULTS

The MTHFR C677T genotype and allele frequencies were determined in 100 chronic pancreatitis patients and 329 individuals as control. The clinical and demographic data of patients and controls are presented in (Table 1). The distribution of genotypes with in patient group was in Hardy- Weinberg equilibrium (p value=.09218), but in the control group it was not in Hardy- Weinberg equilibrium (p value=0.00575). For the 677 locus, the frequencies of the CC, CT, and TT genotypes were 58%, 32% and 10%, respectively, among cases. The corresponding frequencies among the controls were 87.2%, 11.2%, and 1.5%, respectively. The frequency of homozygote (TT) was higher in patients as compared to controls with (P .000) which are highly significant. To our knowledge, this is the first study in which MTHFR C677T polymorphism was analyzed as a potential modulating factor for chronic pancreatitis in Indian CP cases. The T allele frequency was 26% for cases and 7.140% for controls and the difference was statistically significant with an OR of 4.568 (95%CI=2.961-7.046) and (P< 0.001) (Table1). We observed an increased OR value of 4.5 in chronic pancreatitis patients.

These results may implicate a possible role of C677T polymorphism as a risk factor for chronic pancreatitis. On performing multiple logistic regression analysis in order to evaluate the influence of various risk factors on chronic pancreatitis precipitation (Table 2), we found that smoking was significant variable for CP. We additionally observed that smoking increased the risk of chronic pancreatitis by 4.1 fold, whereas in alcoholics the reduced risk was observed but statistically it was significant with an OR of 0.161(95% CI=.084-.310) and (P<0.001), which was viewed as the alcoholic predispose to the development of disease progression. Furthermore, a concurrent increase in MTHFR CT and TT genotypes leads to an even greater

Table 1. Distribution of genotype and allele frequencies in patients and controls.

Genotype/Allele	Patients Group N=100	Control Group N=329	OR 95 CI	p-value
* *CC	58 (58%)	287 (87.2%)		
CT	32 (32%)	37(11.2%)		0.000
TT	10 (10%)	5 (1.5%)		
* *C allele	148 (72.5%)	614(92.7%)	4.5	0.001
T allele	52 (26%)	48(7.3%)	2.961-7.046	

* *Genotype and allele frequencies were compared by χ^2 test between patient and control group in comparison to wild type (CC genotype, C allele) taken as reference, N=Number.

Table 2. Multivariable logistic regression analyses between controls and patients.

Variable	B	P-value	OR	95% CI
Diabetes	-791	0.010	0.453	0.249-0.826
Alcoholic	-1.827	0.000	0.161	0.084-0.310
Smoking	1.420	0.000	4.139	2.23-7.673
MTHFR CT genotype	0.984	0.003	2.674*	1.393-5.134
MTHFR TT genotype	1.389	0.024	4.010*	1.205-13.347

Genotypes were compared in cases and controls; * risk as compared to wild type.

CP risk to the tune of 4.0 and 2.6 folds, respectively. The TT genotype was associated with a 4 fold increased risk of chronic pancreatitis when compared with CC. Interestingly, our results showed a trend of increasing frequency of 677TT genotype in chronic pancreatitis patients.

Interaction of MTHFR genotypes with disease and other factors

Stratified analyses revealed that the C677T genotype increases the risk of chronic pancreatitis with other symptomatic factors like diabetes and pancreas divisum. These factors contribute in the disease progression. In chronic pancreatitis with pancreas divisum the frequency of 677T was 20%, which was same as that in chronic pancreatitis with diabetes (20%). There was increased frequency of C677T genotype (57.60%) in chronic pancreatitis with diabetes mellitus as compared to only chronic pancreatitis cases (27.3%), which was significant (P= .049). The results showed that many factors play roles in disease progression and in enhancement of its complications. Moreover, diabetes and pancreas divisum could be a manifestation of the chronic pancreatitis. Other factors that were associated with an increased risk of chronic pancreatitis were alcoholic, smoking and family history of pancreatitis (Table 3).

On performing independent sample T test with diabetes as symptom in chronic pancreatitis patients with MTHFR (C677T), a positive interaction was found between genotype and diabetes with respect to the risk of chronic pancreatitis. A significant association with Pearson (P< .001) was observed (Table 4). The data indicate that MTHFR with diabetes as a risk factor in CP acts as a modifier and probably as an advancing factor in disease progression. However, no association between MTHFR and Jaundice was found, which proved that jaundice as a factor in obstructive pancreatitis played no role in disease progression. These results may implicate a possible role of C677T polymorphism as a risk factor for chronic pancreatitis. The frequency of CT genotype showed variation with respect to the complications (Table 4).

DISCUSSION

This hospital-based case-control study has shown a significant association between the MTHFR C677T polymorphism and risk of chronic pancreatitis in Indian population. Individuals carrying the homozygous TT variant genotype conferring low enzyme activity had a 4 fold increased risk of developing chronic pancreatitis compared with individuals with the CC wild-type or the heterozygous CT genotype. The results of one study published so far by Ivan and Jelena (12) in 2008 have indicated increased frequency (51%) of the C677T polymorphism in chronic pancreatitis patients in Serbian population but it was not statistically significant with (P=.458) (13). The allele frequency in Europeans was 24%–40% (34), 26%–37% in Japanese (18) and >11% in the African American population (30). Results obtained in our study indicated 32% genotype prevalence of C677T polymorphism and it was significant. The T allele frequency was 26% with OR of 4.568 (95%CI=2.961-7.046) (P< 0.001). The increased incidence of disease caused by the 677TT mutation provided a new insight in the disease pathogenesis. This could allow the C677T mutation to behave as an effectively positive polymorphism so that it could play a crucial role in the

Table 3. Characteristics of controls and patients.

Characteristics	Controls	CP patients	P- value
N	329	100	-
Age (Years)	36.86 ± 1.23*	34.86 ± 1.183*	0.217
Sex (M:F)	243:86	71:29	0.892
Weight (Kg)	68.19 ± 9.634	56.23 ± 13.230	0.001
BMI	25.16 ± 3.152*	21.67 ± 4.534*	0.000
Diabetic:nondiabetic	121:207	47:53	0.080
Smoker:nonsmoker	31:298	39:61	0.004
Alcohalic:non-alcoholic	74:225	20:80	0.333
Triglyceride (45-150 mg/dL)	154.62 ± 39.378*	170.34 ± 49.61*	0.131
Total Cholesterol (125-250mg/dL)	95.34 ± 27.47*	151.18 ± 43.03*	0.000
HDL-cholesterol (23-60mg/dL)	97.72 ± 31.480*	36.62 ± 7.678*	0.000
LDL-cholesterol (92-148mg/dL)	38.41 ± 7.76*	86.35 ± 31.11*	0.000
VLDL- cholesterol (10-30mg/dL)	19.54 ± 6.61*	25.96 ± 12.22*	0.000

*Mean± SD, p-value (with Yates correction), N=Number.

Table 4. Association of genotypes with category distribution of chronic pancreatitis patients.

	MTHFR C677T polymorphism			Total
Category	**CC**	**CT**	**TT**	
Alcoholic chronic pancreatitis (ACP)	8 13.8%	1 3.0%	0 0%	9 8.9%
Chronic pancreatitis (CP)	21 36.2%	9 27.3%	5 50.0%	35 34.7%
CP+ PD (pancreas divisum)	2 3.4%	3 9.1%	2* 20%	7 6.9%
CP+ PD +DM (Diabetes mellitus)	0 0%	1* 3%	1* 10%	2 2%
CP+DM	27 46.6%	19 57.6%	2* 20%	48 47.5%

* Number of TT and CT genotypes, %= frequency of genotypes.

disease progression (22).

Since, numerous environmental factors play important role in the etiologies of chronic pancreatitis (33), therefore, in our study we have observed a clear dose-response relationship in the interaction between cigarette smoking and MTHFR 677 TT genotype in the study population with OR 4.139(95% CI=2.23-7.673) and (P<0.001). The interaction between the MTHFR polymorphism and smoking with respect to disease risk has been observed in many previous investigations. For example, cigarette smoking interacts with the MTHFR polymorphisms resulting in an increased menace of cardiovascular disease (12, 2) and cancer of the colon (34), as well as stomach (7). It acts as a modulating factor in chronic pancreatitis. Many chemical carcinogens present in tobacco smoke are known to cause DNA damage. The folate deficiency has been associated with a reduced capacity of DNA repair, which makes an individual more susceptible to smoking induced DNA damage and gene mutation. The other factors which help increase the disease progression include diabetes.

The explanation of the above results can be deduced from the important role of MTHFR enzyme in the methylation process (5). Aberration of genomic DNA methylation may have association with the cell excess hyperplasia. It was shown that genomic DNA methylation, especially methylation of related gene point, could regulate gene expression. Hypomethylation as a result of reduced MTHFR activity leads to altered gene expression thereby affecting important functions of the cell which could represent a basis for the disease development (8, 28). Chronic pancreatitis increases genomic damage and cellular proliferation leading to the malignant transformation of pancreatic cells and ultimately affecting the development of pancreatic cancer (3, 37).

The MTHFR 677 CT or TT genotype leads to accumulation of 5, 10-methylene tetrahydrofolate which is required for conversion of uridylate to thymidylate; whereas it is thought that individuals harboring the MTHFR 677 CC genotype have less DNA synthesis and repair capacity. In addition, animals treated with ethionine, an inhibitor of cellular methylation reactions, develop acute hemorrhagic pancreatitis as a consequence of autolytic destruction of the pancreas. It is possible that a mutated MTHFR gene leads to an altered regulation of gene for inflammatory mediators which can inflict serious disturbances in inflammatory response and consequently affect the risk of CP.

In conclusion, our study has shown that the MTHFR 677TT genotype has a significant effect on the risk of CP. A strong synergistic interaction of this genotype with cigarette smoking, alcohol consumption and diabetes was detected (33). The complex interactions of these genetic and environmental factors in CP play an important role in disease progression. Such knowledge may have important implications for the primary management of chronic pancreatitis. Further, these results establish a relationship between environmental and genetic factors on the etiopathogenesis of CP.

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Other articles in this theme issue include references (38-65).

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