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ORIGINAL ARTICLE

Liver Cirrhosis Patients Homozygous for MTHFR C677T Develop Portal Vein Thrombosis 8 Years Earlier Than Wild Type

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Abstract

Background and Aim Age at portal vein thrombosis (PVT) in liver cirrhosis (LC) carriers of the methylene tetrahydrofolate reductase (MTHFR) rs1801133 (C → T667 transition) polymorphism has never been addressed; we compared age at PVT in LC patients genotyped for the MTHFR and explored the interrelated clinical and laboratory factors predicting age at PVT.
Approach and Results Retrospective cross-sectional cohort study. PVT participants: MTHFR CC $n = 36$, MTHFR CT $n = 53$, MTHFR TT $n = 19$; age, sex, age at PVT, Child–Pugh score, rs1799963 PT polymorphisms (G → A 20,210 transition), plasma HC and natural anticoagulants available for all participants. Age at PVT was lower in MTHFR TT than CT and CC (56 ± 13 vs. 57 ± 13 vs. 64 ± 9 years, $p = 0.001$); median (IQR) plasma HC was higher in MTHFR TT than in the other groups [(17 (9.4, 23.3) vs 13 (8,14.7) vs 11 (8.9, 12.7) µmol/l, $p = 0.03$)]. MTHFR TT, male gender and protein C predicted age at PVT ($p = 0.02$, p = 0.04 and $p = 0.08$); MTHFR TT and Child–Pugh score predicted plasma HC ($p = 0.005$ and $p = 0.01$) as well as low plasma protein C ($p < 0.0001$ and $p = 0.0002$). Plasma HC inversely related to protein C in the MTHFR TT group ($p < 0.0001$). Compound MTHFR TT with PT GA had lower age at PVT compared to MTHFR TT alone (49 ± 18 vs 58 ± 12 years).
Conclusions MTHFR TT anticipates PVT associated with LC by an average of 8 years; MTHFR TT associates with severity of liver disease and to high plasma HC; the latter may contribute to the prematurity of PVT by interfering with the anticoagulant activity of protein C.

Keywords Homocysteine · Child–Pugh score · Protein C

Introduction

Homocysteine (HC) is a sulfhydryl-containing amino acid the intracellular and plasma concentrations of which are under the control of three enzymes: cystathionine beta synthase, methionine synthase and methylene tetrahydrofolate reductase (MTHFR); reduced enzymatic activity due to inherited deficiencies or genetic polymorphisms of any of these three genes impair the metabolism of HC that may reach toxic concentrations leading to premature atherothrombosis [1].

The rs1801133 polymorphism of the MTHFR gene (C → T transition at position 677) codes for an enzyme with 70% reduced activity carried by almost 25% of the Italian population: this enzymatic phenotype causes mild to moderate increase of plasma HC that favors an increased risk of venous thromboembolism in Caucasians [2]. The onset of venous thromboembolism in carriers of the MTHFR TT genotype occurs 10 years earlier than in heterozygous or wild-type carriers [3] suggesting that clinical phenotype of the MTHFR TT genotype may be a premature age at thrombosis onset.

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A recent meta-analysis on MTHFR and liver cirrhosis (LC) found a 17% prevalence of the MTHFR TT genotype that was not associated with portal vein thrombosis (PVT) [4] at variance with an earlier meta-analysis [5]; however, PVT associated with LC is a potentially life-threatening complication [6] and carriership of the MTHFR TT may have implications for liver transplant candidates [7].

Given that elevated plasma HC is associated with oxidative stress [8], given that both HC and oxidative stress may induce post-translational modifications in proteins [9] and given that plasma prothrombin (PT) may undergo oxidative activation in vitro [10], we investigated whether PVT patients with LC, carriers of the MTHFR TT genotype, underwent the abovementioned anticipation effect, that is, developed PVT at an earlier age than heterozygous and wild-type patients; moreover, we argued whether compound MTHFR TT + the heterozygous rs1799963 PT (G → A transition at position 20,210) or + the homozygous PT AA genotype lowered the age at PVT presentation compared to MTHFR TT in isolation. We also explored possible interrelationships among MTHFR genotypes, Child–Pugh score and the plasma concentration of natural anticoagulants.

Participants and Methods

Study Type and Participants

Retrospective cross-sectional cohort study of LC patients with PVT referred to the Hemostasis Unit of the Ospedali Riuniti di Foggia (Foggia, Italy) for an extensive thrombophilia screen consisting of antithrombin, protein C, protein S, anticardiolipin antibodies, lupus anticoagulant, HC, factor V Leiden, PT 20,201 and MTHFR genotypes. All samples for thrombophilia screens including plasma HC were taken at the time of the PVT diagnosis, before anticoagulation initiation, to minimize the chance of having worsening values of the clotting screens, natural anticoagulants and HC linked with a possible progression or decompensation of LC after six or more months of oral or parenteral anticoagulation.

For the purpose of this retrospective cohort survey the inclusion criteria were: (1) diagnosis of liver cirrhosis by imaging methods and/or by liver biopsy; (2) PVT confirmed by imaging (doppler ultrasound, computerized tomographic scanning, magnetic resonance and and/or their angiographic counterparts); (3) genotyping for MTHFR and PT as part of the thrombophilia screen; (4) complete demographic and clinical data.

Our electronic database held the data of 298 patients presenting with a first PVT who had been genotyped for MTHFR between January 2000 and July 2011; 6 records were excluded because of insufficient data (missing date of birth or date of PVT diagnosis); to focus on PVT occurring in LC, we excluded 38 records of PVT occurring in chronic hepatitis and 125 records of PVT unrelated to LC; from the remaining 129 records dealing with LC and PVT, 21 were excluded because of circumstantial risk factors for PVT: 11 hepatocellular carcinomas, 4 cholangiocarcinomas, 3 post-variceal endoscopic sclerotherapy and 3 post-transjugular intrahepatic portosystemic shunt. The final 108 records represent the data of our final cohort (Table 1).

Genetic, Immune and Clotting Assay

DNA was extracted from peripheral blood leukocytes and submitted to polymerase chain reaction for the detection of the MTHFR as described previously [11]. Plasma HC was measured via a commercially available ELISA (Bio-Rad, Oslo, Norway); according to this method protein-bound HC is hydrolyzed to free HC that is enzymatically converted to S-adenosyl-L-homocysteine (SAH); the sample SAH competes with SAH immobilized on the walls of the microtiter plate for binding sites on a monoclonal anti-SAH antibody. The cut-off for positivity was set at the 95th percentile, 12.5 umol/L; this derived from 90 blood samples of 40 apparently normal subjects and 50 healthy hospital employees who underwent periodical health screens [male 51, female 49, median age (IQR) 33 (22, 54)]; inter and intra-assay coefficient for plasma HC was 3.7% and 4.1%, respectively. The thrombophilia screen was completed by the measurement of antithrombin, protein C (chromogenic assays from Behring, Marburg, Germany) and free protein S antigen (ELISA, Diagnostica Stago, Asnieres, France). The reference ranges (mean ± 2 SD) for antithrombin (66–112 U/dl), protein C (60–118 U/dl), free protein S (60–122 U/dl) were computed from 46 healthy hospital employees [(male 23, female 23, median age (IQR) 37 (9, 18)].

Outcomes

Our first null hypothesis was that MTHFR TT participants with LC showed similar age at incident and first PVT as MTHFR CT and CC participants; our second null hypothesis was that mean age at first PVT in compound MTHFR TT and PT GA or AA patients was similar to that of MTHFR TT in isolation. We also investigated the interrelationship between other clinical and laboratory variables.

Statistics

Normally distributed data are expressed as mean and standard deviation and compared by analysis of variance; non-normally distributed data were compared by Kruskal–Wallis or Mann–Whitney tests; χ-square test compared frequencies across groups. Multivariable analysis was run with backward elimination to minimize issues with variable selection [12].

Table 1 Demographics, clinical and laboratory features liver cirrhosis patients by genotype

MTHFR	CC		CT		TT		*p*
No	36		53		19		
M/F	21/15		39/14		12/7		
Age years ($\bar{x} \pm \sigma$)	82 ± 11		76 ± 12		76 ± 13		
Age at PVT, years ($\bar{x} \pm \sigma$)	64 ± 9		57 ± 13		56 ± 10		0.03
	No	%	No	%	No	%	
Etiology							
HBV	10	27.7	18	33.9	7	36.8	
HCV	23	63.8	30	56.6	8	20.5	
HBV + HCV	2	5.5	3	5.6	1	5.2	
Cryptogenic	1	2.7	2	3.7	3	15.7	
Severity							
Child–Pugh score ($\bar{x} \pm \sigma$)	7.9 ± 2.2		8.7 ± 2.3		11.6 ± 3.1		0.001
Child–Pugh A	11	30.5	9	16.9	0	0	
Child–Pugh B	16	44.5	26	49.0	6	31.6	
Child–Pugh C	9	25.0	18	33.9	13	68.4	0.007*
Clinical features							
Ascites	19	52.7	15	28.3	10	52.6	0.01
Encephalopathy	2	5.5	3	5.6	4	21.0	0.08
Varices	10	27.7	14	26.4	5	26.3	ns
Cavernoma	1	2.8	1	5.6	3	15.7	0.03
PT G20210A	3	8.3	2	3.7	3	15.7	
INR ($\bar{x} \pm \sigma$)	1.2 ± 0.13		1.4 ± 0.27		1.6 ± 0.25		0.001
aPTTr ($\bar{x} \pm \sigma$)	1.12 ± 0.10		1.13 ± 0.14		1.40 ± 0.16		0.001
AT IU/dl ($\bar{x} \pm \sigma$)	86 ± 17		82 ± 20		55 ± 11		0.001
PC IU/dl ($\bar{x} \pm \sigma$)	60 ± 10		52 ± 11		41 ± 9		0.001
PS IU/dl ($\bar{x} \pm \sigma$)	70 ± 12		67 ± 12		60 ± 7		0.02
HC μmol/L, median (IQR)	11 (8.9, 12.7)		13 (8.0, 14.7)		16 (12.1, 18.4)		0.002
HC > 12.5 μmol/L	13	33.3	27	50.9	14	73.6	

MTHFR methylene tetrahydrofolate reductase, *No* number, *M/F* male/female, *PVT* portal vein thrombosis, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *PT* prothrombin, *INR* international normalized ratio, *aPTTr* activated partial thromboplastin ratio, *AT* antithrombin, *PC* protein C, *PS* protein S, *HC* homocysteine

*Refers to the total cross-tabulations

A two-tailed $p < 0.05$ was considered statistically significant for all statistical analyses. All calculations were performed using the MedCalc Statistical Software (MedCalc Software, version 19.2.6, Ostend, Belgium).

Results

Comparative Demographics, Clinical and Laboratory Variables by MTHFR genotypes

MTHFR TT participants were younger than CT and CC participants and their mean age at PVT presentation was lower than CC participants; their average Child–Pugh score was higher as well as the frequency of Child–Pugh score C, and they were more likely to have cavernomas. Moreover, the MTHFR TT group had the highest mean plasma concentration of plasma HC, the highest mean aPTTr and INR, and the lowest mean plasma concentrations of natural anticoagulants (Table 1). According to the prevalence of elevated HC, the relative risk of developing PVT in MTHFR TT compared to MTHFR CC was 1.894 (95% CI 1.114 to 3.222; $p = 0.01$).

Effect of Genotypes and Other Variables on Age at Portal Vein Thrombosis

A first regression model having sex, MTHFR, PT 20,210, etiology of LC, Child–Pugh score and HC as categorical dependent variables, identified MTHFR TT and male gender as an independent predictor of age at PVT (Table 2A). A second regression model having natural anticoagulants, Child–Pugh score and HC as continuous independent

Table 2 Independent predictors of age at portal vein occlusions, plasma protein C and plasma homocysteine

(A) Independent predictors of age at portal vein occlusion				
Independent variables	B	SE	t	P
Categorical				
MTHFR	−3.9584	1.6955	−2.335	0.02
Gender male	4.9763	2.5035	1.988	0.04
Continuous				
Protein C	0.1699	0.09626	1.765	0.08
(B) Independent predictors of plasma protein C				
Categorical				
MTHFR TT	−7.8996	1.5774	−5.008	<0.0001
Child–Pugh score	−3.4159	1.5208	−2.246	0.02
Continuous				
Child–Pugh score	−1.4341	0.3674	−3.904	0.0002
HC	−0.4212	0.0761	−5.529	<0.0001
(C) Independent predictors of plasma homocysteine				
Categorical				
MTHFR TT	5.6124	1.7654	3.1798	0.001
Continuous				
Child–Pugh score	1.1453	0.4550	2.517	0.01

SE standard error, *MTHFR* methylene tetrahydrofolate reductase

variables identified protein C as a weak predictor of age at PVT after adjusting for sex (Table 2A).

Mean age at PVT was lower in compound MTHFR TT + PT G20210A ($n = 3$) compared to MTHFR TT in isolation ($n = 16$) (49 ± 18 vs 58 ± 12 years) and the median (IQR) plasma HC was also lower in the compound group [8.5 (8.4, 13.9) vs 17 (11.8, 21.9) µmol/L, $p = 0.12$)].

Effect of Genotypes and Other Variables on Plasma Protein C Concentration

A first regression model that included sex, MTHFR, etiology of LC, Child–Pugh score and plasma HC as categorical independent variables and protein C as dependent variable, identified MTHFR TT and Child–Pugh score as an independent predictors of plasma protein C (Table 2B). A second regression model that employed MTHFR, Child–Pugh score and plasma HC as continuous independent variables identified Child–Pugh score and plasma HC as positive and negative predictors of plasma protein C respectively, after adjustment for sex. Plasma HC and protein C of the whole cohort were inversely correlated ($n = 108$, $r = -0.518$, $p < 0.001$) but this was accounted mostly by the strong correlation in the MTHFR TT group (Fig. 1).

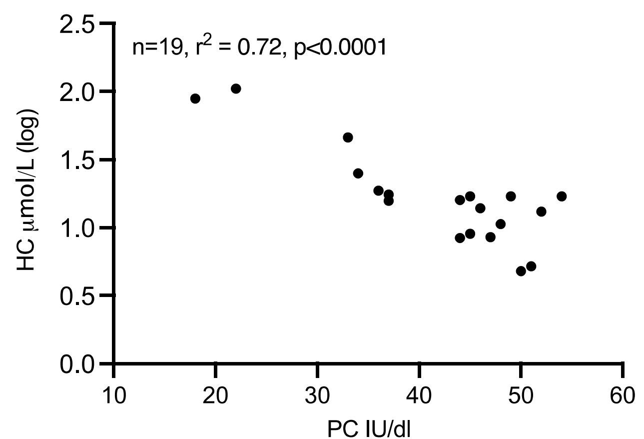

Fig. 1 Correlation between plasma homocysteine (HC) and protein C (PC) in patients with the methylene tetrahydrofolate reductase TT genotype

Effect of Genotypes and Other Variables on Plasma Homocysteine

A first regression model including sex, MTHFR, Child–Pugh score, etiology of liver cirrhosis as categorical independent variables and HC as dependent variable, identified MTHFR TT as independent predictor of plasma HC. A second regression model that employed age at diagnosis and Child–Pugh score as continuous independent variables identified

Child–Pugh score as an independent predictor of plasma HC after adjustment for sex (Table 2C).

Effect of Genotypes and Other Variables on Extent of Portal Vein Thrombosis Cavernoma

A regression model having cavernoma as the dependent variable and sex, MTHFR, PT G20210A, etiology of liver cirrhosis Child–Pugh score and plasma HC as independent categorical variables failed to identify any independent predictors; likewise, a regression employing plasma HC and natural anticoagulant concentrations as continuous variables after correction for gender, failed to identify any predictors.

Discussion

The mean age at PVT in LC carriers of MTHFR TT was 56 years, 8 years earlier than the MTHFR CC, a significant difference that refutes our null hypothesis and confirms the anticipation effect. Interestingly, the MTHFR TT genotype, gender and to a lesser extent protein C predicted age at PVT: one meta-analysis noted that MTHFR TT increased the risk of PVT in LC but did not explore the age at presentation [5]; our study is the first to compare age at presentation of PVT of an entire LC cohort genotyped for MTHFR after exclusion of circumstantial risk factors for LC-related PVT, in order to highlight the effect of the MTHFR genotypes. Indeed, the MTHFT TT genotype independently predicted not only age at PVT but also plasma HC concentration, whereas a previous meta-analysis ruled against an association between plasma HC and PVT in LC [5]. The relative risk of developing PVT in the MTHFR TT group was 1.894, but it should be remembered that our cohort study differs in conception from the case–control studies included in the previous meta-analysis [5].

Neither gender predicted plasma HC in out cohort [13], but the Child–Pugh score did: although the liver does not harbor the highest RNA and protein content related to MTHFR [14], a reduction in the proteo-synthetic activity of the liver may result in decreased MTHFR enzymatic activity that adds to the poor disposal of plasma HC; this in turn contributes to worsening liver damage [15, 16]; however, despite being significant, median values in our MTHFR TT group were not particularly higher than in other groups.

The reduction in the proteo-synthetic activity of the liver was maximal in the MTHFR TT group, that showed the highest INR and aPTTr and the lowest plasma concentrations of natural anticoagulants, particularly protein C; indeed, MTHFR TT, Child–Pugh score and plasma HC were strong independent predictors of plasma protein C. Previous studies reported that protein C predominated among the natural anticoagulant deficiencies in LC [17], it was lowest in patients with Child–Pugh score C [18] and appeared deeply implicated in the development of PVT in LC [19].

The inverse relation between plasma protein PC and HC in the whole cohort, but maximal in the MTHFR TT group is of great interest; elevated intracellular HC, via a thiolactone intermediate, may form an adduct with the ε-amino group of lysyl residues present in protein C, a post-translational modification by which this anticoagulant becomes unable to digest factor Va and VIIIa in vivo, leading to thrombosis, and becomes unable to digest its substrate in the chromogenic assay, leading to low functional levels [20–22].

Additional factors may explain the earlier age at PVT presentation in MTHFR TT carriers: LC is characterized by systemic inflammation in the general circulation [23, 24], whereas leakage of bacterial lipopolysaccharide from the intestine into the portal circulation causes inflammasome activation [25, 26] and thrombin generation [27], the latter further aided by the oxidation of lipids, abundant in the portal affluents [28]; on this background, the additional oxidative effect of elevated HC [29] and that of LC [30] may anticipate the age at PVT in MTHFR TT carriers.

The overall prevalence of cavernoma in our cohort was low at 4.6%, but more common in the MTHFR TT group: the oxidative stress that characterizes LC [31] is invariable accompanied by nitrative stress [32]; the latter may inhibit the activity of cystathionine beta synthase (CBS), the enzyme that converts HC to cystathionine then to cysteine [33], favoring an increased intracellular and plasma concentration of HC, that in turn causes disulfide redox inhibition of CBS [34], perpetuating the elevated HC that could contribute to repetitive portal occlusions and cavernoma formation.

By similar mechanisms, we postulated that the higher concentration of plasma prothrombin in the portal circulation of patients with the PT GA genotype [35] would be more susceptible to activation by the oxidation of low-density lipoprotein [8] in the presence of the double oxidative hit caused by the elevated plasma HC [29, 33, 34] and by the LC proper [30]: although the PT GA did not predict age at PVT, patients with the compound MTHFR TT + PT GA had a lower mean age at PVT compared to MTHFR TT alone, refuting thus our second null hypothesis, but unfortunately the small sample sizes prevents definite conclusions.

The limitations of our cohort survey are severalfold: (1) the retrospective cross-sectional design that prevents causality; (2) the lack of information regarding the duration of the liver disease before PVT developed; (3) the lack of B12/folate measurement and of the genotyping of other genes involved in HC disposal, as 33% of our MTHFR CC presented with an unexplained elevated plasma HC, though at low concentrations; (4) the lack of medium to long term follow-up data that would have informed about the status of the portal vein after oral anticoagulation.

Conversely, our cohort consisting of LC patients who had no circumstantial factors for PVT development, allows us to conclude that: (1) MTHFR TT carriers developed PVT almost 8 years earlier than MTHFR CC; (2) the interplay of disease severity, elevated plasma HC and low, and possibly dysfunctional, plasma protein C, favor the development of PVT.

Some consensus argues against the inclusion of the MTHFR genotypes in the thrombophilia screen [36, 37], though our cohort was tested before the first of these were drawn up [36] and now adds controversy to this topic. Our data suggest that LC patients would benefit from MTHFR genotyping at diagnosis of LC, as primary thromboprophylaxis could delay the emergence of PVT [38], a life-threatening complication [7] that has bearings for a subsequent liver transplant [39] alongside MTHFR itself [40]; this suggestion is even more relevant because folic acid supplementation did not reduce intracellular HC [41] and failed to prevent primary [42] and secondary venous thrombosis [43].

In conclusion, the MTHFR TT genotype is deeply implicated in the severity of LC, in the plasma HC elevation and low plasma protein C concentration that may all contribute to the prematurity of PVT. Whether LC patients should be genotyped for MTHFR is debatable [36, 37] but we would endorse it in this setting.

Author's contribution All authors contributed to the study conception and design. PRJA, FG and VM designed the study; PRJA, LI and AA collected the data; PRJA and MM analyzed the data. The first draft of the manuscript was written by Paul RJ Ames, all authors provided comments on the first draft that was finalized by FG and MM. All authors read and approved the final manuscript.

Declarations

Conflict of interest None of the authors has any financial or non-financial competing interest to declare.

Ethical approval At the time of their attendance for the thrombophilia screen patients gave informed and written consent to the use and storage of their genetic material and of their anonymized clinical information as per approval of the local Ethics Committee.

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