The MTHFR 677C>T polymorphism is associated with an increased risk of hepatocellular carcinoma in patients with alcoholic cirrhosis

Raphael Saffroy1,2, Patrick Pham1,2, Franck Chiappini1,3, Marine Gross-Goupil1,2, Laurent Castera1,2, Daniel Azoulay1,2, Alain Barrier5, Didier Samuel4, Brigitte Debuire1,2 and Antoinette Lemoine1,2,6

1Service de Biochimie et Biologie moléculaire, 2INSERM 602, Hôpital Paul Brousse, 14 Avenue Paul Vaillant Couturier, F-94804 Villejuif Cedex, France, 3Pfizer, Amboise, France, 4Centre Hépato-biliaire, UPRES 3541, Hôpital Paul Brousse, IFR 89, Faculté de Médecine Paris-Sud, Université Paris XI, 14 Avenue Paul Vaillant Couturier, F-94804 Villejuif Cedex, France and 5Inserm U444, CHU St Antoine, Paris, Assistance Publique-Hôpitaux de Paris, France

Email: antoinette.lemoine@pbr.ap-hop-paris.fr

Methylenetetrahydrofolate reductase (MTHFR), a key enzyme in folate metabolism, plays a major role in the provision of methyl groups for DNA methylation and in the production of dTMP for DNA synthesis. Different polymorphisms have been described for this enzyme, the most studied being the C677T, which has been shown to be associated with predisposition to colorectal cancer in patients who consume a high alcohol diet. The aim of this study was to determine whether the MTHFR polymorphism is related to hepatocellular carcinoma (HCC) in patients with alcoholic cirrhosis. MTHFR genotypes were determined in 300 liver transplant patients, 72 of whom had alcoholic cirrhosis with HCC and 122 of whom had alcoholic cirrhosis without HCC. The remaining patients were transplanted for HCC on normal liver (n = 27) or viral cirrhosis with HCC (n = 49) or without HCC (n = 30). We also tested 80 healthy subjects. Among the group of patients transplanted for alcoholic cirrhosis, the frequency of MTHFR variants CC versus CT and TT was significantly higher in patients with HCC than in patients without macroscopic evidence of HCC (P = 0.02). This difference was not observed between patients with and without HCC developed either on viral cirrhosis or on non-cirrhotic liver. If we considered all the patients transplanted for HCC, the MTHFR CC genotype was significantly higher in patients who had developed HCC on alcoholic cirrhosis rather than on viral cirrhosis (P = 0.002) or on non-cirrhotic livers (P = 0.02). The relative risk for HCC in subjects with alcoholic cirrhosis and the CC genotype was 2.03. These results suggest that the MTHFR CC genotype increases the risk to develop HCC in patients who consume a high alcohol diet.

Introduction

Hepatocellular carcinoma (HCC) is the third most frequent cause of death from cancer in men worldwide (1). Although HCC typically occurs in livers that are affected by an underlying disease, such as chronic hepatitis B and/or C, it is generally believed that genetic alterations are the basis for this. Indeed, it has been shown that the risk of HCC is increased in patients with haemochromatosis and alpha-1-antitrypsin deficiency (2,3), and that smoke toxins and/or ethanol consumption play a role in virus-mediated carcinogenesis (4). Furthermore, chromosomal aberrations have been detected in HCC patients (5,6). All of these findings indicate that genetic events play a role in the development of HCC. A number of candidate genes with a weak link have been studied to date. These include glutathione S-transferases, cyp1A1, cyp2C19 and cyp2E1 (7–11). More recently, a genetic link was revealed between HCC and polymorphisms of the UDP-glucuronosyltransferase (UGT1a7) gene (12) and p53 (13).

5,10-Methylenetetrahydrofolate reductase (MTHFR), a key enzyme in folate metabolism was shown recently to present a genetic polymorphism (C677T) associated with predisposition to colorectal cancer (14–16), acute lymphocytic leukemia (17,18), endometrial cancers (19) and oesophageal cancers (20). Other MTHFR gene polymorphisms were described as A1298C (21,22) but these polymorphisms influence specific activity of the enzyme to a lesser extent than the C677T polymorphism. MTHFR plays a central role in the provision of methyl groups and in folate metabolism, reducing 5,10-methylenetetrahydrofolate to 5-methyl-THF. 5-Methyl-THF serves as a substrate for the re-methylation of homocysteine to methionine. The methylation of homocysteine is catalysed by vitamin B12. MTHFR is also linked to the production of dTMP via thymidilate synthase and to purine synthesis. Thus, it plays a role in the provision of nucleotides that are essential for DNA synthesis. Individuals who are heterozygous (CTs) or homozygous (TTs) for this MTHFR polymorphism have an in vitro enzyme activity that is 65 and 30% of that of (CCs) individuals, respectively (23).

A change in MTHFR activity may thus affect both DNA methylation and DNA synthesis; interactions with homocysteine, vitamin B12 and folates should be seen. Low blood concentrations of folates have been shown to be associated with several diseases including alcoholic liver cirrhosis (24) and cancer (25). In alcoholic cirrhosis, folate availability is related to alcohol intake as alcohol affects the intestinal absorption, metabolism and renal excretion of folate (26). Alcohol can also act directly as a methyl group antagonist (27). Moreover, patients with diets high in alcohol face an increased risk of developing HCC or colorectal cancer.

To evaluate the role of folate metabolism in liver carcinogenesis, we examined in patients with and without HCC, MTHFR polymorphism, plasma folate, vitamin B12, homocysteine and the risk of HCC. We found an association between the MTHFR genotype and HCC associated with alcohol-rich diet but not with viral infection.
Materials and methods

Patients

Samples were collected from 80 control subjects and 300 patients who had undergone a liver transplant at our institution. All tissue and blood samples were obtained in accordance with local ethical rules. The mean (±SD) ages of the control subjects and patients are described in Table I; the difference was not significant. All of the patients and healthy subjects were European. The subjects were divided into six groups as detailed in Table I. The first group contained 122 patients who had undergone a transplant for alcoholic cirrhosis, and who showed no evidence of HCC when examined by abdominal ultrasonography. The absence of HCC was confirmed by a pathologist, who thoroughly examined the whole explanted liver by section at 0.5 cm intervals. The second group contained 72 patients, who underwent a transplant for alcoholic cirrhosis and had HCC as confirmed by histological examination of the explanted livers. The diagnosis of alcoholic cirrhosis was based on each patient’s medical history and on the histological observation of Mallory’s hyalin in a liver biopsy specimen. None of the patients in this group were infected by HBV, HCV, delta virus, HGV or HIV. The third group contained 80 healthy subjects. Healthy subjects were cancer-free, virus-free and non-alcoholic individuals. Illness was excluded by medical history, complete physical examination and routine laboratory evaluation. The fourth group contained 27 patients transplanted for HCC on a normal liver. These patients did not have any known aetiology, and were not infected with any known viruses. The fifth group contained 30 patients transplanted for cirrhosis associated with HBV, HCV and delta virus, but without HCC. The sixth group contained 49 patients transplanted for HCC associated with HBV, HCV and delta virus. All of the patients in groups three to six had no history of alcohol abuse.

All of the patients were followed-up for a long period before liver transplantation. This follow-up consisted of regularly measuring serum alphafetoprotein, ultrasonography and several interviews concerning their alcohol abuse.

HCC was diagnosed according to the results of imaging and histology in the explanted liver. In patients with alcoholic or viral cirrhosis but without HCC, the absence of HCC was confirmed by CT scan in the pre-transplantation period and no HCC was observed by thoroughly examining very small slides (~0.5 cm) taken from the explanted liver.

Serological tests (i.e. HCV RNA, hepatitis B surface antigen) were performed using commercially available assays (Amplicor HCV monitor; Roche Diagnostics System, Branchburg, PA). These tests were routinely performed just once before transplantation.

Samples

Blood samples or serum, systematically performed just once before transplantation for routine evaluation, were stored at −80°C until use. They were then used to measure folate, vitamin B12 and homocysteine levels using ion-capture and a microparticle immunoassay in an AXXYM analyser (Abbott Laboratories, Rungis, France). The normal reference ranges were as follows: folate 7.0–28.0 nmol/l; vitamin B12 223–1132 ng/l; homocysteine: 5–14 μmol/l. Blood samples were collected on EDTA from control subjects. Written consent was obtained from all the patients before sampling. DNA was extracted from the white blood cell fraction by use of a standard proteinase K digestion and phenol-chloroform extraction procedure.

MTHFR genotyping

The MTHFR C677T polymorphism was sought using a PCR-RFLP method (modified from Saffroy et al., ref. 28). Briefly, the forward primer (5’TGAAGGAGAAGGTCTGACGGGA3’) was modified so that it introduced an AspI restriction site (underlined), and the reverse primer (5’AGGACGGTGCGGTAGAGTG3’) was labelled so that HX was added to the 5’ end. PCR was performed in a GeneAmp 2400 PCR system (Applied Biosystems, Courtaboeuf, France) in a final volume of 25 μl containing 250 ng of the patient’s DNA, 7.5 pmol of each primer, 50 μM each deoxynucleotide triphosphate, standard PCR reaction buffer containing 1.5 mM MgCl2 and 1 U Taq DNA polymerase (Qbiogene, Illkirch, France). Amplification consisted of one cycle of 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C plus a final extension step of 7 min at 72°C. A reference sequence (288 bp) containing one AspI site amplified as described previously (26) from normal DNA was used as control for digestion.

The digestion mixture contained 1 μl of AspI (10 U/μl) (Roche Diagnostics, Meylan, France), 5 μl of the digestion control PCR product and 10 μl of the patient’s PCR product in a final volume of 25 μl. It was incubated at 37°C for 4 h.

One microlitre of the digested sample was added to a denaturing mixture containing 0.5 μl of Genescan-500 TAMRA size standard (Applied Biosystems, Courtaboeuf, France) and 23.5 μl of deionized formamide. The mixture was heated at 95°C for 5 min and then immediately placed on ice for 10 min. The ABI PRISM 310 Genetic Analyzer was set up for capillary electrophoresis with an injection time of 5 s and electrophoresis was carried out for 30 min at 60°C using the GS POP-4 denatured C module setting. Wild-type (CC) individuals only presented a 198 bp fragment, heterozygotes (CT) presented both a 176 and a 198 bp fragment, and homozygote variants (TT) presented a 176 bp fragment (Figure 1).

Statistical analysis

The Pearson χ2 test was used for comparisons involving nominal data; the Fischer t-test was used for comparisons involving numerical data to determine whether there were any statistical differences between patients. Associations between HCC and MTHFR genotypes were estimated using odds ratio (ORs) and their 95% confidence intervals (CIs). Statistical analysis was performed using the Statview software system (Statview F-4.5).

Results

Prevalence of the MTHFR CC polymorphism in cancerous patients with alcoholic cirrhosis

The group with a long history of alcoholic cirrhosis but no evidence of HCC included 122 patients; the distribution of the MTHFR variants was as follows: CC = 43%, CT = 49% and TT = 8% (Table II). In the group of 72 patients who had alcoholic liver cirrhosis with confirmed HCC, the frequency of the MTHFR variants was 60% for the homozygous CC genotype. The difference in the MTHFR wild-type (CC) genotype versus CT and TT genotypes was significant between the two groups (χ2 test, P = 0.02). The relative risk estimated as an odds ratio for HCC in subjects with alcoholic cirrhosis and the CC genotype is 2.03 (OR 95% CI, 1.10–3.60).

Prevalence of the MTHFR CC polymorphism in cancerous patients with non-cirrhotic liver

Twenty-seven of the 148 patients with confirmed HCC had a non-cirrhotic liver with no known aetiology. There were no HCV, HBV, delta, HIV or HGV viruses found and there was...
no known history of alcohol abuse. The frequencies of the CC, CT and TT genotypes were 33, 59 and 7%, respectively (Table II). These percentages were similar to those observed in healthy controls (CC \(\approx 38\%), CT \approx 46\% and TT \approx 16\%; \chi^2\text{ test, } P = 0.70\). The risk to develop HCC in subjects without cirrhosis was not associated with MTHFR CC genotype (OR = 1).

Prevalence of the MTHFR CC polymorphism in cancerous patients with viral cirrhosis

Forty-nine of the patients transplanted for HCC had viral cirrhosis. In this group, the frequencies of the CC, CT and TT genotypes were 31, 59 and 10%, respectively (Table II).

These frequencies were similar to those in the group of patients transplanted for end-stage liver viral cirrhosis with no evidence of HCC (CC = 33\%, CT = 57\% and TT = 10\%; \chi^2\text{ test, } P = 0.80\), or in the group of healthy controls. The relative risk for HCC in subjects with viral cirrhosis was not associated with the CC genotype (OR = 0.7). No differences in the MTHFR polymorphisms were observed between the HBV- or HCV-related cirrhosis or HCCs.

Prevalence of the MTHFR polymorphisms among different groups of cancerous patients

A total of 148 patients were transplanted for HCC on alcoholic cirrhosis (\(n = 72\)), viral cirrhosis (\(n = 49\)) or non-viral and
non-cirrhotic liver (n = 27). The frequency of the MTHFR wild-type CC genotype versus the CT and TT genotypes was significantly higher in patients who had developed HCC on alcoholic cirrhosis than in those who had developed HCC on viral cirrhosis (P = 0.002) or on non-cirrhotic liver (P = 0.02). We assessed the differentiation grade of the tumours by using the Edmonson classification system. The tumour grade did not differ between the different groups of patients; 8% grade I, 66% grade II, 23% grade III and 3% grade IV. No difference in the MTHFR CC versus CT, TT polymorphisms regarding the tumour differentiation was observed between the different groups of cancerous patients.

**Relationship between plasma folate, vitamin B12, homocysteine and MTHFR genotype**

MTHFR is involved in the folate and methionine metabolic pathways. Table III depicts the interactions between MTHFR genotype and plasma levels of folates, vitamin B12 and homocysteine assessed just before liver transplantation. There were no significant differences observed between the different groups; however, homocysteine levels tended to be elevated in patients who abused alcohol and who possessed the TT genotype.

**Discussion**

For multifactorial diseases such as HCC, the identification of modifier genes should make it possible to define hepatocarcinogenic risk factors. We analysed the MTHFR 677 C>T gene polymorphism and showed that there is a statistically significant association between the homozygous MTHFR CC genotype and the risk of HCC in patients with a high alcohol intake, but not in patients with other aetiologies.

The MTHFR gene is involved in multiple vital cellular metabolic pathways including DNA methylation and nucleic acid synthesis. The change of MTHFR activity, caused by genomic alterations or interactions, could play a role in multistage hepatocarcinogenesis. This hypothesis has never been explored. We hypothesize that there may be a relationship between the MTHFR polymorphism and the risk of HCC in patients with alcoholic cirrhosis because: (i) alcohol intake has been shown to be related to whole body folate availability (26), (ii) folate deficiency has been shown to be associated with the risk of cancer and (iii) the development of HCC, as well as colorectal cancer, has been described in patients with high alcohol diets.

To test this hypothesis, we chose to study liver transplant patients. These patients undergo total hepatectomy, which makes it possible to carry out an exhaustive histological search for HCC using imaging in cirrhotic patients with no evidence of HCC or to confirm the diagnosis of HCC. Moreover, all the patients with alcohol cirrhosis and no HCC underwent regular and complete check-ups for several years in our institution, making it to show that these patients were not at increased risk of developing a primary liver tumour even though they had a long history of alcohol abuse.

Folate levels were lower and vitamin B12 and homocysteine levels higher in our patient group with alcoholic aetiology than in our control group or in the group of patients transplanted for HCC without alcohol history. However, these differences were not significant. This may be due to the small number of patients, but also to the characteristics of our patients. Indeed, all of them had end-stage cirrhosis, often complicated by portal hypertension. Moreover, all of the patients had to wean before they could be transplanted, and weaning is known to increase folate and decrease homocysteine levels compared with non-abstaining patients (22,29,30).

The common C677T transition in the gene encoding MTHFR has been reported to significantly reduce the risk of colorectal cancer (14–16) and acute lymphocytic leukaemia (17,18). In our study, the frequencies of the MTHFR genotypes in the group of healthy subjects were comparable with those described previously in the general population (23). Similar frequencies were also observed in the two populations of patients transplanted either for HCC on normal liver or for HCC associated with viral infection, neither of which had a history of alcohol abuse. These results suggest that the MTHFR metabolic pathway is not implicated in hepatocarcinogenesis on normal liver or associated with viral infection. Similarly, the frequencies of the MTHFR variants in the alcoholic cirrhosis patients with no histological evidence of HCC were not significantly different than those observed in the healthy subjects.

However, we found that the risk to develop a primary liver tumour was significantly increased in the subjects with MTHFR CC genotype and alcoholic cirrhosis. These results are consistent with the previously published results on the association between the MTHFR CC genotype and the increased risk of colorectal cancer (14–16) and with the fact that alcohol is associated with an overall increased risk of cancer in this and other populations (4,26). However, the relative risk for HCC in subjects with alcoholic cirrhosis and CC genotype is weak (2.03). Thus, the influence of other genetic factors cannot be excluded such as MTHFR 1298 A>C genotype or alcohol dehydrogenase 3 (21,22), although these polymorphisms have not been clearly identified as associated risk with cancer.

A change in MTHFR activity may thus influence both DNA methylation and DNA synthesis (Figure 2). We have shown

**Table III. MTHFR genotype and plasma levels of folates, vitamin B12 and homocysteine**

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects (group 3)</th>
<th>Alcoholic cirrhosis (group 1)</th>
<th>HCC on alcoholic cirrhosis (group 2)</th>
<th>HCC without alcohol aetiology (groups 4 + 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>TT</td>
<td>CC</td>
<td>TT</td>
</tr>
<tr>
<td>Folate (nmol/l)</td>
<td>18.7 (6.2)</td>
<td>19.1 (2.3)</td>
<td>16.9 (12.4)</td>
<td>16.5 (5.6)</td>
</tr>
<tr>
<td>Vit B12 (ng/l)</td>
<td>670 (228)</td>
<td>739 (302)</td>
<td>1039 (469)</td>
<td>1060 (602)</td>
</tr>
<tr>
<td>Homocysteine (μmol/l)</td>
<td>9.3 (6.2)</td>
<td>11.2 (3.2)</td>
<td>12.5 (5.6)</td>
<td>15.7 (6.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16.9 (10.6)</td>
<td>17.1 (6.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1030 (687)</td>
<td>1107 (308)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.8 (7.1)</td>
<td>15.3 (4.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>851 (411)</td>
<td>950 (301)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.2 (3.3)</td>
<td>12.1 (3.4)</td>
</tr>
</tbody>
</table>

All data are expressed as the mean (standard deviation).
that decrease in genomic DNA methylation by methionine depletion could be associated with increase in DNA synthesis (31). Thus, the efficiency of the MTHFR pathway could modify the balance between DNA methylation and DNA synthesis. In cancer, and particularly in HCC, a global hypomethylation effect has been shown (32). However, if the change in MTHFR activity leads to hypomethylation, the frequency of the TT genotype should have increased together with homocysteine levels. In our study, the frequencies of the MTHFR genotypes were similar in the patients transplanted for HCC on viral cirrhotic liver or on non-cirrhotic liver and in the group of healthy subjects. Moreover, we did not observe a decrease in vitamin B12 levels even though it is a cofactor of methionine synthase. Thus, the role of the MTHFR pathway in liver carcinogenesis does not support the hypomethylation hypothesis.

The increase in the CC genotype in alcoholic HCC patients could in fact be associated with a defect in nucleotide synthesis due to a lack of folate. Indeed, alcohol decreases the folate levels in blood (24). The MTHFR CC genotype may accentuate the lack of folates available, thus decreasing nucleic acid synthesis, resulting in the excessive incorporation of uracil into DNA (33,34). The simultaneous removal of two uracil bases on opposite DNA strands within a 12 bp region may result in the formation of double-stranded breaks (35). The incorporation of uracil into DNA and the subsequent chromosome breakage caused by double-stranded breaks are important, because the accumulation of chromosome aberrations is a risk factor for cancer (36,37).

In summary, the identification of MTHFR polymorphisms that are associated with HCC provides a potential tool for cancer risk assessment in alcoholic patients and in patients who are at risk of developing HCC.

References


Received December 31, 2003; revised February 20, 2004; accepted March 7, 2004