Polymorphisms of folate metabolism genes in patients with cirrhosis and hepatocellular carcinoma

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AIM
To evaluated the association of the risk factors and polymorphisms in MTHFR C677T, MTHFR A1298C, MTR A2756G and MTRR A66G genes.

METHODS
Patients with cirrhosis (n = 116), hepatocellular carcinoma (HCC) (n = 71) and controls (n = 356) were included. Polymerase chain reaction followed by enzymatic digestion and allelic discrimination technique real-time PCR techniques were used for analysis. MINITAB-14.0
and SNPstats were utilized for statistical analysis.

RESULTS

Showed that age $\geq 46$ years (OR = 10.31; 95%CI: 5.66-18.76; $P < 0.001$) and smoking (OR = 0.47; 95%CI: 0.28-0.78; $P = 0.003$) were associated with cirrhosis. Age $\geq 46$ years (OR = 16.36; 95%CI: 6.68-40.05; $P < 0.001$) and alcohol habit (OR = 2.01; 95%CI: 1.03-3.89; $P = 0.039$) were associated with HCC. MTHFR A1298C in codominant model (OR = 3.37; 95%CI: 1.52-7.50; $P = 0.014$), recessive model (OR = 3.04; 95%CI: 1.43-6.47; $P = 0.0051$) and additive model (OR = 1.71; 95%CI: 1.16-2.52; $P = 0.0072$) was associated with HCC, as well as MTR A2756G in the additive model (OR = 1.68; 95%CI: 1.01-2.77; $P = 0.047$), and MTRR A66G in the codominant model (OR = 3.26; 95%CI: 1.54-6.87; $P < 0.001$), dominant model (OR = 2.55; 95%CI: 1.24-5.25; $P = 0.007$) and overdominant model (OR = 3.05; 95%CI: 1.66-5.62; $P < 0.001$). MTR A2756G in the additive model (OR = 1.54; 95%CI: 1.02-2.33; $P = 0.042$) and smokers who presented at least one polymorphic allele for MTRR A66G (OR = 1.71; 95%CI: 0.77-3.82; $P = 0.0051$) showed increased risk for cirrhosis. There was no association between clinical parameters and polymorphisms.

CONCLUSION

Age $\geq 46$ years, alcohol habit and the MTR A2756G polymorphism are associated with an increased risk of HCC development; age $\geq 46$ years, tobacco habit and the MTR A2756G polymorphism are associated with cirrhosis.

Key words: Polymorphism; Folate metabolism; Liver cirrhosis; Hepatocellular carcinoma

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Core tip: Our study is relevant because we can get better understanding on the mechanisms involved in the development of hepatocellular and Cirrhosis Carcinoma and folate metabolism. It is already known that polymorphisms cause DNA hypomethylation, which cause abnormal changes in gene expression inactivating suppressor genes tumor. In this study we have found some positive associations which was possible to understand the carcinogenesis of this tumor and offer new possibilities for diagnosis. Throughout these results it is possible to achieve better quality of life in early treatments.

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INTRODUCTION

Liver cancer is the second most common cause of death from cancer worldwide. Hepatocellular carcinoma (HCC) is considered the major form of primary liver cancer and is responsible for 70%-85% of all liver cancers[1]. Each year, more than half a million people are diagnosed with HCC. According to the most recent data, 782000 new cases per hundred thousand inhabitants have been diagnosed, with 745000 deaths resulting from this disease. HCC is the fifth most common cancer in men (554000 cases, 7.5% of all cases) and the ninth most common cancer in women (228000, 3.4% of all cases)[2].

The major risk factor for HCC development, present in 90% of HCC patients, is liver cirrhosis, which is characterized by diffuse fibrosis, progressive and irreversible, with the presence of nodules delimited by fibrous septa[13]. There are other risk factors such as hepatitis B and C virus infection, liver disease derived from alcohol consumption, exposure to toxins such as aflatoxins and smoking, non-alcoholic fatty liver, obesity and diabetes[4,5].

Cancer is a multifactorial disease that results from complex interactions between genetic and environmental factors[6]. Some studies have been conducted using genetic polymorphisms involved in folate metabolism in various types of cancers[7-11] because folate metabolism is essential for DNA synthesis and alterations in folate levels are associated with changes in DNA synthesis, methylation and repair; promoting genomic instability that contributes to the process of carcinogenesis[12].

Several enzymes, including methylenetetrahydrofolate reductase enzyme (MTHFR), methionine synthase (MTR) and methionine synthase reductase (MTRR), are involved in folate metabolism[13]. Methylene tetrahydrofolate reductase (MTHFR) is a key regulatory enzyme in folate metabolism, and MTHFR can catalyse 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the predominant circulating form of folate. There are two common functional polymorphisms identified in the MTHFR gene, the MTHFR C677T polymorphism and MTHFR A1298C polymorphism[14].

Moreover, 5-methyl-tetrahydrofolate donates one methyl group for homocysteine remethylation to methionine. The remethylation of this reaction is catalysed by the enzyme methionine synthase (MTR), which requires vitamin B12 as a cofactor. The enzyme methionine synthase reductase (MTRR) is responsible for maintaining the active state of the MTR enzyme. Polymorphisms MTR A2756G and MTRR A66G may cause decreased activity of the enzyme, leading to increased plasma homocysteine and DNA hypomethylation, which causes changes in gene expression, inactivating tumour suppressor genes and activate oncogenesis[16-18].

Studies have confirmed that the genetic polymorphisms involved in folate metabolism may contribute to the development of HCC[16,19]. Therefore, the present
study was aimed to evaluate the association of risk factors and polymorphisms in the genes MTHFR C677T, MTHFR A1298C, MTR A2756G and MTRR A66G involved in folate metabolism with cirrhosis and HCC development in a case control study and to investigate the association of the polymorphisms with the clinical parameters of the disease in patients with cirrhosis and HCC.

**MATERIALS AND METHODS**

**Ethical statement**

Patients at the Liver, Intestine and Pancreas Transplant Unit of a university hospital in the northwest of the state of São Paulo, Brazil were included in the study after the diagnosis of liver cirrhosis and HCC, while the control group consisted of healthy individuals without cancer diagnosis. Individuals with a cancer family history were excluded from the control group. Informed consent was obtained from all subjects in this study, and the research protocol was approved by the Research Ethics Committee of FAMERP (CAAE: 20465713.1.0000.5415).

**Patients**

In this case-control study, 543 subjects (116 patients with liver cirrhosis, 71 patients with HCC and 356 healthy individuals) were included regardless of sex and age, from 2013 to 2015. Patients with cirrhosis were included because it is known as a well-established risk factor in 90% of patients with HCC.[1,3]

The sample calculation was performed according to the reports of Kwak et al[21] and Chang et al[23] which presented a similar sample calculation. Furthermore, no study has evaluated polymorphisms in folate metabolism in HCC and cirrhosis development in the Brazilian population.

The diagnosis of HCC was based on the criteria of the American Association for the Study of Liver Diseases published in 2012,[20] Liver biopsy was performed when the diagnosis was not possible by imaging methods, and the diagnosis of cirrhosis was made by clinical, laboratory, ultrasound and histopathological examinations when possible.

The variables analysed in this study were age, gender, exposure to risk factors (smoking and alcohol habit) and the presence of the MTHFR A1298C, MTHFR C677T, MTR A2756G and MTRR A66G polymorphisms. We considered smokers to be those who consumed at least 100 cigarettes during their lifetime and alcohol consumers to be those who drink more than 4 drinks weekly, corresponding 30 mL of liquor, 102 mL of wine, and 340 mL of beer.[21]

Patients diagnosed with HCC were also classified according to the Barcelona Clinic Liver Cancer (BCLC) classification, which is a staging system that serves mainly for therapeutic guidance, in which the patient is ranked into five stages and includes other classifications. This classification uses variables related to tumour stage, the functional state of the liver, physical condition, and symptoms related to cancer. Patients with stage 0, very early HCC and with only a minor 2-cm tumour were nominated for liver resection. Patients with early HCC phase A with up to 3-cm nodules were eligible for curative therapies (resection, liver transplantation or percutaneous treatments). Patients in phase B with intermediate HCC and this multinodular underwent chemoembolization. Patients in advanced stage C presenting portal invasion and metastases received new agents such as sorafenib, which is a palliative treatment, and patients in stage D with end-stage disease received symptomatic treatment[22].

**Methods**

Genomic DNA was extracted from peripheral blood leukocytes of the cases and controls according to Miller et al[23] and was amplified by multiplex PCR-RFLP to identify the MTHFR C677T (rs1801133) and MTHFR A1298C (rs1801131), MTR A2756G (rs1805087) polymorphisms. The amplification product was subjected to digestion by the restriction enzymes Hinf I, Mbo II, and Hae III, respectively. Electrophoresis was performed in 2.5% agarose gels at 110 volts for 100 min. Allelic discrimination via the Real-Time PCR - SNP Genotyping Assay (Applied Biosystems) was used to identify the MTRR A66G (rs1801394) polymorphism, using primers and probes specific for each allele available by the manufacturer (MTRR A66G: C_3068176_10)[7].

Genotyping confirmation was accomplished in 10% random samples of each group, and 100% concordance was observed.

**Statistical analysis**

Hardy-Weinberg equilibrium (HWE) was performed using $\chi^2$ test. The multiple regression logistic test by the Minitab program - Version 14.0 was used to determine the effects of variables. The model evaluated the following variables: Age (reference: < 46 years; median), smoking habits (reference: No smokers), alcohol habit (reference: Non-consumers) and gender (reference: Female). The polymorphisms were used to adjust the analysis.

The multiple logistic regression model adjusted for age, gender, smoking and alcohol habits was also used to assess the association between polymorphisms and the development of cirrhosis and HCC using the SNPSstats program. The effect of the polymorphisms was evaluated in the following models: (1) codominant (heterozygous vs homozygous wild type and polymorphic homozygous vs homozygous wild type); (2) dominant (heterozygous more polymorphic homozygous vs homozygous wild type); (3) recessive (polymorphic homozygous vs homozygous wild type more heterozygous); (4) overdominant (wild homozygous vs heterozygous more polymorphic homozygote); and (5) additive (weight polymorphic homozygote vs heterozygote 2 more homozygous wild-type).

The SNPSstats program was also used to assess the
Table 1  Relationship between risk factors and hepatocellular carcinoma and liver cirrhosis development

| Variables                          | Controls n (%) | Cirrhosis n (%) | OR (95%CI) | P-value | HCC n (%) | OR (95%CI) | \(^2P\) |
|-----------------------------------|----------------|-----------------|------------|---------|-----------|------------|--------|
| Age                               |                |                 |            |         |           |            |        |
| < 46 anos                         | 238 (67)       | 22 (19)         | 10.31 (5.66-18.76) | \(P < 0.001\) | 8 (11)    | 16.36 (6.68-40.05) | \(P < 0.001\) |
| \(\geq 46\) anos                  | 118 (33)       | 94 (81)         |            |         |           |            |        |
| Gender                            |                |                 |            |         |           |            |        |
| Female                            | 95 (26.7)      | 30 (25.9)       | 0.96 (0.54-1.72) | \(P = 0.893\) | 19 (26.8) | 0.59 (0.29-1.22) | \(P = 0.154\) |
| Male                              | 261 (73.3)     | 86 (74.1)       |            |         |           |            |        |
| Alcohol habit                      |                |                 |            |         |           |            |        |
| Yes                               | 191 (54)       | 54 (46.6)       | 1.55 (0.91-2.63) | \(P = 0.106\) | 27 (38)   | 2.01 (1.03-3.89) | \(P = 0.039\) |
| Not                               | 165 (46)       | 62 (53.4)       |            |         |           |            |        |
| Smoking habit                      |                |                 |            |         |           |            |        |
| Nonsmokers                        | 199 (56)       | 72 (62)         | 0.47 (0.28-0.78) | \(P = 0.003\) | 31 (43.7) | 0.9 (0.48-1.67) | \(P = 0.734\) |
| Smokers                           | 157 (44)       | 44 (38)         |            |         |           |            |        |

\(^{1}\)Odds ratio (OR) adjusted for age, genre, alcohol consumption, smoking habits and polymorphisms; \(^{2}\)P values significant at \(P < 0.05\). HCC: Hepatocellular carcinoma.

potential interaction between the polymorphisms with variables associated with cirrhosis and HCC development (tobacco and alcohol habits) through multiple logistic regression.

The *MTHFR* haplotypes were inferred using the Haplovie 4.2 statistical program, which creates population frequency estimates of the haplotypes.

The association between the clinical parameters and polymorphisms with HCC development were also analysed by multiple logistic regression. The patients were subjected to classification and BCLC staging divided into five stages (0, A, B, C and D). The variables alpha fetoprotein dose values, hepatitis B and C, diabetes mellitus and death were utilized in the adjustment of the analysis. The models included BCLC classification (reference: 0, A, B, C and D), alpha fetoprotein (reference: < 500 ng/mL), hepatitis B (reference: No), hepatitis C virus (reference: No), diabetes (reference: Absence), death (reference: No) and the studied polymorphisms (reference: Wild-type genotype).

The Kaplan-Meier method was applied to evaluate the survival rate by considering the period between the disease diagnosis and death to be the end point.

The results were presented as ORs and 95%CIs. The level of significance was set at 5% (\(P = 0.05\)).

RESULTS

The results for HWE were similar to those expected in both the case and control groups, respectively, for the *MTHFR C677T* (\(\chi^2 = 0.8940, P = 0.3444\) and \(\chi^2 = 3.1218, P = 0.0772\)), *MTR A2756G* (\(\chi^2 = 1.1554, P = 0.2824\) and \(\chi^2 = 1.1929, P = 0.2748\)) and *MTRR A66G* polymorphisms (\(\chi^2 = 3.2227, P = 0.0726\) and \(\chi^2 = 0.0530, P = 0.8018\)). However, the *MTHFR A1298C* polymorphism showed no equilibrium (\(\chi^2 = 8.0244, P = 0.0046\) and \(\chi^2 = 8.6427, P = 0.0033\)) for patients with HCC and/or cirrhosis and controls.

Table 1 shows the results for multiple logistic regression analysis between patients with liver cirrhosis and control subjects to determine the effects of variables. Age \(\geq 46\) years (OR = 10.31; 95%CI: 5.66-18.76; \(P < 0.001\)) and smoking habit were associated with the disease (OR = 0.47; 95%CI: 0.28-0.78; \(P = 0.003\)), and the analysis of patients with HCC and control subjects showed that age \(\geq 46\) years (OR = 16.36; 95%CI: 6.68-40.05; \(P < 0.001\)) and alcohol habit (OR = 2.01; 95%CI: 1.03-3.89; \(P = 0.039\)) were associated with the disease.

Table 2 shows the association of the *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* polymorphisms with HCC, adjusted for gender, age, smoking and alcohol habit according to the heritage models. The *MTHFR A1298C* polymorphism in the codominant model (OR = 3.37; 95%CI: 1.52-7.50; \(P = 0.014\)), recessive model (OR = 3.04; 95%CI: 1.43-6.47; \(P = 0.0051\)) and additive model (OR = 1.71; 95%CI: 1.1929, \(\chi^2 = 0.0033\) for patients with HCC and/or cirrhosis and controls.

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The Kaplan-Meier method was applied to evaluate the survival rate by considering the period between the disease diagnosis and death to be the end point.

The results were presented as ORs and 95%CIs. The level of significance was set at 5% (\(P = 0.05\)).
Table 2: Association of MTHFR C677T, MTHFR A1298C, MTR A2756G and MTRR A66G polymorphisms with hepatocellular carcinoma, adjusted for gender, age, smoking and alcohol consumption

| Model | Genotipe | Control n (%) | Case n (%) | 1OR (95%CI) | P-value | Genotipe | Control n (%) | Case n (%) | 1OR (95%CI) | P-value |
|-------|----------|---------------|------------|-------------|---------|----------|---------------|------------|-------------|---------|
| MTHFR | C677T    |               |            |             |         | A1298C   |               |            |             |         |
| Codominant | C/C | 149 (41.9) | 28 (39.4) | 1           | 0.91 | A/A | 205 (57.6) | 32 (45.1) | 1           | 0.014 |
|          | C/T | 174 (48.9) | 36 (50.7) | 0.93 (0.51-1.68) | 0.01 | A/C | 116 (32.6) | 24 (33.8) | 1.29 (0.69-2.42) | 0.569 |
|          | T/T | 33 (9.3)  | 7 (9.9)   | 1.13 (0.41-3.09) | 0.01 | C/C | 35 (9.8)  | 15 (21.1) | 3.37 (1.52-7.50) | 0.014 |
| Dominant | C/C | 149 (41.9) | 28 (39.4) | 1           | 0.91 | A/A | 205 (57.6) | 32 (45.1) | 1           | 0.014 |
|          | C/T | 174 (48.9) | 36 (50.7) | 0.93 (0.51-1.68) | 0.01 | A/C | 116 (32.6) | 24 (33.8) | 1.29 (0.69-2.42) | 0.569 |
|          | T/T | 33 (9.3)  | 7 (9.9)   | 1.13 (0.41-3.09) | 0.01 | C/C | 35 (9.8)  | 15 (21.1) | 3.37 (1.52-7.50) | 0.014 |
| recessive | C/C | 207 (58.1) | 43 (60.6) | 0.96 (0.54-1.70) | 0.08 | A/C/C/C | 151 (42.4) | 39 (54.1) | 1.71 (0.98-2.99) | 0.06 |
|          | C/C/C/T | 323 (90.7) | 64 (90.1) | 1           | 0.91 | A/A/A/C | 321 (90.2) | 56 (78.9) | 1           | 0.051 |
|          | C/C/T/T | 33 (9.3)  | 7 (9.9)   | 1.18 (0.46-3.05) | 0.73 | C/C/C/C/C | 35 (9.8)  | 15 (21.1) | 3.04 (1.43-6.47) | 0.0051 |
| Overdominant | C/C/T | 182 (51.1) | 35 (49.3) | 1           | 0.91 | A/A/C/C | 240 (67.4) | 47 (66.2) | 1           | 0.0071 |
|          | C/T | 174 (48.9) | 36 (50.7) | 0.91 (0.52-1.59) | 0.73 | A/C | 116 (32.6) | 24 (33.8) | 0.99 (0.55-1.78) | 0.98 |
| additive | -- | -- | -- | 1.01 (0.64-1.58) | 0.97 | -- | -- | -- | 1.71 (1.16-2.52) | 0.007 |

*Odds ratio (OR) adjusted for age, gender and alcohol consumption and smoking habits; *P* values significant at *P* ≤ 0.05.

Table 3: Association of MTHFR C677T, MTHFR A1298C, MTR A2756G and MTRR A66G polymorphisms with Liver Cirrhosis, adjusted for gender, age, smoking and alcohol consumption

| Model | Genotipe | Control n (%) | Case n (%) | 1OR (95%CI) | P-value | Genotipe | Control n (%) | Case n (%) | 1OR (95%CI) | P-value |
|-------|----------|---------------|------------|-------------|---------|----------|---------------|------------|-------------|---------|
| MTHFR | C677T    |               |            |             |         | A1298C   |               |            |             |         |
| Codominant | C/C | 149 (41.9) | 48 (64.8) | 1           | 0.91 | A/A | 105 (29.5) | 12 (16.9) | 1           | 0.014 |
|          | C/T | 174 (48.9) | 55 (74.7) | 1.58 (0.84-2.98) | 0.13 | A/G | 179 (50.3) | 50 (70.4) | 3.26 (1.54-6.87) | < 0.001 |
|          | T/T | 33 (9.3)  | 13 (11.2) | 3.29 (0.81-13.30) | 0.56 | G/G | 72 (20.2) | 9 (12.7) | 1.16 (0.44-3.11) | 0.569 |
| Dominant | C/C | 205 (57.6) | 46 (64.8) | 1           | 0.91 | A/A | 105 (29.5) | 12 (16.9) | 1           | 0.014 |
|          | C/T | 251 (70.5) | 25 (32.5) | 1.73 (0.95-3.15) | 0.078 | A/G/G/G | 251 (70.5) | 59 (83.1) | 2.55 (1.24-5.25) | 0.0072 |
|          | T/T | 346 (97.2) | 67 (94.4) | 1           | 0.91 | A/G/G/G | 346 (97.2) | 67 (94.4) | 1           | 0.0072 |
| recessive | C/G | 10 (2.8) | 4 (5.6) | 2.91 (0.73-11.59) | 0.15 | G/G | 72 (20.2) | 9 (12.7) | 0.51 (0.23-1.12) | 0.077 |
|          | T/T | 273 (76.7) | 70 (74.0) | 1           | 0.91 | A/G/G/G | 273 (76.7) | 70 (74.0) | 1           | 0.077 |
|          | A/G | 83 (23.3) | 21 (26.6) | 1.49 (0.80-2.79) | 0.22 | G/G | 179 (50.3) | 50 (70.4) | 3.05 (1.66-5.62) | < 0.001 |
| additive | -- | -- | -- | 1.68 (1.01-2.77) | 0.047 | -- | -- | -- | 1.16 (0.77-1.73) | 0.48 |

*Odds ratio (OR) adjusted for age, gender and alcohol consumption and smoking habits; *P* values significant at *P* ≤ 0.05.

(40.6%) of the AC haplotype observed in both groups (Case group: 0.403, Control group: 0.407; *χ*² = 0.01, *P* = 0.9194). The haplotype frequencies of AT (Case group: 0.283, Control group: 0.312; *χ*² = 0.843, *P* = 0.3584), haplotype frequencies of CC (Case group: 0.269, Control group: 0.247; *χ*² = 0.573, *P* = 0.4491), and haplotype frequencies of CT (Case group: 0.045, Control group: 0.035; *χ*² = 0.569, *P* = 0.4505) did not show significant
results.
There was no association in the multiple logistic regression analysis of the analysed clinical parameters and polymorphisms in patients with HCC stratified into tumours in stages 0 and A, and in tumours in stages B, C and D, according to the BCLC criteria (Table 6).

The Kaplan-Meier survival curves for genotype showed no association of polymorphisms and overall survival with HCC development. No polymorphism was associated with overall survival.

**DISCUSSION**

The results showed that age $\geq$ 46 years and alcohol habits were associated with an increased risk of HCC development, similar to the results of Fassio et al., Munaka et al., Carrilho et al., Donato et al., Hamed et al. and Mittal et al.

Brazilian publications have reported that the mean age of the HCC patients is 54.6 years[3], in Latin
America, the average age in one published study was 64 years[24]; in another multicentre study in Spain, the reported average age was 65.6 years[25], and Mittal et al[1] concluded that a more recent increase in the incidence of HCC in the United States population was seen in Hispanics and blacks between the ages of 45 and 65 years, results that are similar to ours.

Regarding the result that alcohol consumption was also significant and more frequent in patients with HCC, a prospective case-control study from Japan has observed that heavy alcohol drinkers had a five-fold increase in the risk of HCC compared with non-drinkers[26]. Donato et al[27] 2002 in Italy, with a sample size of 464 cases and 824 controls individuals, found a positive relationship between alcohol consumption and HCC. The latter findings were confirmed in review of Hamed et al[28].

Evidence of a positive association between heavy alcohol drinking and liver cancer is derived mainly from case-control studies. The increased risk of those drinking 6 or more drinks per day compared with non-drinkers was 22%. Alcohol was the only cause present in 14% of cases in the 2010 Brazilian study by Carrilho et al[3]. Thus, the significance indexes are increased because drinking alcohol causes poor absorption of vitamin B complex, changing the folate metabolism and causing oxidative damage and breaks in the DNA strands[29]. In our study we found the association between alcohol and HCC development, this may be due the fact described above.

Regarding cirrhosis, we found that age ≥ 46 years and smoking habit was associated with risk of cirrhosis. As previously mentioned, 85%-90% of primary liver cirrhosis causes cancer, and multiple nonviral factors that are concerned with the development of liver cancer include iron overload syndromes, alcohol use, tobacco, oral contraceptives, aflatoxin, and pesticide exposure, which is prevalent in the developing world[28]. Regarding the tobacco habit, the data showed that 38% of individuals with cirrhosis had a tobacco habit. In addition to the liver, which is the target of chemical compounds in tobacco that can progress to cirrhosis, it was also observed in the literature that the development of diseases related to the progression to cirrhosis occurs more frequently in older individuals[30].

In addition to the association that we found between tobacco habit and cirrhosis development, there can be a relationship of tobacco with the dysfunction of genes as

| Variables                      | Stage 0 e A Pacientes n (%) | Stage B, C e D Pacientes n (%) | OR (95%CI)¹ | P     |
|--------------------------------|-----------------------------|--------------------------------|-------------|-------|
| Alpha fetoprotein              |                             |                                |             |       |
| > 500 ng/mL                    | 22 (84.6)                   | 21 (46.7)                      | Reference   | Reference |
| < 500 ng/mL                    | 4 (15.4)                    | 24 (53.3)                      | 2.66 (0.55-12.72) | 0.22 |
| Hepatitis B virus              |                             |                                |             |       |
| Absence                        | 25 (96.2)                   | 38 (84.4)                      | Reference   | Reference |
| Presence                       | 1 (3.85)                    | 7 (15.6)                       | 4.06 (0.34-48.29) | 0.27 |
| Hepatitis C virus              |                             |                                |             |       |
| Absence                        | 12 (46.2)                   | 22 (48.9)                      | Reference   | Reference |
| Presence                       | 14 (53.8)                   | 23 (41.1)                      | 1.43 (0.35-5.78) | 0.61 |
| Steatohepatitis                |                             |                                |             |       |
| Absence                        | 26 (100)                    | 42 (93.3)                      | Reference   | Reference |
| Presence                       | 00 (00)                     | 3 (6.7)                        | Reference   | Reference |
| Diabetes                       |                             |                                |             |       |
| Absence                        | 18 (69.2)                   | 32 (71.1)                      | Reference   | Reference |
| Presence                       | 8 (30.8)                    | 13 (28.9)                      | 0.25 (0.03-1.63) | 0.15 |
| Death                          |                             |                                |             |       |
| No                             | 24 (92.3)                   | 17 (37.8)                      | Reference   | Reference |
| Yes                            | 2 (7.7)                     | 28 (62.2)                      | 25.3 (0.67-174.38) | 0.09² |
| MTHFR A1298C                   |                             |                                |             |       |
| AA                             | 10 (38.5)                   | 22 (48.9)                      | Reference   | Reference |
| AC/CC                          | 16 (61.5)                   | 23 (51.1)                      | 0.93 (0.22-3.86) | 0.92 |
| MTHFR C677T                    |                             |                                |             |       |
| CC                             | 11 (42.3)                   | 17 (37.8)                      | Reference   | Reference |
| CT/TT                          | 15 (57.7)                   | 28 (62.2)                      | 1.65 (0.34-7.97) | 0.53 |
| MTR A2756G                     |                             |                                |             |       |
| AA                             | 18 (69.2)                   | 28 (62.2)                      | Reference   | Reference |
| AG/GG                          | 8 (30.8)                    | 17 (37.8)                      | 0.78 (0.18-3.43) | 0.75 |
| MTRRA666G                      |                             |                                |             |       |
| AA                             | 4 (15.4)                    | 8 (17.8)                       | Reference   | Reference |
| AG/GG                          | 22 (84.6)                   | 37 (82.2)                      | 1.09 (0.16-7.30) | 0.93 |

¹OR: Odds ratio, CI: Confidence interval; ²Statistically significant at P ≤ 0.05; ³Could not calculate due to numerical proximity.

Table 6  Regression analysis of data from multiple logistic analyzed clinical parameters and polymorphisms in patients with hepatocellular carcinoma tumors divided into stages 0 and tumore in stages A and B, C and D according barcelona clinic liver cancer criteria
well as enzymes involved in the detoxification of nicotine, consequences that generate various types of liver-related diseases such as fibrosis, alcoholic hepatitis, cirrhosis and HCC\[^{33}\]. Although alcohol habit is a well-established risk factor for cirrhosis development\[^{22}\], our study did not find this association. However, 53.4% of cirrhosis patients in the present study were alcohol consumers.

In relation to the genetic characteristics, the present study was the first to be performed in a Brazilian population with HCC and cirrhosis and revealed that the MTR A2756G, MTHFR A1298C and MTRR A66G polymorphisms were associated with an increased risk of HCC development, results similar to the studies of Kwak et al\[^{37}\] and Yu et al\[^{33}\].

Chang et al\[^{39}\], with a sample of 204 patients with liver cancer and 415 controls found an association between MTR A2756G and increased risk for the disease, as well the meta-analysis performed by Yu et al\[^{33}\] that reported a significantly higher association between the genotype and 2756GG cancer risk in Asian populations.

There are studies involving other cancers that have found a positive association with at least one polymorphic allele 2756G and an increased risk of the development of disease. For example, the Hosseini et al\[^{8}\] that evaluated 592 individuals in Iran found an association between MTR GG genotype and breast cancer; Galbiatti et al\[^{33}\] also concluded that MTR A2765G polymorphism is involved in the risk of head and neck cancer; de Lima et al\[^{38}\] suggested an association between the MTR A2756G polymorphism and retinoblastoma susceptibility in a northeast population of Brazil, Ouerhani et al\[^{39}\] found that MTR A2756G affecting bladder cancer risk.

Regarding the MTRR A66G polymorphism, Kwak et al\[^{37}\] studied 96 patients and 201 controls and observed an association between the polymorphism and an increased risk of HCC, a finding that has also been found in other types of cancer; the Wu et al\[^{11}\] study demonstrated a positive relationship with the MTRR A66G polymorphism and breast cancer, and a meta-analysis performed by Zhou et al\[^{30}\] also found an association between this polymorphism and colorectal cancer, which is in agreement with our study. However, the study of Zhang et al\[^{35}\] did not find an association of this polymorphism with HCC development.

We also found an association between the MTHFR A1298C polymorphism and an increased risk of HCC development. Two meta-analyses reported an association of this polymorphism with a decreased risk of HCC, demonstrating a protective effect\[^{36,37}\]. However, Liang et al\[^{39}\] meta-analysis of a total of seven studies showed that the homozygote genotype CC of the MTHFR rs1801131 polymorphism was significantly associated with a decreased risk of liver cancer (for CC vs AA: OR = 0.65, 95%CI: 0.47-0.89, P = 0.007; for CC vs AA + AC: OR = 0.65, 95%CI: 0.48-0.89, P = 0.006), similar our study.

Our results for cirrhosis and polymorphisms showed an association between MTR A2756G and an increased risk of the disease. There are no studies in the literature that have evaluated the association between the MTR A2756G polymorphism and cirrhosis development. The present study is the first to investigate the MTRA A2756G polymorphism and cirrhosis development, and the association that was found can be related to alteration of the MTR enzyme that occurs due to the presence of the MTR A2756G polymorphism. The alteration of the MTR enzyme causes elevation in the homocysteine levels and DNA hypomethylation, leading to chromosomal instability, mutations and the overexpression of proto-oncogenes that can be associated with the development of several types of diseases, including cirrhosis. However, more studies in different populations of individuals with cirrhosis are needed\[^{39}\].

Regarding the potential interaction among the polymorphisms with variables associated with the diseases, we found that smoking in those with the heterozygote genotype (AG) or polymorphic homozygote genotype (GG) for MTRR gene was associated with liver cirrhosis. There are no studies that have investigated this interaction, however, tobacco habit can be related to cirrhosis because the chemical compounds can modify the liver and lead to cirrhosis\[^{39}\] independently of the MTRR A66G polymorphism.

Regarding BCLC classification, we did not find an association with the polymorphisms evaluated. Our data showed that 7% of patients in stage 0, 29.6% in stage A, 22.5% in stage B, 31% in stage C and 9.8% in stage D. The study of Varela et al\[^{25}\] reported that 49.8% of 705 cases were in the initial stage (A), 19.8% in the intermediate stage (B), 18.8% in the advanced stage (C) and 11.6% in the terminal phase (D). Additionally, the study of Raphe et al\[^{30}\] reported that 32.7% were in stage A, 22% in stage B, 30.4% in stage C, 14% in stage D. Current published data show that patients who are in stage A are asymptomatic and have preserved liver function have a 5-year survival of 50%-75%. Patients who are in stage B have a median survival of 20 mo; those who are already in the C and D stages have severe liver dysfunction and extrahepatic metastases reach an 11-mo survival, and only 10% of patients in the D stage survive more than a year with an average survival of 3-4 mo\[^{26}\].

In conclusion, age \(\geq 46\) years, alcohol habit and the MTR A2756G, MTHFR A1298C and MTRR A66G polymorphisms are associated with an increased risk of HCC development; age \(\geq 46\) years, tobacco habit and the MTR A2756G polymorphism are associated with cirrhosis development. There is an interaction between the MTRR A66G polymorphism and tobacco consumers with liver cirrhosis. The present study can collaborate to establish the etiologic factors related to HCC and cirrhosis development and to contribute to strategies related to health care.

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**Peres NP et al.** Polymorphisms in patients with liver diseases
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