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ORIGINAL ARTICLE

Case Control Study

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

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Abstract

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 ⩾ 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 ⩾ 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 ⩾ 46 years, alcohol habit and *MTR A2756G*, *MTHFR A1298C* and *MTRR A66G* polymorphisms are associated with an increased risk of HCC development; age ⩾ 46 years, tobacco habit and the *MTR A2756G* polymorphism are associated with cirrhosis.

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

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[1,3]. 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]. Methylenetetrahydrofolate 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[15-19].

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 the reports of Kwak *et al*[17] and Chang *et al*[19] 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 χ^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 SNPStats 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 SNPStats 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* (%)	[1]OR (95%CI)	*P*-value	HCC *n* (%)	OR (95%CI)	[2]*P*
Age							
< 46 anos	238 (67)	22 (19)	10.31 (5.66-18.76)	$P < 0.001$	8 (11)	16.36 (6.68-40.05)	$P < 0.001$
⩾ 46 anos	118 (33)	94 (81)			63 (89)		
Genre							
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)			52 (73.2)		
Alcoholic habit							
Not	191 (54)	54 (46.6))	1.55 (0.91-2.63)	$P = 0.106$	27 (38)	2.01 (1.03-3.89)	$P = 0.039$
Yes	165 (46)	62 (53.4)			44 (62)		
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)			40 (56.3)		

[1]Odds ratio (OR) adjusted for age, genre, alcohol consumption, smoking habits and polymorphisms; [2]*P* values significant at $P \leqslant 0.05$. 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 Haploview 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), 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 ⩾ 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 ⩾ 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: 1.16-2.52; $P = 0.0072$), the *MTR A2756G* polymorphism in the additive model (OR = 1.68; 95%CI: 1.01-2.77; $P = 0.047$), the *MTRR A66G* polymorphism 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; $P < 0.001$) were associated with an increased risk of HCC development.

Table 3 shows the association of the *MTHFR C677T, MTHFR A1298C, MTR A2756G* and *MTRR A66G* polymorphisms with liver cirrhosis, adjusted for gender, age, smoking and alcohol habit according to the heritage models. The *MTR A2756G* polymorphism was associated with an increased risk of liver cirrhosis in the additive model (OR = 1.54; 95%CI: 1.02-2.33; $P = 0.042$).

Regarding the potential interaction among the polymorphisms with variables associated with the diseases, there was no interaction among the *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* polymorphisms and smoking habit or alcohol habit regarding the risk of HCC (Table 4). However, smokers who presented with the heterozygous genotype (AG) or polymorphic homozygote genotype (GG) for the MTRR gene (OR = 1.71; 95%CI: 0.77-3.82; $P = 0.0051$) was associated with liver cirrhosis (Table 5).

The haplotype analysis showed a higher frequency

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* (%)	[1]OR (95%CI)	[2]*P*-value	Genotipe	Control *n* (%)	Case *n* (%)	[1]OR (95%CI)	[2]*P*-value
	MTHFR C677T					*MTHFR A1298C*				
Codominant	C/C	149 (41.9)	28 (39.4)	1		A/A	205 (57.6)	32 (45.1)	1	
	C/T	174 (48.9)	36 (50.7)	0.93 (0.51-1.68)	0.91	A/C	116 (32.6)	24 (33.8)	1.29 (0.69-2.42)	0.014
	T/T	33 (9.3)	7 (9.9)	1.13 (0.41-3.09)		C/C	35 (9.8)	15 (21.1)	3.37 (1.52-7.50)	
Dominant	C/C	149 (41.9)	28 (39.4)	1		A/A	205 (57.6)	32 (45.1)	1	
	C/T-T/T	207 (58.1)	43 (60.6)	0.96 (0.54-1.70)	0.88	A/C-C/C	151 (42.4)	39 (54.9)	1.71 (0.98-2.99)	0.06
Recessive	C/C-C/T	323 (90.7)	64 (90.1)	1		A/A-A/C	321 (90.2)	56 (78.9)	1	
	T/T	33 (9.3)	7 (9.9)	1.18 (0.46-3.05)	0.73	C/C	35 (9.8)	15 (21.1)	3.04 (1.43-6.47)	0.0051
Overdominant	C/C-T/T	182 (51.1)	35 (49.3)	1		A/A-C/C	240 (67.4)	47 (66.2)	1	
	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
Aditive	---	---	---	1.01 (0.64-1.58)	0.97	---	---	---	1.71 (1.16-2.52)	0.007
	MTR A2756G					*MTRR A66G*				
Codominant	A/A	263 (73.9)	46 (64.8)	1		A/A	105 (29.5)	12 (16.9)	1	
	A/G	83 (23.3)	21 (29.6)	1.58 (0.84-2.98)	0.13	A/G	179 (50.3)	50 (70.4)	3.26 (1.54-6.87)	< 0.001
	G/G	10 (2.8)	4 (5.6)	3.29 (0.81-13.30)		G/G	72 (20.2)	9 (12.7)	1.16 (0.44-3.11)	
Dominant	A/A	263 (73.9)	46 (64.8)	1		A/A	105 (29.5)	12 (16.9)	1	
	A/G-G/G	93 (26.1)	25 (35.2)	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
Recessive	A/A-A/G	346 (97.2)	67 (94.4)	1		A/A-A/G	284 (79.8)	62 (87.3)	1	
	G/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
Overdominant	A/A-G/G	273 (76.7)	50 (70.4)	1		A/A-G/G	177 (49.7)	21 (29.6)	1	
	A/G	83 (23.3)	21 (29.6)	1.49 (0.80-2.79)	0.22	A/G	179 (50.3)	50 (70.4)	3.05 (1.66-5.62)	< 0.001
Aditive	---	---	---	1.68 (1.01-2.77)	0.047	---	---	---	1.16 (0.77-1.73)	0.48

[1]Odds ratio (OR) adjusted for age, gender and alcohol consumption and smoking habits; [2]*P* values significant at $P \leqslant 0.05$.

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* (%)	[1]OR (95%CI)	[2]*P*-value	Genotipe	Control *n* (%)	Case *n* (%)	[1]OR (95%CI)	[2]*P*-value
	MTHFR C677T					*MTHFR A1298C*				
Codominant	C/C	149 (41.9)	48 (41.4)	1		A/A	205 (57.6)	57 (49.1)	1	
	C/T	174 (48.9)	55 (47.4)	0.90 (0.56-1.45)	0.56	A/C	116 (32.6)	43 (37.1)	1.27 (0.78-2.07)	0.21
	T/T	33 (9.3)	13 (11.2)	1.37 (0.64-2.95)		C/C	35 (9.8)	16 (13.8)	1.85 (0.92-3.71)	
Dominant	C/C	149 (41.9)	48 (41.4)	1		A/A	205 (57.6)	57 (49.1)	1	
	C/T-T/T	207 (58.1)	68 (58.6)	0.97 (0.62-1.52)	0.89	A/C-C/C	151 (42.4)	59 (50.9)	1.40 (0.90-2.18)	0.14
Recessive	C/C-C/T	323 (90.7)	103 (88.8)	1		A/A-A/C	321 (90.2)	100 (86.2)	1	
	T/T	33 (9.3)	13 (11.2)	1.45 (0.71-2.99)	0.32	C/C	35 (9.8)	16 (13.8)	1.68 (0.86-3.29)	0.14
Overdominant	C/C-T/T	182 (51.1)	61 (52.6)	1		A/A-C/C	240 (67.4)	73 (62.9)	1	
	C/T	174 (48.9)	55 (47.4)	0.85 (0.54-1.33)	0.47	A/C	116 (32.6)	43 (37.1)	1.14 (0.72-1.82)	0.58
Aditive	---	---	---	1.07 (0.75-1.51)	0.72	---	---	---	1.33 (0.97-1.83)	0.079
	MTR A2756G					*MTRR A66G*				
Codominant	A/A	263 (73.9)	79 (68.1)	1		A/A	105 (29.5)	37 (31.9)	1	
	A/G	83 (23.3)	32 (27.6)	1.52 (0.91-2.53)	0.13	A/G	179 (50.3)	55 (47.4)	0.94 (0.56-1.56)	0.95
	G/G	10 (2.8)	05 (4.3)	2.47 (0.75-8.12)		G/G	72 (20.2)	24 (20.7)	0.91 (0.49-1.71)	
Dominant	A/A	263 (73.9)	79 (68.1)	1		A/A	105 (29.5)	37 (31.9)	1	
	A/G-G/G	93 (26.1)	37 (31.9)	1.60 (0.98-2.61)	0.06	A/G-G/G	251 (70.5)	79 (68.1)	0.93 (0.58-1.50)	0.77
Recessive	A/A-A/G	346 (97.2)	111 (95.7)	1		A/A-A/G	284 (79.8)	92 (79.3)	1	
	G/G	10 (2.8)	5 (4.3)	2.19 (0.68-7.09)	0.21	G/G	72 (20.2)	24 (20.7)	0.95 (0.55-1.64)	0.86
Overdominant	A/A-G/G	273 (76.7)	84 (72.4)	1		A/A-G/G	177 (49.7)	61 (52.6)	1	
	A/G	83 (23.3)	32 (27.6)	1.46 (0.88-2.41)	0.15	A/G	179 (50.3)	55 (47.4)	0.97 (0.62-1.52)	0.9
Aditive	---	---	---	1.54 (1.02-2.33)	0.042	---	---	---	0.95 (0.70-1.30)	0.77

[1]Odds ratio (OR) adjusted for age, gender and alcohol consumption and smoking habits; [2]*P* values significant at $P \leqslant 0.05$.

(40.6%) of the AC haplotype observed in both groups (Case group: 0.403, Control group: 0.407; $\chi^2 = 0.01$, $P = 0.9194$). The haplotype frequencies of AT (Case group: 0.283, Control group: 0.312; $\chi^2 = 0.843$, $P = 0.3584$), haplotype frequencies of CC (Case group: 0.269, Control group: 0.247; $\chi^2 = 0.573$, $P = 0.4491$), and haplotype frequencies of CT (Case group: 0.045, Control group: 0.035; $\chi^2 = 0.569$, $P = 0.4505$) did not show significant

Table 4 Interaction between *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* polymorphisms and smoking habits or alcohol drinking on the risk of hepatocellular carcinoma

	Smoking habits							Alcoholic habit						
	No smoker			Smoker			^2P interaction	Non-alcoholic			Alcoholic			^2P interaction
	Case	Control	^{1}OR (95%CI)	Case	Control	^{1}OR (95%CI)		Case	Control	^{1}OR (95%CI)	Case	Control	^{1}OR (95%CI)	
MTRA2756G														
A/A	22	142	1.00	24	121	1.00	0.81	17	137	1.00	29	126	1.00	0.43
A/G-G/G	10	57	1.59 (0.65-3.87)	15	36	1.85 (0.81-4.21)		11	53	2.29 (0.91-5.72)	14	40	1.40 (0.63-3.12)	
MTRRA66G														
A/A	4	55	1.00	8	50	1.00	0.7	3	53	1.00	9	52	1.00	0.34
A/G-G/G	28	144	3.04 (0.95-9.71)	31	107	2.27 (0.90-5.72)		25	137	4.16 (1.11-15.55)	34	114	1.98 (0.83-4.75)	
MTHFRC677T														
C/C	15	85	1.00	13	64	1.00	0.55	14	85	1.00	14	64	1.00	0.75
C/T-T/T	17	114	0.80 (0.35-1.81)	26	93	1.13 (0.51-2.52)		14	105	0.86 (0.36-2.06)	29	102	1.04 (0.48-2.22)	
MTHFRA1298C														
A/A	13	119	1.00	19	85	1.00	0.61	11	113	1.00	21	91	1.00	0.56
A/C-C/C	19	80	2.00 (0.88-4.56)	20	72	1.49 (0.69-3.20)		17	77	2.10 (0.86-5.10)	22	75	1.49 (0.72-3.07)	

^{1}Odds ratio (OR) adjusted for age, gender and alcohol consumption and smoking habits; ^2P values significant at $P \leqslant 0.05$.

Table 5 Interaction between *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* polymorphisms and smoking habits or alcohol drinking on the risk of liver cirrhosis

	Smoking habits							Alcoholic habit						
	No smoker			Smoker			^2P interaction	Non-alcoholic			Alcoholic			^2P interaction
	Case	Control	^{1}OR (95%CI)	Case	Control	OR$^+$ (95%CI)		Case	Control	^{1}OR (95%CI)	Case	Control	^{1}OR (95%CI)	
MTRA2756G														
A/A	50	142	1.00	29	121	1.00	0.39	36	137	1.00	43	126	1.00	0.73
A/G-G/G	21	57	1.35 (0.72-2.53)	16	36	2.07 (0.97-4.41)		17	53	1.46 (0.72-2.97)	20	40	1.74 (0.89-3.38)	
MTRRA66G														
A/A	26	55	1.00	11	50	1.00	0.051	14	53	1.00	23	52	1.00	0.42
A/G-G/G	45	144	0.63 (0.35-1.17)	34	107	1.71 (0.77-3.82)		39	137	1.17 (0.56-2.45)	40	114	0.78 (0.42-1.48)	
MTHFRC677T														
C/C	31	85	1.00	17	64	1.00	0.96	24	85	1.00	24	64	1.00	0.76
C/T-T/T	40	114	0.96 (0.54-1.71)	28	93	0.98 (0.48-2.02)		29	105	1.05 (0.54-2.02)	39	102	0.91 (0.49-1.69)	
MTHFRA1298C														
A/A	33	120	1.00	24	85	1.00	0.32	23	113	1.00	34	92	1.00	0.56
A/C-C/C	38	79	1.68 (0.95-3.00)	21	72	1.06 (0.53-2.13)		30	77	1.83 (0.95-3.54)	29	74	1.11 (0.61-2.03)	

^{1}Odds ratio (OR) adjusted for age, gender and alcohol consumption and smoking habits; ^2P values significant at $P \leqslant 0.05$.

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 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 (*MTHFR C677T*, $P = 0.5483$; *MTHFR A1298C*, $P = 0.3861$; *MTR A2756G*, $P = 0.6765$; *MTRR A66G*, $P = 0.3840$) with overall survival.

DISCUSSION

The results showed that age $\geqslant$ 46 years and alcohol habit were associated with an increased risk of HCC development, similar to the results of Fassio *et al*[24], Varela *et al*[25], Munaka *et al*[26], Carrilho *et al*[3], Donato *et al*[27], Hamed *et al*[28] and Mittal *et al*[1].

Brazilian publications have reported that the mean age of the HCC patients is 54.6 years[3]; in Latin

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

Variables	Stage 0 e A Pacientes *n* (%)	Estage 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)	[3]	0.99
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 (3.67-174.38)	0.001[2]
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
MTRRA66G				
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

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

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 $\geqslant$ 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

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[31]. Although alcohol habit is a well-established risk factor for cirrhosis development[32], 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*[17] and Yu *et al*[33].

Chang *et al*[19], 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 in MTR GG genotype and breast cancer; Galbiatti *et al*[7] also concluded that *MTR A2765G* polymorphism is involved in the risk of head and neck cancer; de Lima *et al*[9] suggested an association between the *MTR A2756G* polymorphism and retinoblastoma susceptibility in a northeast population of Brazil, Ouerhani *et al*[10] found that *MTR A2756G* affecting bladder cancer risk.

Regarding the *MTRR A66G* polymorphism, Kwak *et al*[17] 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*[34] also found an association between this polymorphism and colorectal cancer, which is in agreement with to 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*[38] 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 heterozygous 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*[40] 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 $\geqslant$ 46 years, alcohol habit and the *MTR A2756G*, *MTHFR A1298C* and *MTRR A66G* polymorphisms are associated with an increased risk of HCC development; age $\geqslant$ 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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COMMENTS

Background

Age ⩾ 46 years, alcohol habit and the *MTR A2756G*, *MTHFR A1298C* and *MTRR A66G* polymorphisms are associated with an increased risk of hepatocellular carcinoma (HCC) development; age ⩾ 46 years, tobacco habit and the *MTR A2756G* polymorphism are associated with cirrhosis development.

Research frontiers

It is already known that polymorphisms cause DNA hypomethylation, which cause abnormal changes in gene expression inactivating suppressor genes tumor.

Innovations and breakthroughs

The authors confirm the literature data that report a positive association between the presence of polymorphisms and consumption of alcohol and tobacco to the development in an cirrhosis and later HCC.

Applications

These results may offer new possibilities of diagnosis with early initiation of treatment reflecting the improved quality of life.

Peer-review

This is a good descriptive study in which the authors analysed patients with cirrhosis, HCC and healthy individuals in the 2013-2015 period, the authors evaluated the association of the risk factors and polymorphisms in *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* genes involved in folate metabolism.

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