Methylene tetrahydrofolate reductase (MTHFR) and vascular endothelial growth factor (VEGF) polymorphisms in Brazilian patients with Hepatitis C virus (HCV)–related hepatocellular carcinoma (HCC)

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Carvalho SCR, Vasconcelos LRS, Fonseca L, Carmo RF, Tomitão MT, Aroucha DCBL, et al. Methylene tetrahydrofolate reductase (MTHFR) and vascular endothelial growth factor (VEGF) polymorphisms in Brazilian patients with Hepatitis C virus (HCV)–related hepatocellular carcinoma (HCC). Clinics (Sao Paulo). 2021;76:e2881

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OBJECTIVE: The folate pathway is involved in hepatic carcinogenesis and angiogenesis. Polymorphisms in genes related to such processes, including methylene tetrahydrofolate reductase (MTHFR) and vascular endothelial growth factor (VEGF) may play an important role in the development of hepatocellular carcinoma (HCC). The objective of this study was to evaluate MTHFR and VEGF polymorphisms in Brazilian patients with hepatitis C virus (HCV)–related HCC.

METHODS: A total of 119 patients diagnosed with confirmed HCC and HCV were included in the study. SNP genotyping assays were performed using real-time PCR. VEGFA (rs2010963, rs3025039, and rs833061) and MTHFR C677T (rs1801133, rs1801131) polymorphisms were evaluated.

RESULTS: The C alleles of MTHFR (rs1801131) and VEGF (rs2010963) were associated with protection against the development of multinodular HCC, while the T allele of MTHFR (rs1801133) was associated with a higher risk of multinodular presentation [p=0.04 OR 1.835 CI (1.022–3.297)]. Multivariate analysis revealed that the GG/GC genotypes of VEGF rs2010963 were independently associated with multinodular tumors at diagnosis (p=0.013; OR 4.78 CI (1.38–16.67)).

CONCLUSION: Our results suggest that these polymorphisms may increase the risk of rapid tumor progression in patients with HCV infection. This subgroup of patients with HCC and who present polymorphism is more likely to be diagnosed with multinodular disease and not be amenable to receiving curative treatments. These data must be validated in larger cohorts, and the screening intervals can be customized based on genetic history.

KEYWORDS: Polymorphisms; Methylene Tetrahydrofolate Reductase; Vascular Endothelial Growth Factor; Hepatocellular Carcinoma; Hepatitis C.

INTRODUCTION

Hepatitis C virus (HCV) chronic infection is one of the leading causes of chronic liver disease worldwide (1). It is estimated that 20% of the chronically infected patients will develop liver cirrhosis within 13 to 23 years, and among these, 1-4% will develop hepatocellular carcinoma (HCC) each year (2).

All etiologies leading to cirrhosis may be further complicated by HCC development, but the risk is higher in patients with viral hepatitis. One-third of the cirrhotic patients develop HCC in their lifetime (1). In Brazil, HCC has been associated with liver cirrhosis in 98% of the cases, and HCV coexists in 54% of these cases. Other etiologies of HCC-associated liver disease include hepatitis B virus (HBV) (16% of the cases), alcohol consumption (14%), non-alcoholic fatty liver disease (3%), and hemochromatosis (1%). Other etiologies include autoimmune hepatitis and primary biliary cirrhosis (4%) (3,4).
Several hallmarks of carcinogenesis remain unclear, although there is solid evidence for the role of vascular endothelial growth factor (VEGF) signaling (5). Angiogenesis is an important step in cancer development and is necessary for primary tumor cell growth, invasion, and metastasis (6). Polymorphisms in the regulatory regions of VEGF are associated with the occurrence of HCC and have been suggested as biomarkers of HCV-related chronic liver disease. Another protein potentially associated with HCC development is methylene tetrahydrofolate reductase (MTHFR), which plays an important role in the folate pathway; MTHFR is also involved in the maintenance of new cells and in DNA methylation, synthesis, and repair (7). Prior studies have evaluated the association between MTHFR polymorphisms and HCC, but the results remain controversial (8,9).

The prognosis of HCC is closely related to the stage at diagnosis. Patients with single tumors who are treated by resection or ablation present long-term survival, as do patients satisfying the Milan criteria who underwent liver transplantation. In contrast, patients with multinodular spread or extrahepatic disease have a poor prognosis despite the recent advances in intra-arterial and systemic treatments (10). Therefore, the search for biomarkers that can help identify more aggressive tumor behavior and early spread is of utmost importance.

In the present study, we aimed to verify the association of MTHFR and VEGF polymorphisms with tumor burden at diagnosis and clinical outcomes in patients with HCV-related HCC.

## METHODS

### Population and study design

This study included 119 patients with chronic HCV infection and HCC who were recruited between January 2014 and December 2016. Patient follow-up was conducted at the hepatology unit of the Oswaldo Cruz University Hospital of the University of Pernambuco and at the Institute of Liver and Transplants of Pernambuco, Northeast Region, Brazil. All patients were over 18 years of age, and chronic HCV infection was confirmed by polymerase chain reaction (PCR) (HCV-RNA +). HCC was diagnosed by computed tomography (CT) and/or nuclear magnetic resonance imaging (MRI). The radiological criteria used for HCC diagnosis were the presence of intense contrast enhancement in the arterial phase and washout of contrast in the venous-delayed phases, according to the American Association for the Study of Liver Diseases (AASLD) (11). Tumor staging was performed using the Barcelona Clinic Liver Cancer staging system (12). The exclusion criteria were human immunodeficiency virus (HIV) and/or HBV coinfection and cirrhosis of any NON-HCV etiology. To characterize the immunodeficiency virus (HIV) and/or HBV coinfection and staging system (12). The exclusion criteria were human immunodeficiency virus (HIV) and/or HBV coinfection and cirrhosis of any NON-HCV etiology. To characterize the frequency of genotypes in our population, we used blood bank material from 128 individuals with normal liver enzymes.

### Ethics approval and consent to participate

This study was conducted in accordance with the principles of the Declaration of Helsinki. The study protocols were approved by the Committee of the Clinics Hospital of the University of São Paulo School of Medicine (No. 349/14).

Informed consent was obtained from all participants included in the study.

### Blood collection and DNA extraction

Peripheral blood samples (10 mL) were collected by peripheral vein puncture in a tube containing ethylenediaminetetraacetic acid (EDTA). Genomic DNA was isolated using the PureLink™ Genomic DNA Mini Kit (Invitrogen-Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. DNA concentrations were quantified using a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA); the following formula was used: 1 OD260=50 μg/mL DNA. DNA purity was assessed based on the ratio of absorbance at 260 and 280 nm, and only DNA samples with a ratio between 1.8 and 2.0 were used in this study.

### Genotyping VEGF and MTHFR single nucleotide polymorphisms (SNPs)

Genotyping was performed using the TaqMan® SNP Genotyping Assays technique (Thermo Fisher Scientific, CA, USA) through base discrimination using fluorophore-labeled probes, with real-time PCR amplification. Polymorphisms were detected using the following assays: VEGFA -634G>C (rs2010963) assay (C_8311614_10) VEGFA 936C>T (rs3025039) assay (C_16198794_10) VEGFA -460C>T (rs833061) assay (C_1647381_10), MTHFR C677T (rs1801133) assay (C_1202883_20), and MTHFR A1298C (rs1801131) assay (C_850486_20).

Real-time PCR was performed on a Real Time PCR Step One Plus system (Applied Biosystems, Life Technologies, Foster City, USA) with TaqMan Genotyping 20× master mix (Thermo Fisher Scientific, CA, USA).

### Statistical analysis

The data were statistically analyzed using SPSS v.17 software. Allele frequencies were estimated by the gene counting method using PLINK. The chi-square test was used to determine if the genotypic distribution was in accordance with the Hardy–Weinberg equilibrium. Associations between categorical variables were evaluated using Pearson’s chi-square and Fisher’s exact tests, as appropriate. For the quantitative variables, the t-test or Mann–Whitney test was used to determine whether the variables followed a normal distribution. Differences were considered significant at \( p < 0.05 \), and Bonferroni correction for multiple associations was used to confirm the association in a univariate model. The magnitude of these associations was estimated as odds ratios (ORs) using 95% confidence intervals (CIs). The ORs were adjusted for possible confounding variables using multivariate logistic regression.

## RESULTS

### Clinical and laboratory characteristics

A total of 119 patients with chronic HCV infection and HCC were evaluated and followed up at the hepatology unit of Oswaldo Cruz University Hospital of the University of Pernambuco and at the Institute of Liver and Transplants of Pernambuco. Among the individuals with HCC, 70.59% were men and 29.41% were women. The mean age of the participants was 62.3 years (range: 46-86 years) and the mean body mass index (BMI) was 25.6 kg/m² (range: 15.2-38.5). Type 2 diabetes mellitus (T2DM) occurred in 33.61% of the patients.
Based on the Child–Pugh classification, the patients were found to be distributed in three groups (A, 60.9%; B, 36.5%; C, 2.6%). Tumor size was assessed based on the largest diameter of the largest lesion. The mean tumor size was 4.4 cm (range: 0.5-13.8 cm). Unifocal disease was observed in 46.21% of the patients, and multinodular disease was observed in 52.10% of the patients; in 2 (1.68%) patients, the number of lesions was not characterized.

The frequency of genotype 1 was found to be 67%. There were no significant differences in age, sex, BMI, and DM or in laboratory variables (total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransferase; ALT, gamma glutamyltransferase; GGT, alkaline phosphatase; ALP, alpha 1 fetoprotein; HCV, hepatitis C virus; T2DM, type 2 diabetes.

\[ \text{Analysis of genotype frequency and distribution of} \]
\[ \text{SNPs in MTHFR and VEGF in patients with HCC and} \]
\[ \text{HCV and in control subjects} \]

The chromosomal positions of the MTHFR and VEGF polymorphisms, expected and observed frequencies, and information on Hardy-Weinberg equilibrium, minor allele, and major allele frequencies are described in Table 1. In the present study, the observed and expected genotype frequencies of the MTHFR and VEGF SNPs were consistent with the Hardy–Weinberg equilibrium ($p > 0.05$).

\[ \text{Association between MTHFR and VEGF and tumor} \]
\[ \text{characteristics} \]

When analyzing the relationship between SNPs and the number of tumors, we observed that the MTHFR and VEGF were correlated with the presentation of the tumors as uninode and multinodular. The MTHFR C (rs1801131) and VEGF C (rs2010963) alleles were inversely associated with multinodular HCC at diagnosis. The MTHFR C allele frequency (rs1801131) was 0.18 vs 0.32 for patients with multinodular and uninodular HCC, respectively [$p=0.012$ OR 0.46 CI (0.25-0.85)]. The VEGF C allele frequency (rs2010963) was 0.27 vs 0.43 [$p=0.009$ OR 0.48 CI (0.28-0.84)] for patients with multinodular and uninodular HCC, respectively. In contrast, the MTHFR T allele (rs1801133) was associated with multinodular HCC at diagnosis, with an allele frequency of 0.33 for patients with multinodular tumors and 0.21 for patients with a uninodular tumor [$p=0.040$ OR 1.83 CI (1.02-3.29)]. After Bonferroni correction for multiple associations, only one VEGF allele (rs2010963) was significantly associated with multinodular tumors at diagnosis (Table 3).

Investigation of an association between the MTHFR polymorphism and presentation of uninodeal and multinodular tumors revealed a significant association for MTHFR rs1881131 in the genotype and dominant models [$p=0.036$ and $p=0.042$, OR 2.18 CI (1.04-4.59), respectively]. For this SNP, the AA genotype was associated with the development of multinodular HCC. For the MTHFR rs1881133, the dominant genetic model exhibited a significant association, where the CT/TT genotype was associated with the development of multinodular HCC [$p=0.04$ OR 2.13 CI (1.01-4.54)]. With respect to VEGF rs2010963, a significant association was observed in the recessive and genotypic models, and the GG/GC genotypes were associated with the development of multinodular HCC [$p=0.018$ and $p=0.005$, OR 4.95 (CI 1.52-16.13), respectively] (Table 4).

We also evaluated the clinical and laboratory characteristics of the MTHFR (rs1801131 and rs1801133) and VEGF (rs2010963) tumors that showed significant differences in the BCLC classifications shown in Table 1, namely mean serum AFP level, Child–Pugh score, mean tumor size, and the relationship to the best genetic model (Table 5). However, no significant differences were observed in any of the aforementioned variables (Table 5).

In the binary logistic regression analysis, after adjusting for sex and age, we observed that the GG/GC genotypes for VEGF rs2010963 were an independent risk factor for multinodular HCC at presentation [$p=0.013$, OR 4.78 CI (1.38-16.67)] (Table 6).

**Table 1 - Clinical and laboratory characteristics of Brazilian patients with HCV-related HCC, according to the BCLC (Barcelona Clinic Liver Cancer) classification.**

| VARIABLES          | HCV-CHC (N=119) | BCLC-0/A (N=60) | BCLC-B (N=32) | BCLC-C (N=25) | $p$-value |
|--------------------|----------------|----------------|---------------|---------------|-----------|
| **Clinical**       |                |                |               |               |           |
| Age (years)        | 62.30 ± 8.63   | 62.17 ± 8.79   | 62.5 ± 9.13   | 63.08 ± 7.74  | 0.79      |
| Male sex           | 84 (70.59%)    | 40 (66.67%)    | 23 (71.9%)    | 21 (84%)      | 0.27      |
| BMI (kg/m²)        | 25.60 ± 4.32   | 26.1 ± 4.79    | 24.8 ± 3.2    | 25.34 ± 4.19  | 0.70      |
| T2DM               | 40 (33.61%)    | 20 (33.32%)    | 9 (28.12%)    | 8 (32%)       | 0.87      |
| Tumor size         | 4.4 ± 2.6      | 2.8 ± 1.2      | 5.9 ± 2.7     | 6.15 ± 2.96   | $<0.0001^*$ |
| **Laboratory**     |                |                |               |               |           |
| TB [mg/dL] (min-max)| 1.6 (0.4-7.2)  | 1.7 (0.5-7.22) | 1.4 (0.5-3.7) | 1.74 (0.36-5.51) | 0.70 |
| AST (U/L) (min-max)| 98.9 (16-237)  | 103.9 (28-237) | 93.2 (31-183.9)| 96 (16-225)   | 0.46      |
| ALT (U/L) (min-max)| 90.2 (18-249)  | 100.7 (19-240) | 84.4 (26-208) | 75.92 (16-225) | 0.05      |
| GGT (U/L) (min-max)| 239 (26-1629)  | 232.8 (26-1133)| 184.7 (41-532) | 320.61 (50-1629)| 0.10      |
| ALP (U/L) (min-max)| 168.4 (36-630)| 151.2 (51-320) | 192.3 (36-618.3)| 170.74 (61-630)| 0.40      |
| Platelets (min-max)| 119037 (12000-324000)| 107701.7 (12000-230000)| 131645.2 (46000-247000)| 131260.9 (55000-324000)| 0.18 |
| AFP ng/mL (min-max)| 1492.5 (1.7-60500)| 660 (1.8-19000)| 691.9 (1.7-9604) | 4468.85 (1.97-60500)| 0.002* |
| VHC genotype 1 (%) | 67%            | 63.32%         | 50%           | 52%           | 0.30      |

**Abbreviations:** HCV, hepatitis C virus; HCC, hepatocellular carcinoma; BCLC, Barcelona Clinic Liver Cancer; BMI, body mass index; TB, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyltransferase; ALP, alkaline phosphatase; AFP, alpha 1 fetoprotein; HCV, hepatitis C virus; T2DM, type 2 diabetes.

*Statistically significant.*
Table 2 - Distribution of SNPs in Brazilian patients with HCV-related HCC and in the control group, testing for Hardy-Weinberg equilibrium (HWE).

| CHR | SNP    | Gene | Group | Minor allele | Major allele | Genotypes | O(HET)   | E(HET)   | p-value (HWE) |
|-----|--------|------|-------|--------------|--------------|-----------|----------|----------|---------------|
| 1   | rs1801131 | MTHFR | HCC   | C            | A            | 9/45/65   | 0.3782   | 0.3893   | 0.8136        |
| 1   | rs1801133 | MTHFR | Control | C            | A            | 10/39/79 | 0.3047   | 0.3547   | 0.1319        |
| 1   | rs1801133 | MTHFR | HCC   | T            | C            | 12/42/65 | 0.3529   | 0.4008   | 0.2502        |
| 1   | rs1801133 | MTHFR | Control | T            | C            | 10/60/58 | 0.4688   | 0.4297   | 0.4103        |
| 6   | rs2010963 | VEGF  | HCC   | C            | G            | 18/48/53 | 0.4034   | 0.4567   | 0.2282        |
| 6   | rs2010963 | VEGF  | Control | C            | G            | 16/44/46 | 0.5      | 0.4761   | 0.7102        |
| 6   | rs3025039 | VEGF  | HCC   | T            | C            | 0/31/88  | 0.2605   | 0.2266   | 0.2138        |
| 6   | rs3025039 | VEGF  | Control | T            | C            | 0/37/91  | 0.2891   | 0.2473   | 0.07268       |
| 6   | rs833061  | VEGF  | HCC   | C            | T            | 16/59/44 | 0.4958   | 0.4723   | 0.6987        |
| 6   | rs833061  | VEGF  | Control | C            | T            | 2/54/53  | 0.4219   | 0.4688   | 0.2611        |

Abbreviations: SNP, single nucleotide polymorphism; CHR, chromosome; O(HET), observed relative frequency of heterozygotes; E(HET), expected relative frequency of heterozygotes; HWE, Hardy-Weinberg equilibrium; MTHFR, methylenetetrahydrofolate reductase; VEGF, vascular endothelial growth factor.

Table 3 - Correlation between MTHFR and VEGF SNPs and number of tumors in Brazilian patients with HCV-related HCC.

| CHR | SNP    | Gene | Minor allele | Multi nodular | Uninodular | Major allele | p-value | OR (95%) |
|-----|--------|------|--------------|---------------|------------|--------------|---------|----------|
| 1   | rs1801131 | MTHFR | C            | 0.1855        | 0.3273     | A            | 0.01267 | 0.4681 (0.2561-0.8555) |
| 1   | rs1801133 | MTHFR | T            | 0.3387        | 0.2182     | C            | 0.04086 | 1.835 (1.022-3.297) |
| 6   | rs2010963 | VEGF  | C            | 0.2742        | 0.4364     | G            | 0.009456* | 0.488 (0.2828-0.842) |
| 6   | rs3025039 | VEGF  | T            | 0.1129        | 0.1455     | C            | 0.4573  | 0.7477 (0.3468-1.612) |
| 6   | rs833061  | VEGF  | C            | 0.4032        | 0.3636     | T            | 0.5344  | 1.182 (0.6968-2.007) |

Abbreviations: CHR, chromosome; SNP, single nucleotide polymorphism; COX2, cyclooxygenase 2; MTHFR, methylenetetrahydrofolate reductase; VEGF, vascular endothelial growth factor; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; OR, odds ratio; CI, confidence interval.*Statistically significant after Bonferroni correction for multiple comparisons (p <0.01).
To our knowledge, this is the first study to analyze tumor characteristics in HCC-infected HCC patients and their association with MTHFR and VEGF polymorphisms, thereby revealing the relationship between these polymorphisms and tumor presentation.

Our cohort shares a close distribution of demographics with larger studies in the Brazilian population in terms of sex (59% men) and age (mean 62.3 years) (4). We also validated the values of well-known prognostic factors, such as AFP levels, which are directly related to BCLC stages (13). More advanced BCLC stages—characterized by gradual tumor growth along with an increase in nodule number, vascular invasion, and extrahepatic disease—are associated with high AFP levels.

Angiogenesis—a fundamental process that is required for the proper functioning of organs—is involved in tissue repair and a variety of pathological processes (14). VEGF can directly stimulate the growth of new blood vessels, (15) and plays an important role in HCC angiogenesis (16). Yao et al. suggested that the serum VEGF level correlates with tumor stage and HCC aggressiveness (17). Changes in plasma VEGF levels may be influenced by VEGF polymorphisms, and a correlation has been observed between VEGF polymorphisms and HCC onset (18).

In this study, we did not find a significant association between the BCLC (B/C vs. A) groups. Baitello et al. evaluated a VEGF polymorphism (C936T-rs3025039) in 102 patients from São Paulo with an HCC diagnosis and BCLC classification, but did not find any association (19). Wu et al. showed an association of HCC in HBV patients with a VEGF polymorphism (rs1801133) but did not analyze the correlation with the BCLC stage (18). Kong et al. evaluated 416 Korean patients with a diagnosis of HCC of different etiologies and analyzed 19 VEGF polymorphisms. They found that for VEGF rs2010963, the GG genotype was associated with increased tumor size and more advanced BCLC stage (p=0.025), but no association was found with the number of nodules (20).

In contrast, we observed that the GG/GC genotype of VEGF rs2010963 was a risk factor for multinodular HCC at diagnosis. This SNP has already been shown to affect gene expression patterns and consequently alter the synthesis of active VEGF. The CC genotype of this SNP has been found to be associated with decreased VEGF expression. Thus, the GG/GC genotypes are associated with increased VEGF expression and angiogenesis, leading to more pronounced tumorigenesis and an increase in the multinodular forms (21,22). Park et al. observed that VEGF levels progressively increased as the nodules progressed from low-grade to high-grade dysplasia and early HCC (16). Deli et al. reported detection of VEGF expression in 72 patients out of the investigated 105 HCC patients (68.6%) using immunohistochemistry (22). The expression of VEGF in HCC tissues with microscopic venous invasion is significantly higher than that in HCC tissues without microscopic venous invasion (23).

The relationship between MTHFR polymorphisms (rs1801133) and HCC has been well studied. A meta-analysis by Jin et al. conducted showed that an MTHFR polymorphism, i.e., rs1801133 increases the risk of developing HCC, especially in European patients with chronic liver disease; in this meta-analysis, ten studies showed that compared to the CT genotype, the TT genotype increased the risk of developing HCC (8). Kwak et al. showed no association between MTHFR polymorphisms, i.e., rs1801133 and rs1801131 and the risk of developing HCC (24).

We analyzed the association of MTHFR SNPs, i.e., rs1881131 and rs1801133 with all clinical variables in patients with HCC and HCV. We identified a significant association of these SNPs with tumor presentation (uninodular vs. multinodular) in the univariate model. The AA genotype of rs1801131 and the CT/TT genotype of rs1801133 were risk factors for the multinodular form. Functional studies on MTHFR variants have shown that the variant rs1801133-TT is related to decreased activity of the enzyme responsible for

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**Table 4 - Analysis of the correlation between MTHFR and VEGF and the number of tumors in Brazilian patients with HCV-related HCC using genetic association models.**

| Gene (SNP) | Multinodular (62) | Uninodular (55) | Association model | p-value OR (95%CI) |
|-----------|-------------------|-----------------|-------------------|-------------------|
| MTHFR (rs1881131) | | | | |
| AA | 40 (0.64) | 25 (0.45) | AA vs AC vs CC | p=0.036 |
| AC | 21 (0.34) | 24 (0.44) | AA/AC vs CC | p=0.505, 7.47 (0.87-64.17) |
| CC | 1 (0.02) | 6 (0.11) | AA vs AC/CC | p=0.042, 2.18 (1.04-4.59) |
| CC (rs1801133) | | | | |
| CC | 28 (0.45) | 35 (0.64) | CC vs CT vs TT | p=0.150 |
| CT | 26 (0.42) | 16 (0.29) | CC/CT vs TT | p=0.372, 0.53 (0.15-1.87) |
| TT | 8 (0.13) | 4 (0.07) | CT/TT vs CC | p=0.04, 2.13 (1.01-4.54) |
| VEGF (rs2010963) | | | | |
| GG | 32 (0.52) | 21 (0.38) | GG vs GC vs CC | p=0.018 |
| GC | 26 (0.42) | 20 (0.36) | GG/GC vs CC | p=0.005, 4.95 (1.52-16.13) |
| CC | 4 (0.08) | 14 (0.26) | GG vs GC/CC | p=0.192, 1.73 (0.82-3.61) |
| CC (rs3025039) | | | | |
| CC | 48 (0.77) | 39 (0.71) | CC vs CT vs TT | p=0.525 |
| CT | 14 (0.23) | 16 (0.29) | CC/CT vs TT | - |
| TT | 0 (0) | 0 (0) | CC vs CT/TT | p=0.525, 1.41 (0.61-3.23) |
| VEGF (rs833061) | | | | |
| TT | 21 (0.34) | 22 (0.40) | TT vs TC vs CC | p=0.815 |
| TC | 32 (0.52) | 26 (0.47) | TT/TC vs CC | p=0.999, 0.85 (0.30-2.48) |
| CC | 9 (0.14) | 7 (0.13) | TT vs TC/CC | p=0.565, 0.77 (0.36-1.63) |
DNA repair, and consequently, to the onset of HCC (24-26). Additionally, a meta-analysis showed that the CC genotype of rs1881131 is a protective factor in patients with HCC (27) and other cancers (28).

Our results suggest that these polymorphisms may increase the risk of rapid tumor progression in HCC patients with HCV infection. This subgroup of patients is more likely to be diagnosed with multinodular disease and not be amenable to receiving curative treatments. If these data are further validated in larger cohorts, screening intervals can be personalized based on the genetic background. In addition, our findings may aid the development of a risk stratification model to prioritize liver transplant recipients on waiting lists based on an increased risk of HCC progression and dropout.

Although our study was the first to analyze tumor characteristics in HCV-infected HCC patients and their association with \textit{MTHFR} and \textit{VEGF} polymorphisms in an ethnically mixed population, more studies are needed to confirm this hypothesis in other populations with other etiologies and stratification for tumor presentation. Further, functional studies should be performed to determine the impact of these variants on the production of active molecules. Therefore, this approach may provide new insights into the clinical management of HCC patients infected with HCV, and consequently, may aid better prediction of prognosis and development of novel therapeutic measures.

Our results must be interpreted within the limitations of cross-sectional studies, i.e., small sample size and heterogeneity between studies with respect to allele frequencies and penetrance in different ethnicities, in addition to variations in age, lifestyle behaviors, and medication usage.

**CONCLUSIONS**

In conclusion, we have determined that the AA genotype of \textit{MTHFR} SNPs, i.e., rs1801131, rs1801133 and the CT/TT genotypes of \textit{VEGF} rs2010963 are associated with multinodular HCC at diagnosis. The GG/GC genotypes of \textit{VEGF} rs2010963 were shown to be independent risk factors for the development of the multinodular form of HCC. Individuals with these genotypes may have impaired cellular DNA repair and might exhibit tumor progression.

**AUTHOR CONTRIBUTIONS**

Carvalho SCR, Vasconcelos LRS, Carmo RF, Tomitão MT, Aroucha DCBL, Pereira LMMB and Stefano JT contributed to the acquisition, analysis and interpretation of experimental data. Fonseca L, Ribeiro-Júnior U, Oliveira CP and Carrilho FJ contributed to the design and critical review of the manuscript.
REFERENCES

1. Stasi C, Silvestri C, Voller F. Update on Hepatitis C: Epidemiology, Unaware and Untreated Infected Population Could Be the Key to Elimination. SN Compr Clin Med. 2020;7:1-8. https://doi.org/10.1007/s42399-020-00586-3

2. Fedeli U, Grande E, Grippio F, Grova L. Mortality associated with hepatitis C and hepatitis B virus infection: A nationwide study on multiple causes of death data. World J Gastroenterol. 2017;23(10):i866-71. https://doi.org/10.3748/wjg.v23.i10.1866

3. Ikuchi L, Chagas AL, Alencar RS, Paranaagu-Vezozzo DC, Carrilho FJ. Clinical and epidemiological aspects of hepatocellular carcinoma in Brazil. Antivir Ther 2015;16(3 Pt B):445-9. https://doi.org/10.3851/IMP2602

4. Carrilho FJ, Ikuchi L, Branco F, Goncalves CS, Mattos AA; Brazilian HCC Study Group. Clinical and epidemiological aspects of hepatocellular carcinoma in Brazil. Clinics (Sao Paulo). 2010;65(12):1285-90. https://doi.org/10.1590/S1807-93522010001200010

5. Mise M, Arri S, Higashijichi H, Furutani M, Niwano M, Harada T, et al. Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. Hepatology. 1996;23(3):455-64. https://doi.org/10.1002/hep.510230309

6. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9(9):669-76. https://doi.org/10.1038/nm0903-669

7. Duthie SJ. Folate and cancer: how DNA damage, repair and methylation impact on colon carcinogenesis. J Inherit Metab Dis. 2011;34(1):101-9. https://doi.org/10.1007/s10545-010-9128-0

8. Jin F, Qu LS, Shen XZ. Association between the methylenetetrahydrofolate reductase C677T polymorphism and hepatocellular carcinoma risk: a meta-analysis. Diagn Pathol. 2009;4:39. https://doi.org/10.1186/1746-1596-4-39

9. Qin X, Peng Q, Chen Z, Deng Y, Huang S, Xu J, et al. The association between MTHFR gene polymorphisms and hepatocellular carcinoma risk: a meta-analysis. PLoS One. 2013;8(2):e56070. https://doi.org/10.1371/journal.pone.0056070

10. Former A, Reig M, Bruix J. Hepatocellular carcinoma. Lancet. 2018;391(10127):1301-14. https://doi.org/10.1016/S0140-6736(18)30010-2

11. Bruix J, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. Hepatology. 2011;53(3):1020-2. https://doi.org/10.1002/hep.24199

12. Forner A, Reig ME, de Lope CR, Bruix J. Current strategy for staging and treatment: the BCLC update and future prospects. Semin Liver Dis. 2011;31(3):1301-14. https://doi.org/10.1055/s-0031-1247133

13. Liu C, Xiao QG, Yan LN, Li B, Jiang L, Wen TF, et al. Value of a-fetoprotein in association with clinicopathological features of hepatocellular carcinoma. World J Gastroenterol. 2013;19(11):1811-9. https://doi.org/10.3748/wjg.v19.i11.1811

14. Folkman J, Shing Y. Angiogenesis. J Biol Chem. 1992;267(16):10931-4. https://doi.org/10.1016/S0021-9258(19)49853-0

15. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989;246(4935):1306-9. https://doi.org/10.1126/science.2479986

16. Park YN, Kim YB, Yang KM, Park C. Increased expression of vascular endothelial growth factor and angiogenesis in the early stage of multistep hepatocarcinogenesis. Arch Pathol Lab Med. 2000;124(7):1061-5. https://doi.org/10.1043/0003-8884-124.7.1061

17. Yao DF, Wu XH, Zhu Y, Shi GS, Dong ZZ, Yao DB, et al. Quantitative analysis of vascular endothelial growth factor, microvacular density and their clinicopathologic features in human hepatocellular carcinoma. Hepatobiliary Pancreat Dis Int. 2005;4(2):220-6.

18. Wu X, Yin Z, Zhang W, Wu J, Chen K, Wang H, et al. Polymorphisms in the VEGFA promoter are associated with susceptibility to hepatocellular carcinoma by altering promoter activity. Int J Cancer. 2013;133(5):1085-93. https://doi.org/10.1002/ijc.28109

19. Baitello ME, Tenani GD, Ferreira RF, Nogueira V, Pinhel MA, da Silva RC, et al. VEGF Polymorphisms Related to Higher Serum Levels of Protein Identify Patients with Hepatocellular Carcinoma. Can J Gastroenterol Hepatol. 2016;2016:9607054. https://doi.org/10.1155/2016/9607054

20. Kong SY, Park JW, Lee JA, Park JE, Park KW, Hong EK, et al. Association between vascular endothelial growth factor gene polymorphisms and survival in hepatocellular carcinoma patients. Hepatology. 2007;46(2):446-55. https://doi.org/10.1002/hep.21720

21. Ratnasari N, Nurdianah S, Sadewa AH, Hakimi M. The role of vascular endothelial growth factor -634 G/C and its soluble receptor on chronic liver disease and hepatocellular carcinoma. Arab J Gastroenterol. 2016;17(2):61-6. https://doi.org/10.1016/j.ajg.2016.06.005

22. Deli G, Jin CH, Mu R, Yang S, Liang Y, Chen D, et al. Immunohistochemical assessment of angiogenesis in hepatocellular carcinoma and surrounding cirrhotic liver tissues. World J Gastroenterol. 2005;11(7):960-3. https://doi.org/10.3748/wjg.v11.i7.960

23. Huang GW, Yang LY, Lu WQ. Expression of hypoxia-inducible factor 1alpha and vascular endothelial growth factor in hepatocellular carcinoma: Impact on neovascularization and survival. World J Gastroenterol. 2005;11(17):1705-8. https://doi.org/10.3748/wjg.v11.i17.1705

24. Kwak SY, Kim UK, Cho HJ, Lee HK, Kim HJ, Kim NK, et al. Methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) gene polymorphisms as risk factors for hepatocellular carcinoma in a Korean population. Anticancer Res. 2008;28(5A):2807-11.

25. Cui LJ, Song Y, Si H, Shen F, Shiin MH, Kim HY, et al. Folate metabolism-related gene polymorphisms and susceptibility to primary liver cancer in North China. Med Oncol. 2012;29(3):1837-42. https://doi.org/10.1007/s12032-011-0466-y

26. D’Amico M, Pasta L, Sammarco P, MTHFR C677TT, PAI-1 4G-4G, V Lei- den Q506, and prothrombin C20210A in hepatocellular carcinoma with and without portal vein thrombosis. J Thromb Thrombolysis. 2009;28(1):70-3. https://doi.org/10.1007/s11239-008-0246-6

27. Qi YH, Yao LP, Cui GB, Liang J, Shao QJ, Yan LF, et al. Meta-analysis of MTHFR C677T and A1298C gene polymorphisms: association with the risk of hepatocellular carcinoma. Clin Res Hepatol Gastroenterol. 2014;38(2):172-80. https://doi.org/10.1016/j.clre.2013.10.002

28. Zhao X, Ling J, Zhou Y, Zhao H, Song Y, Tan Y. Polymorphisms of MTHFR C677T and A1298C association with oral carcinoma risk: a meta-analysis. Cancer Invest. 2012;30(6):447-52. https://doi.org/10.3109/07357907.2012.681820