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ORIGINAL ARTICLE

Association of *MTHFR* and *TYMS* gene polymorphisms with the susceptibility to HCC in Egyptian HCV cirrhotic patients

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Abstract

Identification of host genetic factors influencing the risk of developing hepatocellular carcinoma (HCC) in patients with chronic hepatitis C virus (HCV) infection may help to refine patients' selection to benefit from specific preventative measures and/or adapted screening policies. Thus, this study aimed to investigate the association of *MTHFR* c.677C > T and c.1298A > C in addition to *TYMS* 3′-UTR 6-bp ins/del polymorphisms with the susceptibility to HCV-related HCC in an Egyptian population. Polymerase chain reaction–restriction fragment length polymorphism was performed to genotype the polymorphisms in 194 HCV-infected patients subdivided into liver cirrhotic (LC, $n = 104$) and HCC ($n = 90$) patients as well as 100 healthy subjects. In healthy controls, the *MTHFR* c.677C > T polymorphism under the homozygous and recessive models ($p = 0.005$) and the c.1298A > C polymorphism under all the tested genetic models (p-values range from < 0.001 to 0.007) were associated with an increased risk of HCC. In LC patients, the *MTHFR* c.677C > T polymorphism under the homozygous, dominant, and recessive models (p-values range from 0.001 to 0.007), as well as *MTHFR* c.1298A > C under the homozygous model only ($p = 0.014$), increased the susceptibility to HCC. The C/C and T/C haplotypes of *MTHFR* c.677C > T and *MTHFR* c.1298A > C polymorphisms were contributed to an increased risk of healthy subjects to develop HCC (p-values range from < 0.001 to 0.015), while only the T/C haplotype was associated with the progression of HCC in LC patients ($p = 0.001$). In conclusion, *MTHFR* c.677C > T and c.1298A > C in addition to their haplotypes may contribute to the development of HCV-related HCC in an Egyptian population. These findings may aid in the early diagnosis and management of HCC.

Keywords Hepatitis C virus (HCV) · Hepatocellular carcinoma (HCC) · Methylenetetrahydrofolate reductase (*MTHFR*) · Thymidylate synthase (*TYMS*) · Polymorphism

Introduction

With about 840,000 new cases diagnosed every year worldwide, liver cancer was estimated to be the fifth most common malignancy in men and the eleventh in women. Moreover, it is the third leading cause of cancer deaths globally [1]. Hepatocellular carcinoma (HCC) represents almost 80% of primary liver cancer cases and therefore attracts widespread attention due to its high occurrence, recurrence, and mortality rate [2].

Although the major risk factors for HCC vary somewhat with its geographical distribution, chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) are by far the most important risk factors and the primary cause of HCC [3]. Egypt has the highest HCV prevalence in the world, where 10% of the population is estimated to be chronically infected, mostly with genotype 4 [4, 5]. Given that 15–20% of patients exposed to chronic HCV infection will eventually develop HCC, the conventional risk factors appear to only explain in part the pathogenesis of HCC while other cofactors (environmental and/or genetic) are more likely to be involved in its development [6, 7].

Compared with the well-established associations between environmental risk factors and the development of HCC,

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genetic susceptibility factors of HCC are not as extensively studied or understood. Therefore, recent studies aim to identify the etiology-specific genetic variants predictive of HCC risk focusing on polymorphisms in candidate genes [8, 9].

Methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme in the complex metabolic entity involved in folate metabolism and the generation of S-adenosylmethionine (SAM). It catalyzes the irreversible conversion of 5, 10-methylenetetrahydrofolate into 5-methyltetrahydrofolate and thus plays a crucial role in DNA synthesis as well as the methylation of DNA and other large molecules [10–12]. *MTHFR* gene contains two common functional polymorphisms: a C–T transition at nucleotide 677 results in an alanine-to-valine exchange at position 222 (c.677C > T, p.Ala222Val), and an A–C transversion at position 1298 causes glutamine-to-alanine change at position 429 (c.1298A > C, p.Glu429Ala) [13]. Several studies investigated the possible associations between *MTHFR* c.677C > T and c.1298A > C polymorphisms and the risk of HCC development [14–18]. However, the results were inconsistent and inadequate to draw a definite conclusion.

Thymidylate synthase (TYMS) is another key enzyme involved in folate metabolism; it catalyzes the synthesis of deoxythymidine monophosphate from deoxyuridine monophosphate using 5, 10-methylenetetrahydrofolate as a methyl donor. Therefore, TYMS activity is important in dNTP balance and consequently DNA synthesis and repair because a sufficient pool of thymidylate is essential for minimizing the mis-incorporation of uracil into DNA, chromosomal breakage, and fragile site induction [19]. *TYMS* gene contains 3 functional polymorphisms in its 5'- and 3'-untranslated regions (UTRs) among which the 6-bp insertion/deletion of the sequence AAGTTA at nucleotide 1494 in the 3′-UTR (g.20844_20849ins/del) is thought to affect TYMS mRNA expression and stability [20]. *TYMS* polymorphisms were associated with the risk of solid tumors that indicates their essential role in the development of malignancies [21–23].

In light of the abovementioned facts, understanding the HCC development in HCV cirrhotic patients and its associated risk factors would be of great importance. Therefore and taken into account their critical role, the current study was designed to assess the association of the genetic single-nucleotide polymorphisms (SNPs) of *MTHFR* c.677C > T and c.1298A > C in addition to *TYMS* 6-bp ins/del polymorphism with the susceptibility to HCC in Egyptian HCV cirrhotic patients.

Subjects and methods

Study population

This is a case–control study conducted in the period between August 2016 and February 2019. A total of 194 HCV-infected patients including 104 with liver cirrhosis (LC) and 90 with HCC were sequentially recruited from inpatients and the outpatient clinic of the Tropical Medicine and Hepatology Department, Faculty of Medicine, Mansoura University. Patients suffering from a different type of viral hepatitis, other liver diseases, HCC due to other causes, or other organ malignancy, as well as those who drink alcohol, were excluded. Additionally, 100 age- and sex-matched participants with no underlying liver diseases recruited from healthy blood donors at Mansoura University Hospitals were included in the study. Of note, Egypt is quite a homogeneous society, with 99.6% being ethnically Egyptian.

The presence of HBV surface antigen, anti-HCV antibody, and HCV-RNA was tested in all study samples. HCC was diagnosed by serum α-fetoprotein (AFP), in addition to the diagnostic imaging by abdominal color Doppler ultrasound and spiral computed tomography according to the standards with high specificity for HCC ≥ 10 mm established by the American College of Radiology through its Liver Imaging Reporting and Data System (LI-RADS) and the guidelines of the European Association for the Study of the Liver (EASL). All HCV cirrhotic patients with or without HCC were further classified according to Child–Pugh classification.

From each participant, 5 ml of venous blood was collected. A part of the sample (3 ml) was collected into dry tubes, left to clot, and centrifuged to obtain sera in which biochemical parameters were assessed. The other part of the sample (2 ml) was collected into ethylene diamine tetraacetic acid (EDTA) coated tubes for molecular analyses.

Laboratory analyses

Liver functions including the activities of alanine transaminase (ALT) and aspartate transaminase (AST) as well as albumin and total bilirubin levels were determined in sera of the participants using available commercial kits provided by Spectrum-diagnostics (Cairo, Egypt). Moreover, serum AFP level was measured using an enzyme-linked immunosorbent assay (ELISA) kit purchased from DRG International Inc. (NJ, USA).

Molecular analyses

The c.677C > T and c.1298A > C polymorphisms of the *MTHFR* and the 3′-UTR 6-bp ins/del polymorphism of the *TYMS* gene were genotyped using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique. Briefly, genomic DNA was extracted from the whole blood using QIAamp® DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and the purity of the isolated DNA were assessed by measuring the

optical density at 260 and 280 nm using NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific).

PCRs were performed in a final volume of 25 µl with ~ 20 ng of DNA as template, 12.5 µl of 2 × PCR master mix (Thermo Fisher Scientific), and 2 µl of each of the forward and the reverse primers (LGC, Biosearch Technologies, Hoddesdon, UK) for the gene of interest; the reaction's volume was then completed with nuclease-free water. PCR tubes were loaded into Biometra Uno II thermal cycler (Göttingen, Germany), and the conditions were as follows: 10 min at 95 °C for initial denaturation followed by 40 amplification cycles of denaturation at 95 °C for 1 min, annealing at 63 °C (for *MTHFR*) or 61 °C (for *TYMS*) for 1 min, and extension at 72 °C for 1 min. The primers' sequences used were as follows.

MTHFR c.677C > T	Forward primer, 5′ TGAAGGAGAAGG TGTCTGCGGGA-3′ Reverse primer, 5′-AGGACGGTGCGT GAGAGTG-3′
MTHFR c.1298A > C	Forward primer, 5′-CAAGGAGGAGCT GCTGAAGA-3′ Reverse primer, 5′-CCACTCCAGCAT CACTCACT-3′
TYMS 3′-UTR 6-bp ins/del	Forward primer, 5′-CAAATCTGAGGG AGCTGAGT-3′ Reverse primer, 5′-CAGATAAGTGGC AGTACAGA-3′

The PCR products were analyzed by electrophoresis on a 2% agarose gel. The size of the PCR amplified products was 198 bp for *MTHFR* c.677C > T and 128 bp for *MTHFR* c.1298A > C. On the other hand, the size of *TYMS* 3'-UTR ins/del 6-bp amplified product was 152 bp for 6-bp deletion and 158 bp for 6-bp insertion.

10 µl of the amplified PCR products was then subjected to restriction digestion at 37 °C for 2 h using 1 µl of *HinfI* (10U/µl) for *MTHFR* c.677C > T, *MboII* (5U/µl) for *MTHFR* c.1298A > C, and *DraI* (10U/µl) for *TYMS* 3′-UTR 6-bp ins/del; the enzymes were purchased from Thermo Fisher Scientific (MA, USA). The digested products were visualized on 4% agarose gel stained with ethidium bromide.

On the digestion with *HinfI*, the amplified product of *MTHFR* c.677C > T produced 175- and 23-bp fragments for TT genotype (homozygous mutant) and 198-, 175-, and 23-bp fragments for CT genotype (heterozygous). An undigested product length of 198 bp was retained by the wild-type CC genotype (Fig. 1a). The 23-bp fragment was not retained on the gel.

On the other hand, the digestion of the amplified product of *MTHFR* c.1298A > C by *MboII* yielded three fragments of 28, 28, and 72 bp for AA genotype (homozygous wild type), three fragments of 28, 72, and 100 bp for AC genotype (heterozygous), and two fragments of 28 and 100 bp for CC genotype (homozygous mutant) (Fig. 1b).

Regarding *TYMS* 3′-UTR 6-bp ins/del, the presence of the 6-bp insertion (+6pb/+6-bp homozygous genotype) creates a *DraI* restriction site, and the expected fragment sizes were 88 and 70 bp while the heterozygous genotype (+6 bp/-6 bp) produced three fragments of 88, 70, and 152 bp (Fig. 1c).

More than 10% of the samples were randomly selected for repeated assays, and the results were 100% concordant.

Statistical analysis

Data distribution was determined using the Shapiro–Wilk test; after that, Gaussian data were expressed as mean ± SD, non-Gaussian data were expressed as median and interquartile range (25th and 75th percentile), and categorical variables are expressed as frequencies (percentages). Continuous variables were compared between groups using one-way ANOVA followed by Tukey post hoc or Kruskal–Wallis test followed by Dunn post hoc for multiple comparisons as appropriate. Chi-square test was used to compare categorical variables and to assess departures from Hardy–Weinberg equilibrium. For each polymorphism, homozygous for the allele with greater frequency among controls (the major allele) was used as the reference genotype. Unconditional logistic regression analyses were used to investigate the strength of the association between the *MTHFR* and *TYMS* gene polymorphisms and the susceptibility to HCC using various genetic models. Additionally, the association of *MTHFR* and *TYMS* haplotypes with HCC risk was tested. The strength of association was measured by the adjusted odd ratio (OR) for sex and age, and their corresponding 95% confidence interval (CI). All *p*-values were 2-sided, and a *p*-value < 0.05 was considered statistically significant. For association analyses, the statistical significance threshold was set to $p < 0.0167$ after Bonferroni correction. Statistical analyses were performed using SPSS version 20.0 (IBM Corp, NY, USA). Linkage disequilibrium was examined using Haploview 4.2 software (MIT/Harvard Broad Institute, Cambridge, USA).

Results

Basic characteristics of the study population

Basic characteristics of the study population are shown in Table 1. There were no significant differences among studied groups regarding age and sex. There was no significant difference between LC and HCC patients regarding the Child–Pugh score with most patients classified as score A (53.85% in LC vs. 60% in HCC, $p = 0.388$). Further, 80%

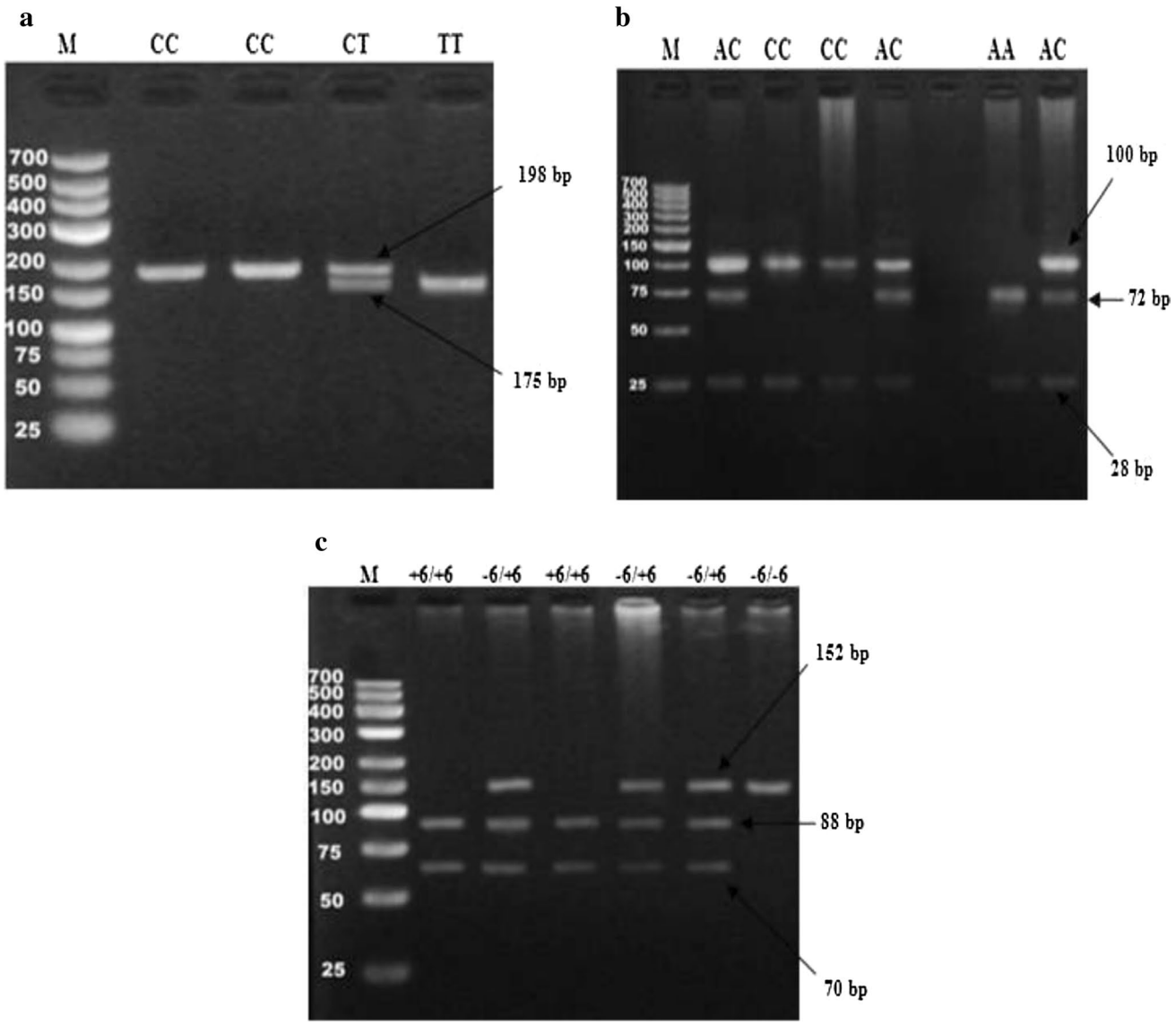

Fig. 1 Detection of the studied variants' genotypes using agarose gel electrophoresis after performing polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). **a** *MTHFR* c.677C > T, **b** *MTHFR* c.1298A > C, and **c** *TYMS* 3′-UTR 6-bp ins/del. M: Molecular weight marker

of HCC patients had a tumor size < 5 cm, and only 13.33% suffered from multifocal lesions.

Compared to the control group, LC and HCC patients had significantly higher ALT and AST activities in addition to total bilirubin and AFP levels ($p < 0.001$); in contrast, a significant decrease in albumin concentration was noted ($p < 0.001$). In comparison with LC patients, HCC patients had significantly higher ALT activity in addition to albumin and AFP levels ($p < 0.001$).

Genotypic distribution and allelic frequencies

The genotypic distribution and allelic frequencies of the studied polymorphisms among normal, LC, and HCC subjects are summarized in Table 2. The genotypic distribution of the three polymorphisms was consistent with HWE in all groups ($p > 0.05$).

For *MTHFR* c.677C > T SNP, there was no significant difference in the genotype distribution and allele frequency

Table 1 Basic characteristics of the study population

	Control group ($n=100$)	LC patients ($n=104$)	HCC patients ($n=90$)	p-value
Age (years)	54.40 ± 8.61	56.56 ± 7.31	54.76 ± 9.61	0.155
Gender (Male/female)	70 (70.00)/30 (30.00)	62 (59.60)/42 (40.40)	58 (64.40)/32 (35.60)	0.300
ALT activity (U/L)	25.00 (21.00–28.00)	43.00 (25.50–58.25)[a]	55.00 (45.00–85.00)[a,b]	0.002
AST activity (U/L)	21.00 (20.00–25.00)	44.50 (33.75–58.75)[a]	52.00 (39.75–64.50)[a]	< 0.001
Albumin (g/dl)	4.10 (4.00–4.40)	2.62 ± 0.63[a]	3.31 ± 0.57[a,b]	< 0.001
Total bilirubin (mg/dl)	0.80 (0.70–0.90)	1.60 (1.12–3.30)[a]	1.43 (1.10–2.32)[a]	0.350
AFP (µg/L)	3.95 (3.10–5.00)	6.35 (5.22–10.00)[a]	48.00 (13.70–140.67)[a,b]	< 0.001

Data are expressed as mean ± SD for Gaussian variables, median (interquartile range) for non-Gaussian variables and frequencies (percentages) for categorical variables

LC liver cirrhosis, *HCC* hepatocellular carcinoma, *ALT* alanine transaminase, *AST* aspartate transaminase, *AFP* alpha-fetoprotein

In multiple comparisons, [a]$p<0.05$ versus normal control group, and [b]$p<0.05$ versus HCV patients

Table 2 Genotype distribution and allele frequency of the studied genes among different groups

Variants		Groups			p-value		
		Control	LC patients	HCC patients	LC versus control	HCC versus control	HCC versus LC
MTHFR c.677C > T							
Genotype distribution *n* (%)	CC	34 (34.00)	44 (42.31)	20 (22.22)	0.452	0.014	0.002
	CT	50 (50.00)	44 (42.31)	40 (44.44)			
	TT	16 (16.00)	16 (15.38)	30 (33.33)			
	p-HWE	0.738	0.371	0.343			
Allele frequency (%)	C	59.00	63.46	44.44	0.355	0.005	< 0.001
	T	41.00	36.54	55.56			
MTHFR c.1298A > C							
Genotype distribution *n* (%)	AA	42 (42.00)	26 (25.00)	12 (13.33)	0.019	< 0.001	0.117
	AC	44 (44.00)	52 (50.00)	50 (55.57)			
	CC	14 (14.00)	26 (25.00)	28 (31.11)			
	p-HWE	0.652	0.843	0.162			
Allele frequency (%)	A	64.00	50.00	41.11	0.004	< 0.001	0.080
	C	36.00	50.00	58.89			
TYMS 3′-UTR 6-bp ins/del							
Genotype distribution *n* (%)	−6/−6	24 (24.00)	24 (23.08)	12 (13.33)	0.867	0.110	0.198
	−6/+6	56 (56.00)	56 (53.85)	52 (57.78)			
	+6/+6	20 (20.00)	24 (23.08)	26 (28.29)			
	p-HWE	0.223	0.433	0.080			
Allele frequency (%)	−6	52.00	50.00	42.22	0.686	0.057	0.126
	+6	48.00	50.00	57.78			

Data are expressed as frequencies (percentage)

LC liver cirrhosis, *HCC* hepatocellular carcinoma, *MTHFR* methylenetetrahydrofolate reductase, *TYMS* thymidylate synthase, 3′-UTR: 3′-untranslated region, p-HWE: p-value of Hardy–Weinberg Equilibrium

when LC patients were compared to the control group ($p=0.452$ and $p=0.355$, respectively). On the other hand, HCC patients showed a significantly higher frequency of TT genotype with the decrease in CC and CT genotypes compared to controls ($p=0.014$). In the same context and compared to LC patients, HCC patients showed a significantly higher frequency of TT genotype with the decrease in CC genotype ($p=0.002$). Accordingly, HCC patients had higher *MTHFR* 677 T allele frequency than normal subjects and LC patients ($p=0.005$ and $p<0.001$, respectively).

Regarding *MTHFR* c.1298A > C SNP, LC and HCC patients showed a significantly higher frequency of CC and AC genotype with the decrease in AA genotype compared to the control group ($p=0.019$ and $p<0.001$, respectively).

Consequently, LC and HCC patients had higher *MTHFR* 1298C allele frequency than normal subjects ($p=0.004$, and $p<0.001$, respectively). In contrast, no statistically significant differences were detected between LC and HCC patients in either the genotypic or allelic frequencies ($p=0.117$ and $p=0.080$, respectively).

When considering *TYMS* polymorphism, the results showed that there was no significant difference in the genotype distribution and allele frequency between the studied groups.

Gene polymorphisms and HCC risk

Homozygous, heterozygous, dominant, and recessive genetic models were applied to test the associations of the *MTHFR* and *TYMS* polymorphisms with HCC risk (Table 3).

The *MTHFR* c.677C > T SNP was associated with an increased risk of HCC in controls under the homozygous and recessive models ($p=0.005$). The same results were observed in LC patients with an increased risk to develop HCC under the homozygous, dominant, and recessive models ($p=0.001$, $p=0.003$, and $p=0.007$, respectively).

The *MTHFR* c.1298A > C SNP in controls was associated with an increased risk of HCC under the homozygous, heterozygous, and dominant models ($p<0.001$) as well as recessive model ($p=0.007$). In LC patients, a significant association with HCC risk was found under the homozygous model only ($p=0.014$).

Concerning *TYMS* polymorphism, no significant association was found with HCC risk in controls under any of the tested genetic models. Further, it showed a borderline significant association with HCC risk under the homozygous model only in LC patients ($p=0.028$).

Linkage disequilibrium and haplotype analyses

Pairwise linkage disequilibrium estimates obtained for the three gene polymorphisms of *MTHFR* c.677C > T, *MTHFR* c.1298A > C in addition to *TYMS* 6-bp ins/del in the study population showed that there was almost no disequilibrium between the three polymorphisms. The D' value (%) and r^2 for *MTHFR* c.677C > T-*MTHFR* c.1298A > C pairwise were 0.1 and 0.008, respectively. For *MTHFR* c.677C > T-*TYMS* 3′-UTR 6-bp ins/del pairwise, D′ value was 0.147 with $r^2=0.016$. Finally, the D′ value (%) and r^2 for *MTHFR* c.1298A > C-*TYMS* 3′-UTR 6-bp ins/del pairwise were 0.112 and 0.011, respectively.

Haplotypes of the *MTHFR* polymorphisms were constructed and analyzed for their association with HCC (Table 4). The C/C and T/C haplotypes of *MTHFR* c.677C > T and *MTHFR* c.1298A > C polymorphisms were contributed to an increased risk of healthy subjects to develop HCC by 2.249- and 3.496-fold, respectively. In contrast, only the T/C haplotype was associated with the progression of HCC in LC patients by 2.415-fold.

Table 3 *MTHFR* and *TYMS* variants and HCC risk according to genetic association models

	HCC versus control		HCC versus LC	
	†Adjusted OR (95% CI)	*p*-value	†Adjusted OR (95% CI)	*p*-value
MTHFR c.677C > T				
Homozygous model (TT versus CC)	3.300 (1.430–7.614)	0.005	4.134 (1.832–9.329)	0.001
Heterozygous model (CT versus CC)	1.337 (0.668–2.677)	0.412	2.142 (1.070–4.287)	0.032
Dominant model (TT/CT versus CC)	1.790 (0.936–3.421)	0.078	2.643 (1.396–5.004)	0.003
Recessive model (TT versus CT/CC)	2.718 (1.351–5.468)	0.005	2.619 (1.305–5.254)	0.007
MTHFR c.1298A > C				
Homozygous model (CC versus AA)	7.111 (2.621–19.294)	< 0.001	3.434 (1.290–9.145)	0.014
Heterozygous model (AC versus AA)	4.865 (2.189–10.813)	< 0.001	1.902 (0.852–4.250)	0.117
Dominant model (CC/AC versus AA)	4.829 (2.290–10.182)	< 0.001	2.069 (0.961–4.453)	0.063
Recessive model (CC versus AC/AA)	2.725 (1.321–5.621)	0.007	1.380 (0.716–2.657)	0.336
TYMS 3′-UTR 6-bp ins/del				
Homozygous model (+6/+6 versus −6/−6)	2.196 (0.860–5.608)	0.100	3.114 (1.128–8.600)	0.028
Heterozygous model (−6/+6 versus −6/−6)	2.117 (0.973–4.784)	0.071	1.575 (0.692–3.586)	0.279
Dominant model (+6/+6 & −6/+6 versus −6/−6)	2.140 (0.992–4.616)	0.052	1.994 (0.911–4.366)	0.084
Recessive model (+6/+6 versus −6/+6 & −6/−6)	1.608 (0.801–3.229)	0.182	1.274 (0.663–2.447)	0.467

HCC hepatocellular carcinoma, *LC* liver cirrhosis, *MTHFR* methylenetetrahydrofolate reductase, *TYMS* thymidylate synthase, 3′-UTR: 3′-Untranslated region, *OR* odd ratio, 95% *CI* 95% confidence interval

†Adjusted for sex and age. $n=100$ for healthy controls, 104 for LC patients, and 90 for HCC patients

Table 4 Frequencies of *MTHFR* c.677C > T and *MTHFR* c.1298A > C haplotypes in the studied groups as well as their association with HCC risk

Haplotype	HCC versus control				HCC versus LC			
	Control ($n=100$)	HCC ($n=90$)	†Adjusted OR (95% CI)	p-value	LC ($n=104$)	HCC ($n=90$)	†Adjusted OR (95% CI)	p-value
C/A	43 (43.00)	23 (25.56)	Ref	–	38 (36.54)	23 (25.56)	Ref	–
C/C	16 (16.00)	17 (18.89)	2.249 (1.173–4.311)	0.015	28 (26.92)	17 (18.89)	0.966 (0.545–1.712)	0.905
T/A	21 (21.00)	14 (15.56)	1.452 (0.762–2.766)	0.257	14 (13.46)	14 (15.56)	1.605 (0.843–3.058)	0.150
T/C	20 (20.00)	36 (40.00)	3.496 (1.992–6.138)	< 0.001	24 (23.08)	36 (40.00)	2.415 (1.433–4.071)	0.001

Data are expressed as frequencies (percentages)

MTHFR methylenetetrahydrofolate reductase, *HCC* hepatocellular carcinoma, *LC* liver cirrhosis, *OR* odd ratio, 95% *CI* 95% confidence interval

†Adjusted for sex and age

Discussion

Being the most common cancer among Egyptians, HCC represents a major health concern in Egypt [24]. Several lines of evidence indicate that the development of HCC is a complex and multistep process in which both environmental and genetic features interfere and contribute to malignant transformation [25]. Further, it is well known that HCV infection is one of the main risk factors in HCC development and the progression rate of the latter varies greatly among chronic HCV-infected patients probably due to the existence of a complex interplay between host, viral, and environmental factors [26, 27]. Thus, the identification of host genetic factors influencing the risk of developing HCC in patients with chronic HCV infection is crucial and may improve the stratification of those patients and help refine their selection to benefit from specific preventative measures and/or adapted screening policies.

Because of the important role of the folate metabolism pathway in the synthesis and methylation of DNA, accumulating investigations highlighted the association of *MTHFR* and *TS* genetic polymorphisms, as members in this pathway, with cancer risk [28]. However, the findings were conflicting regarding HCC. Accordingly, this study was conducted to verify the association of *MTHFR* c.677C > T and c.1298A > C as well as *TYMS* 6-bp ins/del polymorphisms with the risk of HCV-related HCC in an Egyptian population.

In the current work, no deviation from HWE was found in the genotype distribution of the gene loci under study. *MTHFR* 677C and 1298A alleles were the most prominent in controls which is in agreement with the results observed in previous studies performed on the Egyptian population [29, 30]. These figures are similar to, but yet different from, prior studies in populations with different ethnic backgrounds [31, 32]. Regarding *TYMS* 3′-UTR 6-bp ins/del polymorphism, there was no enough data, to the best of our knowledge, about the allele frequency of this polymorphism in the Egyptian population. However, the results of the present study were in the line with those observed by Manche et al. [33] and Qasem et al. [34] who reported that the 6-bp del (−6) allele was the most frequent in their controls. On the other hand, Fujishima et al. [35] and Ruiz-Tovar et al. [36] found that the 6-bp ins (+6) allele was the most common in their controls. The fact that the genetic characteristics of the modern Egyptian population are a mixture of European, Middle Eastern, and African populations [37] could partially explain the similarities/differences between the results of current work and published data on other populations.

Some case–control studies and meta-analyses indicated the protective role of *MTHFR* c.677C > T and c.1298A > C SNPs in the carcinogenesis of HCC. Yuan et al. [38] determined *MTHFR* genotypes on HCC cases and healthy control subjects. The authors found that individuals possessing homozygous mutant alleles at either the 677 or 1298 loci of the *MTHFR* gene had a 30–50% reduction in the risk of developing HCC compared with those with homozygous wild-type alleles. Moreover, Zhang et al. [39] conducted a meta-analysis in which an association between *MTHFR* c.677C > T polymorphism and the decreased risk of HCC in hepatitis/virus related patients was found under the recessive model with OR of 0.85 and 95% CI = 0.72–0.99. In another meta-analysis, a significantly decreased HCC risk in the overall population was found under the homozygous model of the *MTHFR* c.1298A > C polymorphism with OR of 0.66 and 95% CI = 0.46–0.946 [40].

On the contrary, the results of the current study showed that the *MTHFR* 677TT and 1298CC carriers, either healthy or HCV cirrhotic, had an increased risk to develop HCC compared to their corresponding wild-type carriers consistently with previous reports and pooled analyses suggesting the association between these SNPs and the high risk to develop HCC. Zhang et al. [41] examined the association of *MTHFR* c.677C > T polymorphism with the risk of HCC in a Chinese population composed

of HCC patients and cancer-free controls matched by age, gender, and the status of HCV and HBV infection. The authors detected an increased risk of HCC in individuals carrying the mutant homozygous genotype compared to those with the wild homozygous genotype (OR = 1.27, 95% CI = 1.02–1.58). Further, Mu et al. [42] conducted a population-based case–control study in which primary HCC cases and potential healthy controls were enrolled to assess the association between the MTHFR c.677C > T polymorphism and the risk of HCC. Taken into account that HBsAg and anti-HCV markers for chronic infections of HBV and HCV were much more prevalent among cases than controls, Mu and colleagues observed that the MTHFR 677 CT genotype was associated with an increased risk of primary liver cancer with an adjusted OR (for age, gender, education, and HBsAg) of 1.66 (95% CI = 1.06–2.61). Moreover, the results of a meta-analysis found that the *MTHFR* c.677C > T polymorphism increased HCC risk in a recessive model when HCC cases were compared to chronic liver disease (CLD) patients with a random-effect OR reached 1.85 (95% CI = 1.00–3.42) [43]. Another meta-analysis involving HCC cases and healthy controls showed a significant association between the *MTHFR* c.1298A > C polymorphism and increased risk of HCC under the dominant model (OR = 1.94, 95% CI = 1.24–3.02) [44]. Additionally, Qi et al. [45] performed a meta-analysis to comprehensively assess the association of *MTHFR* genetic polymorphisms with HCC risk in Chinese population. The authors revealed a significant association of the *MTHFR* c.677C > T polymorphism with susceptibility to HCC in Chinese population under the dominant (OR = 1.17, 95% CI = 1.00–1.38) and recessive (OR = 1.12, 95% CI = 1.00–1.26) models.

Both 677 T and 1298C variant alleles of the *MTHFR* gene result in a thermolabile enzyme with reduced activity. In fact, the TT carriers of *MTHFR* c.677C > T SNP have 30% of the MTHFR enzymatic activity compared to the CC carriers. The c.1298A > C SNP was found to influence the enzyme activity but to a lesser extent than the c.677C > T SNP as the CC carriers of the former have only 60% of the normal enzyme activity [46–48]. Low MTHFR enzymatic activity may affect DNA synthesis or cause abnormal DNA methylation leading to gene instability that affects the expression of oncogenes or tumor suppressor genes and thereby gives rise to the development of various kinds of cancers [13, 49]. These considerations alongside the notion that *MTHFR* c.677C > T SNP seems to influence fibrosis mainly through a condition of hyperhomocysteinemia capable of inducing liver steatosis at least in chronic HCV-infected patients in addition to the role of *MTHFR* 677 T allele in the development of liver fibrosis through the modulation of proteins involved in collagen degradation [50, 51] could provide a satisfactory explanation of increased HCC risk among the participants of this study carrying *MTHFR* c.677C > T and c.1298A > C mutant alleles.

Interestingly, the results of the current work showed that *MTHFR* c.1298A > C was associated with liver cirrhosis as evidenced by the significantly higher frequency of the *MTHFR* 1298CC and AC genotypes in LC patients compared to the controls that in turn led to a higher frequency of the mutant C allele in those patients.

Hyperhomocysteinemia status that results from the decreased MTHFR activity may explain this association. Hyperhomocysteinemia is highly prevalent in LC but not in other chronic liver diseases and may contribute to fibrogenesis and vascular complications of LC [52]. Moreover, hyperhomocysteinemia causing oxidative stress determines the loss of the normal phenotype of liver sinusoidal endothelial cells (LSEC); the consequent cross-talk between the LSEC and the hepatic stellate cells induces the activation of the latter which in turn proliferate, migrate, and increase collagen deposition around the sinusoids contributing to fibrogenesis, architectural disruption, and angiogenesis [53].

When considering *TYMS* polymorphism, the 6-bp del variant in the 3′-UTR is thought to affect RNA stability and translation. Mandola et al. [20] suggested that the 6-bp deletion (−6) allele is associated with decreased mRNA stability in vitro and lower gene expression in vivo. Epidemiological studies found inconsistent associations between the *TYMS* 3′-UTR 6-bp ins/del polymorphism and the risk of various diseases. On the one hand, several studies reported that the −6/−6 genotype was associated with a reduced risk of lung and gastric cancers [34, 54], in agreement with the results of the present study which revealed a borderline significant association between the +6/+6 genotype and the high risk to develop HCC in cirrhotic HCV-infected patients. The same results were obtained by Yuan et al. [38] who found that individuals with 1 or 2 copies of the deletion (−6) allele had a statistically significant 50% reduction in the risk of HCC compared to the carriers of +6/+6 genotype. On the other hand, some studies reported that individuals carrying the −6/−6 genotype were at a higher risk of colorectal adenoma and breast cancer [55, 56].

It is still unclear why the −6/−6 genotype appears to be beneficial in some circumstances compared to the +6/+6 genotype even though the latter results in higher gene expression and mRNA stability. A possible explanation could be attributable to the fact that TYMS is an auto-regulatory protein that binds directly to its mRNA and consequently inhibits translation. Therefore, while high gene expression level and mRNA stability would result in a direct increase in protein production, the increased binding of the protein to its own mRNA can lead to greater suppression under certain cellular conditions [57].

The variability between the results of the current work and other studies can be attributed to the differences in

genetic backgrounds, environmental exposures, social life patterns and habits, and disease etiology that may have an impact on the association between polymorphisms of the studied genes and HCC risk. Actually, it is not uncommon that the same polymorphism may play different roles in cancer susceptibility among different ethnic populations.

Herein, linkage disequilibrium analysis has revealed almost no disequilibrium between the three polymorphisms. Further, haplotype analysis has revealed that the C/C and T/C haplotypes of *MTHFR* c.677C > T and *MTHFR* c.1298A > C polymorphisms were contributed to increasing the risk of healthy subjects to develop HCC. On the other hand, the T/C haplotype alone was associated with the progression of HCC in LC patients.

In conclusion, the present study identified the association of gene variants of *MTHFR* and their haplotypes with increased susceptibility to HCV-related HCC in an Egyptian population. These results might be of potential importance to be used in the programs of the early cancer diagnosis as well as cancer prevention.

Study limitation

Several limitations should be acknowledged in this study. First, the effect of gene-environmental interactions was not identified because of the lack of information about environmental factors such as diet (folate intake) and behavior (smoking). Second, some disease factors were not considered in the present study, such as the duration of HCV infection, which are closely associated with the infection outcome. Third, this was a study with a modest sample size and limited SNPs in folate metabolism-related genes. Finally, there are likely selection bias and limitation of the generalizability of the findings because this was a single-center study; for that, multicenter studies with larger sample size are needed to confirm the results of the present work.

Authors' contribution SAE-M, WEZ, and AFS involved in conception; ME-B and HMMAA took part in provision of study materials or patients; HMMAA, WEZ, and AFS involved in collection and assembly of data; WEZ and AFS took part in preparation of the manuscript; SAE-M, WEZ, and ME-B involved in revision for important intellectual content; all authors participated in final approval of manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate Written informed consent was obtained from each participant before the start of the study. This work was carried out *per* the Declaration of Helsinki and approved by the local Ethics Committee of Faculty of Medicine, Mansoura University, Egypt.

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