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The emerging role of lncRNA MEG3 and MEG3 rs7158663 in hepatocellular carcinoma

S.R. MOHAMMED[1], O.G. SHAKER[2], M.M. MOHAMED[3], M.N. ABDELHAFEZ MOSTAFA[4], S.N. GABER[5], D.Y. ALI[6], H.A. HUSSEIN[7], A.M. ELEBIARY[8], A.A. ABONAR[9], H.M. EID[5], H.S. EL SAYED ALI[1]

[1]Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Fayoum University, Fayoum, Egypt
[2]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Cairo, Egypt
[3]Department of Internal Medicine, Faculty of Medicine, Cairo University, Cairo, Egypt
[4]Department of Medical Oncology, National Cancer Institute, Cairo University, Cairo, Egypt
[5]Department of Medical Microbiology and Immunology Department, Faculty of Medicine, Fayoum University
[6]Department of Clinical and Chemical Pathology, Faculty of Medicine, Fayoum University, Fayoum, Egypt
[7]Department of Internal Medicine, Faculty of Medicine, Fayoum University, Fayoum, Egypt
[8]Department of Medical Physiology, Faculty of Medicine, Fayoum University, Fayoum, Egypt
[9]Department of Clinical Pathology, Faculty of Medicine, Al-Azhar University, Egypt

Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC) is the most common primary liver malignancy in Egypt. Genetic and environmental factors play a role in its development. This study explored the association between the long non-coding RNA (lncRNA) MEG3 rs7158663 polymorphism, MEG3 expression, and the risk of HCC and other clinicopathologic characteristics in an Egyptian population.

PATIENTS AND METHODS: This case-control study included 114 patients with HCC and 110 healthy controls. TaqMan Real-time PCR was used to analyze lncRNA MEG3 rs7158663. Serum MEG3 expression levels were measured using RT-PCR.

RESULTS: The AA, GA+AA, and A alleles were associated with increased risk for HCC (adjusted odds ratio (OR) 11.84%, 95% CI 4.07–34.45, $p < 0.0001$; adjusted OR 3.18, 95% CI 1.79–5.67, $p < 0.0001$; and adjusted OR 2.87, 95% CI 1.91–4.34, $p < 0.0001$, respectively). The mutant genotype and allele were linked to an increased risk in male patients and patients ≥ 50 years old. MEG3 serum expression level was downregulated in HCC patients. The rs7158663 G > A polymorphism and downregulated MEG3 were significantly associated with larger tumor size and advanced disease stage.

CONCLUSIONS: MEG3 rs7158663 single nucleotide polymorphisms and downregulated lncRNA MEG3 were associated with HCC risk and may represent diagnostic and bad prognostic factors for HCC patients.

Key Words:
Hepatocellular carcinoma, LncRNA MEG3, Maternally expressed gene 3 polymorphism, Rs7158663.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and is considered one of the most frequent causes of death worldwide[1]. HCC is a major public health issue in Egypt. In males and females, it accounts for 33.63% and 13.54% of all malignancies, respectively[2]. Despite improvements in diagnosis and treatment, the mortality rate has increased worldwide. A variety of treatments, such as resection, radiofrequency, chemoembolization, or transplantation, are available for HCC; however, recurrence and metastasis are inevitable[3]. Although early diagnosis and developing effective therapeutic strategies to increase survival rates is paramount, it is also necessary to elucidate the molecular mechanisms underlying the etiology and progression of HCC to identify relevant biomarkers.

Genetic alterations and epigenetics may be involved in the complexity of human carcinogenesis[4]. Long non-coding RNAs (LncRNAs),

Corresponding Author: Shereen Rashad Mohammed, MD; e-mail: srk00@fayoum.edu.eg

a heterogeneous group of ncRNAs that are longer than 200 nucleotides, can regulate gene expression at the epigenetic, transcriptional, and post-transcriptional levels[5]. Many lncRNAs are frequently expressed in human cancers in which they may serve as oncogenes or tumor suppressors, suggesting that they may act as drivers of tumorigenesis[6].

The lncRNA maternally expressed gene 3 (MEG3) is a tumor suppressor gene. It has a role in the carcinogenesis and progression of various cancers, including HCC[7]. LncRNA MEG3 encodes an imprinted lncRNA that is commonly lost or downregulated in many human cancers[8]. LncRNA MEG3 is also expressed in many normal tissues and contributes to various biological processes through diverse mechanisms[9]. Hypermethylation of the lncRNA MEG3 promoter has been shown to contribute to loss of lncRNA MEG3 expression in human cancer cells, resulting in permanent transcriptional silencing and the consequent loss of its antiproliferative function, thus contributing to oncogenesis[9]. The first evidence for a contribution of lncRNA MEG3 to human cancer was obtained from pituitary non-functioning adenomas, where expression of the gene is lost[10]. Expression of lncRNA MEG3 was also found to be decreased in high-grade meningiomas[11]. However, the role and function of lncRNA MEG3 in HCC has yet to be established and correlated with clinical and pathological characteristics.

Single nucleotide polymorphisms (SNPs) are the most common form of sequence variation in eukaryotic genomes. SNPs can affect the expression of genes and are associated with genetic susceptibility to many diseases, including cancer[12]. SNPs in lncRNA MEG3 (rs7158663 G > A) were reported to affect the phenotypes of cells and increase the risk of developing cancer[13]. LncRNA MEG3 rs7158663 G > A was shown to be associated with a high risk of developing colorectal[14], breast[15], and gastric[16] cancers. However, there are no studies published to date regarding an association between the lncRNA MEG3 rs7158663 G > A polymorphism and HCC.

Considering the data showing a role for dysregulated lncRNA MEG3 in cancer and an emerging role of rs7158663 on lncRNA MEG3 expression level, we hypothesize that rs7158663 SNP also contributes to HCC susceptibility by affecting the expression level of lncRNA MEG3. Therefore, we examined the association between the lncRNA MEG3 rs7158663 polymorphism, the risk of developing HCC, and clinicopathologic characteristics of HCC in an Egyptian population. LncRNA MEG3 expression was also measured and correlated with various genotypes and alleles.

Patients and Methods

This case-control study included 114 adult Egyptian patients with HCC who were recruited from the National Cancer Institute, Cairo University, as well as the Internal Medicine Department of Fayoum University Hospital, Fayoum University. The initial diagnosis of the patients depended on history as some patients presented with nonspecific symptoms, including fever, jaundice, anorexia, malaise, and a history of hepatitis C infection. Diagnostic imaging was done using abdominal ultrasound, multiphasic liver CT, and MRI. In most of the patients, the diagnosis of HCC was noninvasive, and no biopsy confirmation was needed. All patients were newly diagnosed and had not received chemotherapy or radiotherapy treatment before enrollment in this study. Patients were staged according to the TNM staging for HCCs[17]. Patients were excluded if they had non-alcoholic steatohepatitis, malignancies other than HCC, diabetes, or autoimmune hepatitis.

The control group consisted of 110 healthy individuals of matched age and sex with no history of cancer. The protocol was approved by the Medical Ethics and Human Clinical Trial Committee of the Faculty of Medicine, Fayoum University, and conformed to the Ethical guidelines of Helsinki Declaration. Written informed consent was obtained from subjects before entering the study, and all participants signed an informed consent agreement after the purpose of the study was explained.

Blood Sample Collection and Storage

Peripheral blood samples (5 ml) were drawn from each subject and into two plain tubes. Then, 2.5 ml was transferred to a plain tube, permitted to clot for 15 min, and the separated serum was used for laboratory measurements. Serum was stored at −80°C for further RNA extraction and measurement of lncRNA MEG3. The other 2.5 ml of whole blood was mixed with EDTA for further DNA extraction and SNP genotyping using TaqMan probe-fluorescence PCR.

LncRNA MEG3 Fold Change Estimation

The serum expression level of lncRNA MEG3 was performed as previously described[18,19].

RNA Extraction

Total RNA was extracted from 200 µl serum using an RNeasy extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. RNA quantitation and purity assessment were done using a NanoDrop® (ND)-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, New Hanover, NC, USA).

Reverse Transcription and Real-Time Quantitative PCR

Reverse transcription (RT) was done with 60 ng of total RNA in a final volume of 20 µl using the RT2 First Strand Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Serum expression levels of lncRNA MEG3 were measured using GAPDH as an internal control[20-22]. GAPDH was amplified with the following primers: forward F: 5′-GTCTCCTCTGACTTCAACAGCG-3′ and reverse R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′. LncRNA MEG3 primers were purchased from Qiagen (UniGene No. Hs.654863, Catalog No. 4331182, LPH02974A-200, and Lot No. 201710030001). cDNA was stored at −80°C until RT-PCR analysis. Customized ready-made primers and the Maxima SYBR Green PCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) were used according to the manufacturer's protocol. The reaction mixture (25 µl) for Real-time PCR was prepared by combining 12.5 µl SYBR Green, 1 µl primer, 2 µl cDNA, and 9.5 µl RNAase-free water, and the Rotor-gene Q System was used with the following cycling conditions: 95°C for 10 min, 45 cycles at 95°C for 15 s, and 60°C for 60 s. The expression levels of both GAPDH and lncRNA MEG3 were determined using the $2^{-\Delta\Delta Ct}$ method. A melt curve was also used for the RT-PCR analysis. The cycle threshold (Ct) is the cycle's number needed for the fluorescence to pass a definite threshold. ΔCt and ΔΔCt were calculated according to the following equations[22]:

$$\Delta CT = CT \text{ (lncRNA MEG3)} - CT \text{ (GAPDH)}.$$

$$\Delta\Delta CT = \Delta CT \text{ test sample} - \Delta CT \text{ calibrator sample}.$$

The range for the target relative to a calibrator sample was calculated by $2^{-\Delta\Delta Ct}$.

DNA Extraction and SNP Genotyping by the TaqMan™ SNP Genotyping Assay

Whole EDTA blood was used to extract genomic DNA by QIAamp® Whole Blood Genomic DNA Purification Kit (Qiagen, Valencia, CA, USA). The NanoDrop1-1000 spectrophotometer was used for DNA quantitation (NanoDrop innovations, Inc., Wilmington, NC, USA). The TaqMan allelic discrimination assay was used to genotype purified DNA in a PCR reaction (Applied Biosystems, Foster City, CA, USA) using lncRNA MEG3 rs7158663 (G > A) with a pre-designed fluorescent primer/probe (Catalog No. 4351379, probe ID C_9693465_10, SNP ID rs7158663, Lot No. P171206_012E08) (Thermo Fisher Scientific, Waltham, MA, USA). The sequence was "VIC/FAM":

A T G G C A C A A A A G C C A G A G A T A-A A A C[A/G]TCCTTCACGTGCTCCCTAC-CCGGT.

The PCR reaction (25 µl) mixture (Qiagen, Valencia, CA, USA) contained a mixture of 12.5 µl TaqMan master mix, 2 µl (1-20 ng) purified genomic DNA, 1.25 µl genotyping assay mix, and 9.25 µl nuclease-free water. Thermal cycling conditions were as follows: 95°C for 10 min for polymerase activation followed by 45 cycles at 95°C for 15 s for denaturation and 60°C for 60 s for annealing and extension.

Statistical Analysis

Data were coded and entered using the statistical package SPSS version 25 (IBM, Armonk, NY, USA). Data were summarized using mean, standard deviation, median, minimum, and maximum for quantitative data and frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal–Wallis and Mann-Whitney tests. For comparing categorical data, a Chi square (χ^2) test was performed and the exact test was used when the expected frequency was less than 5. Genotype and allele frequencies were compared between the disease and the control groups using Chi-square tests. The odds ratio (OR) with 95% confidence intervals was also calculated. Correlations between quantitative variables were done using Spearman correlation coefficients. To estimate the cut-off point at which the highest sensitivity and specificity of MEG3 was reached for differentiating between different conditions, a receiver operating characteristic (ROC) curve analysis was established. The good-of-fit χ^2 test was used to perform the Hardy–Weinberg equilibrium in both cases and controls. *p*-values less than 0.05 were considered statistically significant.

Results

Demographic, Laboratory Findings, and Pathological Characteristics of the Study Groups

This study was conducted on 114 patients with HCC (24 females, 90 males) with a mean age 60.3 ± 7.4 years and 110 healthy control subjects (15 females, 95 males) with a mean age of 58.6 ± 7 years. Sex was not significantly different between patients with HCC and the controls (p = 0.14); however, there was a predominance of males with HCC (males to females, 78.9% to 21.1%). The liver portal vein was patent in 76.3% of the patients, whereas it was thrombosed in 23.7%. Metastasis was not present in 97.4% of the patients, whereas only 2.6% had portahepatis and pancreaticoduodenal lymph nodes. In addition, 47.4% of the patients had a tumor size ≥5 cm, 39.5% were diabetic, and 47.4% were hypertensive. Regarding tumor stage, 6 patients (5.3%) were stage IA, 21 patients (18.4%) were stage IB, 30 patients (26.3%) were stage II, 27 patients (23.7%) were stage IIIA, 27 patients (23.7%) were stage IIIB, and 3 patients (2.6%) were stage IVA. With respect to TNM staging, the percentage of patients with T 1, 1a, 1b, 2, 3, and 4 were 2.6, 2.6, 18.4, 26.3, 23.7, and 26.3%, respectively, whereas the percentage of patients with N1 was 2.6% (Table I).

Table I. Clinical and pathological features of the HCC group.

Parameters	HCC cases (N, %)
Liver size	
Average	45 (39.5%)
Enlarged	54 (47.4%)
Shrunken	15 (13.2%)
Lesion number	
1	66 (57.9%)
2	18 (15.8%)
Multiple	30 (26.3%)
Site	
Right	78 (68.4%)
Left	24 (21.1%)
Both	12 (10.5%)
Size (cm)	
≥5	54 (47.4%)
<5	60 (52.6%)
Liver IHBR	
Intra and extra	6 (5.3%)
No	108 (94.7%)
Liver PV	
Patent	87(76.3%)
Thrombosed	27 (23.7%)
Spleen size	
Average	66 (57.9%)
Enlarged	48 (42.1%)
Metastasis	
No	111 (97.4%)
Yes (porta hepatis and pancreatico duodenal LMNS)	3 (2.6%)
Ascites	
Marked	9 (7.9%)
Mild	15 (13.2%)
Moderate	18 (15.8%)
No	72 (63.2%)
DM	
No/yes	69 (60.5%)/45 (39.5%)
HTN	
No/yes	60 (52.6%)/54 (47.4%)
Staging	
Stage IA	6 (5.3%)
Stage IB	21 (18.4%)
Stage II	30 (26.3%)
Stage IIIA	27 (23.7%)
Stage IIIB	27 (23.7%)
Stage IVA	3 (2.6%)

Frequency Distribution of the Genotypes and Alleles of the LncRNA MEG3 rs7158663 Polymorphism in HCC Patients and Healthy Controls

To assess the genetic association of the lncRNA MEG3 rs7158663 polymorphism with HCC susceptibility, we compared HCC cases to healthy controls. The results indicated that there was a significant difference in the frequency of the lncRNA MEG3 rs7158663 G/A genotype between HCC cases and controls (p < 0.001). The incidences of the GG, GA, and AA genotypes were 28.9%, 47.4%, and 23.7%, respectively, in HCC patients, and 54.5%, 40.9%, and 4.5%%, respectively, in the controls. To characterize the genotype that carries a risk for developing HCC, we classified patients into four models: genotypic, dominant, recessive, and allelic. We found that the mutant genotype, and/or the heterozygote genotype, or the mutant allele (AA, AA+GA, or A allele) were risk factors for developing HCC.

Regarding the genotypic and dominant models, the GG genotype represents the reference genotype for both models. The frequency of the AA and GA+AA genotypes was significantly increased in HCC patients compared with that in the controls (23.7/4.5%,71.1/45.5%, p < 0.0001). AA and GA+AA were related to increased risk of HCC after adjusting for age and sex (adjusted OR 11.84 %, 95% CI 4.07–34.45, p < 0.0001; and adjusted OR 3.18, 95% CI 1.79–5.67, p < 0.0001, respectively). In the recessive model, the GG+-GA genotypes were set as a reference group. The

Table II. Genotype and allelic distribution of MEG3 rs7158663 G/A in HCC patients and controls.

Genotypes and alleles	HCC cases N (%)	Control N (%)	[a]Adjusted OR (95% CI), *p*-value
Genotypic model			
GG	33 (28.9%)	60 (54.5%)	1
GA	54 (47.4%)	45 (40.9%)	2.318 (1.267–4.241), 0.006
AA	27 (23.7%)	5 (4.5%)	11.842 (4.070–34.453), <0.0001
Dominant			
GG	33 (28.9%)	60 (54.5%)	1
GA+AA	81 (71.1%)	50 (45.5%)	3.182 (1.787–5.665), <0.0001
Recessive			
GG+GA	87 (76.3%)	105 (95.5%)	7.409 (2.705–20.294), <0.0001
AA	27 (23.7%)	5 (4.5%)	
Allele			
G	120 (52.6%)	165 (75.0%)	2.874 (1.905–4.337), <0.0001
A	108 (47.4%)	55 (25.0%)	

[a]adjusted *p*-value done by multivariate logistic regression (adjusted for age and sex). Adjusted for age and sex, HWE in control: Chi-square value = 0.909, $p = 0.340$, HWE in cases: Chi-square value = 0.285, $p = 0.593$. HCC, hepatocellular carcinoma. CI, confidence interval. OR, odds ratio.

AA genotype showed significant differences with an increased distribution in HCC patients compared with the controls (adjusted OR 7.41, 95% CI 2.71–20.29, $p < 0.0001$). Regarding the allelic model, the A allele was associated with a significant increase for developing HCC (adjusted OR 2.87, 95% CI 1.91–4.34, $p < 0.0001$). Therefore, the presence of the mutant genotype or allele is associated with increased risk of HCC in all models (Table II).

Stratification of the LncRNA MEG3 rs7158663 Polymorphism Genotypes and Alleles According to Sex and Age in HCC Patients and Controls

The lncRNA MEG3 rs7158663 polymorphism was stratified according to sex and age. AA and GA in the genotypic model, GA+AA in the dominant model, AA in the recessive model, and A in the allelic model were all associated with increased risk of HCC in male patients (adjusted OR 3.49, 95% CI 1.76–6.93, $p <0.0001$; adjusted OR 19.88, 95% CI 6.12–64.58, $p < 0.0001$; adjusted OR 5.05, 95% CI 2.62–9.73, $p < 0.0001$; adjusted OR 9.75, 95% CI 3.2529.24, $p < 0.0001$; and adjusted OR 3.83, 95% CI 2.46–5.96, $p < 0.0001$, respectively). Also, patients ≥ 50 years old, expressing the same genotypes and alleles, showed an increased risk of HCC (adjusted OR 2.78, 95% CI 1.47–5.27, $p = 0.002$; adjusted OR 14.34, 95% CI 4.51–45.61, $p < 0.0001$; adjusted OR 3.86, 95% CI 2.09–7.11, $p < 0.0001$; adjusted OR 8.1, 95% CI 2.71–24.19, $p < 0.0001$; and adjusted OR 2.18, 95% CI 1.22–3.9, $p = 0.008$, respectively).

Relationship Between the LncRNA MEG3 rs7158663 Polymorphism and Clinical Data of Patients with HCC

We assessed the association between lncRNA MEG3 rs7158663 polymorphisms and tumor size, portal vein thrombosis, and tumor stage in HCC patients (Table III). We found significant associations between GA+AA in the dominant model and A in the allelic model and large tumor size, in which 55.6% of the patients carrying GA+AA genotypes and 52.8% of patients carrying A allele, had a tumor size greater than 5 cm (OR 9.480, 95% CI 2.574–34.915, $p = 0.001$). Also, there were significant associations between A allele in the allelic model and advanced tumor stage when comparing stages I and II *vs.* stages III and IV (OR 1.894, 95% CI 1.119–3.207, $p = 0.017$). We did not find any association between the mutant genotypes and portal vein thrombosis or disease stage in the dominant model, although the OR was 1.182 and 1.90, respectively, but the resulting *p*-value was insignificant.

LncRNA MEG3 Fold Change in the Study Group

The expression of lncRNA MEG3 in the serum was significantly decreased in patients with HCC compared with that of controls [median (IQR) = 0.43 (0.02–3.52)] ($p < 0.0001$).

LncRNA MEG3 Fold Change in Relation to Polymorphisms

The correlations between the expression of lncRNA MEG3 and its polymorphisms are present-

Table III. Relationship between MEG3 rs7158663 polymorphism and the clinical data of HCC patients.

	GG N, %	GA+AA N, %	OR (95% CI); *p*-value#	G N, %	A N, %	OR (95% CI); *p*-value#
Tumor Size						
< 5	24 (72.7%)	36 (44.4%)	**1**	69 (57.5%)	51 (47.2%)	**1**
≥ 5	9 (27.3%)	45 (55.6%)	**9.480 (2.574-34.915); 0.001**	51 (42.5%)	57 (52.8%)	**2.002 (1.133–3.538); 0.017**
Liver PV						
Patent	27 (81.8%)	60 (74.1%)	1	87 (72.5%)	87 (80.6%)	1
Thrombosed	6 (18.2%)	21 (25.9%)	1.182 (0.393-3.559); 0.766	33 (27.5%)	21 (19.4%)	0.479 (0.246-0.933); 0.031
Stage						
Stage I and II	21 (63.7%)	36 (44.4%)	1	69 (57.5%)	45 (41.7%)	1
Stage III 12 and IV	(36.4%)	45 (55.6%)	1.900 (0.755–4.656); 0.161	51 (42.5%)	63 (58.3%)	**1.894 (1.119–3.207); 0.017**

p-values in bold are statistically significant ($p < 0.05$).

ed in Table IV. Compared with the expression levels of lncRNA MEG3 in the different genotypes, we found that the wild-type GG genotype exhibited higher expression levels followed by the AG genotype, whereas the mutant AA genotype had the lowest expression level (median = 0.53, 0.37, and 0.32, respectively; $p = 0.001$). Furthermore, the mutant A allele exhibited a lower expression level than did the wild-type G allele (median = 0.37, 0.46, respectively; $p < 0.0001$).

LncRNA MEG3 Expression in Relation to Clinical Characteristics

We studied the relationship between lncRNA MEG3 expression and clinical characteristics in HCC patients. The results indicated that low expression of lncRNA MEG3 was associated with tumor size >5 cm, porta hepatis and pancreaticoduodenal LMNS metastasis, thrombosed portal vein, ascites, hypertension, the presence of intrahepatic and extrahepatic biliary radicles, and advanced TNM stage ($p = 0.079$, 0.001, 0.001, 0.001, 0.002, 0.07, 0.006, respectively), but not associated with sex, liver size, number of lesions, or tumor location.

Univariate and Multivariate Logistical Regression Analysis

To identify factors associated with increased risk of HCC, logistical regression analysis was performed (Table V). Decreased expression levels of lncRNA MEG3 and the GA and AA genotypes of lncRNA MEG 3 rs7158663 were all positively associated with increased risk of HCC in both univariate and multivariate analyses.

ROC Curve for LncRNA MEG3 Fold Change

To help determine the diagnostic potential of lncRNA MEG3 as a biomarker for HCC, we performed a ROC analysis. We found that the best cut-off value was 0.98 (AUC = 0.722, 95% CI 0.638–0.807, $p \leq 0.0001$), yielding a 72.2% sensitivity and 100% specificity.

Discussion

HCC is considered the third major cause of cancer-related deaths worldwide[23,24]. Late diagnosis and hence limited therapeutic choices con-

Table IV. LncRNA MEG3 fold-change in relation to polymorphism in HCC.

	Genotype	Fold change of MEG-3, median (IQR)	*p*-value
MEG 3 G>Ars7158663	GG (N = 33)	0.53 (0.3–0.71)	**0.001[a,b]**
	GA (N = 54)	0.37 (0.23–0.47)	
	AA (N = 27)	0.32 (0.19–0.33)	
Allele	G (N = 120)	0.46 (0.29–0.65)	**< 0.0001**
	A (N = 108)	0.37 (0.24–0.42)	

[a]differences between GG and AG, [b]differences between GG and AA. HCC, hepatocellular carcinoma.

Table V. Logistic regression analysis to predict the risk of HCC development.

	B	S.E.	Adjusted OR (95% CI)	*p*-value
Univariate[a]				
MEG3 Fold Change	−0.437	0.217	0.646 (0.423–0.988)	0.044
PolyMEG3 GArs7158663 (GA vs. GG)	0.841	0.308	2.318 (1.267–4.241)	0.006
PolyMEG GA rs7158663 (AA vs. GG)	2.472	0.545	11.842 (4.07–34.453)	<0.0001
Multivariate[b]				
FoldChangeMEG3	−0.478	0.229	0.62 (0.396–0.971)	0.037
PolyMEG GA rs7158663 (GA vs. GG)	0.812	0.312	2.253 (1.222–4.154)	0.009
PolyMeg GA rs7158663 (AA vs. GG)	2.435	0.565	11.418 (3.775–34.533)	<0.0001
Constant	−1.375	1.255	0.253	0.273

[a]Crude *p*-value done by univariate logistic regression.
[b]Adjusted p-value done by multivariate logistic regression (adjusted for age and sex).
p-values in bold are statistically significant ($p < 0.05$). HCC, hepatocellular carcinoma. CI, confidence interval. OR, odds ratio.

tribute to the high rate of mortality[6]. Therefore, it is essential to clarify the molecular bases for initiation and development of HCC to improve the survival of HCC patients.

Genetic and dysregulated epigenetic factors are known to influence cancer development[25]. LncRNAs are expressed in many human cancers, where they act as tumor suppressor genes or oncogenes[5,26,27]. SNPs can disturb their function and expression and thus affect tumorigenesis[6]. The expression of MEG3 occurs in normal tissue and is lacking in many cancer cell lines. Previous data have suggested a role for MEG3 as a tumor suppressor[28-30]. Accordingly, decreased expression of MEG3 may have a role in the pathogenesis of HCC. Prediction of RNAsnp utility has shown a role for lncRNA MEG3 rs7158663 on MEG3 folding[31,32]. Therefore, we assumed that this MEG3 SNP may act as a regulatory SNP that modulates LncRNA expression and therefore affects HCC pathogenesis. To date, the relationship between lncRNA MEG3 and its polymorphism(s) is not well understood in HCC patients. We examined the association between lncRNA MEG3 rs7158663 polymorphism and the risk of HCC and clinicopathologic characteristics of HCC in an Egyptian population. We also measured lncRNA MEG3 expression, which was analyzed for an association with different genotypes and alleles.

Our results indicated that lncRNA MEG3 rs7158663 is associated with HCC risk. We also discovered that the mutant homozygous AA and/or the heterozygote GA and A allele were all associated with an increased risk of HCC. Furthermore, the mutant genotypes were linked with increased risk of HCC in male patients and patients ≥50 years old. Logistical regression analysis also revealed that AA and GA genotypes were risk factors for HCC (adjusted OR 2.318, 95% CI 1.267–4.42, $p = 0.006$; adjusted OR 11.842, 95% CI 4.07–34.45, $p < 0.0001$). These results suggest that this SNP may represent a predisposition SNP for HCC development.

Consistent with our results, Cao et al[14] identified six tag SNPs of MEG3 in Chinese colorectal cancer patients and controls. They found that carriers of the mutant AA genotype of MEG3 rs7158663 had a higher risk for colorectal cancer compared with controls. Moreover, Hou et al[33] found a significant association between an MEG3 polymorphism and oral squamous cell carcinoma. Ali et al[13] also demonstrated a role for MEG3 rs7158663 in breast cancer patients. They found that mutant genotypes of rs7158663 were risk factors for breast cancer in patients with fibroadenoma.

Zhuo et al[34] identified two SNPs, rs4081134 and rs7158663, in neuroblastoma children and controls. Although no link was identified between the two SNPs and the risk of neuroblastoma, an association between rs4081134 and early onset of disease and progressive clinical stage was evident. They proposed rs7158663 as a risk locus for neuroblastoma. Furthermore, both SNPs were analyzed in Chinese patients with lung cancer, and rs4081134, but not rs7158663, was associated with lung cancer risk[35]. Finally, a study of patients with nasopharyngeal cancer revealed a correlation between MEG3 rs10132552 and a greater risk of grade 3–4 anemia following chemoradiotherapy[36]. In contrast, Xu et al[37] reported that the MEG3 polymorphisms (rs11627993 and rs7158663) may have no effect on prostate cancer susceptibility.

We found a significant association between the mutant genotype of rs7158663 and tumor size. Also, there were significant associations between A allele in the allelic model and advanced tumor

staging when comparing stages I and II *vs.* stages III and IV. We did not find an association between the mutant genotypes and portal vein thrombosis or disease stage, although the OR was 1.182 and 1.90, respectively, but the *p*-value was insignificant. Consistent with our study, Ali et al[13] found that mutant genotypes (GA+AA) and the A allele of rs7158663 were significantly associated with large tumor size in patients with breast cancer; however, they found no correlation between the SNP and tumor grade. In addition, Yang et al[38] also found no significant association between the mutant genotypes of tag SNPs of lncRNA H19 and the clinicopathological characteristics of gastric cancer. Cao et al[14] also found no association between mutant genotypes and tumor site or stage. Variations in the genetic background of the studied population, differences in sample sizes, cancer type, and varieties of genotyping techniques, as well as random errors, may contribute to the substantial differences observed in the results of the studies.

To explain our results, Han et al[32] elucidated the relationship between lncRNA MEG3 rs7158663 and cancer risk. They showed that the A allele of lncRNA MEG3 rs7158663 can bind to transcriptional factors [C/EBPα, oct-1, and TATA binding protein (TBP)], whereas the G allele cannot. This binding can modify the assembly to a response element causing changes in gene expression[39]. Also, SNPs may disturb the structural characteristics of lncRNAs affecting their molecular function[14,40].

Yin et al[41] reported that lncRNA MEG3 SNPs affect cancer risk by regulating long non-coding MEG3 gene expression and thus influencing cell proliferation. They demonstrated that MEG3 polymorphism carriers have a poor cancer prognosis. Also, Yang et al[38] showed that polymorphisms in lncRNA H19 affect gastric cancer risk by modulating H19 gene expression.

Some studies have shown the potential role of long non-coding SNPs in numerous biological processes. Yang et al[38] revealed a role for H19 and its variants in the risk for developing gastric cancer. They revealed that the H19 SNPs, rs217727 and rs2839698, were significantly correlated with an increased risk of gastric cancer. Moreover, Zhang et al[42] revealed that rs920778 influences the expression of lncRNA HOTAIR and affects the growth of esophageal squamous cell carcinoma. Xue et al[43] also found that SNPs in HOTAIR were correlated to colorectal cancer risk and rs7958904 could be a possible biomarker for predicting colorectal cancer risk. Bayram et al[44] found that rs920778 of HOTAIR could play a significant role in the development and aggressiveness of breast cancer in a Turkish population.

Several studies also examined this SNP in diseases other than cancer. Han et al[32] showed that MEG3 rs7158663 was associated with increased ischemic stroke. Furthermore, Ghaedi et al[40] found that the mutant AA genotype was associated with risk for type 2 diabetes mellitus.

We studied for the first time the correlation between lncRNA MEG3 rs7158663 and MEG3 expression in HCC patients and controls. We first measured lncRNA MEG3 expression in the sera of patients and controls. LncRNA MEG3 serum expression levels were found to be significantly decreased in patients with HCC compared with controls, suggesting its role as a tumor suppressor. Furthermore, logistical regression analysis revealed that decreased lncRNA MEG3 expression is a risk factor for HCC.

In addition, we found that downregulation of lncRNA MEG3 was correlated with tumor size >5 cm, porta hepatis and pancreaticoduodenal LMNS metastasis, thrombosed portal vein, ascites, the presence of intrahepatic and extrahepatic biliary radicles, and advanced TNM staging, indicating that lncRNA MEG3 may be implicated in the HCC carcinogenesis and metastasis.

Our results are consistent with previous studies. For example, Li et al[45] showed that MEG3 expression levels were downregulated in HCC patients. Braconi et al[46] found that the expression of MEG3 was markedly downregulated in four HCC cell lines compared with normal hepatocytes. Moreover, Chang et al[47] designed a vector that delivered lncRNA MEG3 to HCC cell lines. The results indicated that lncRNA MEG3 significantly diminished tumor cell growth.

Further evidence revealed that the expression of MEG3 is decreased in many human tumors and tumor cell lines[7]. Tian et al[48] showed that lncRNA MEG3 is downregulated in patients with osteosarcoma, and it was associated with poor overall survival. They concluded that lncRNA MEG3 may be a useful prognostic biomarker for osteosarcoma.

Lu et al[49] revealed that MEG3 was downregulated in non-small cell lung cancer and correlated with advanced clinical features and poor prognosis. Moreover, Yin et al[41] demonstrated that it was markedly decreased in colorectal cancer tissues compared with normal control tissues and associated with poor prognosis. Sun et al[50] reported that downregulated MEG3 enhanced cell proliferation

and migration and increased tumor growth and metastasis in gastric cancer. MEG3 overexpression inhibited cell proliferation and induced apoptosis in several cancer cell lines, including lung[29], squamous cell[51], and gastrointestinal[52] cancers.

To date, the underlying mechanism of downregulated MEG3 is not well understood. We found that the wild-type GG genotype exhibited higher expression levels followed by the AG genotype, whereas the mutant AA genotype had the lowest. Furthermore, the mutant A allele showed lower expression levels than did the wild-type G allele.

Accordingly, recent studies have shown that rs920778 and rs12826786 polymorphisms in the HOTAIR gene affect HOTAIR expression according to the genotypes of these polymorphisms[42,43]. However, previous studies[10,11,53] demonstrated that promotor hypermethylation is the fundamental cause of downregulation in many carcinomas.

We examined the relationship between rs7158663 SNP and clinical features of HCC patients as well as the expression of MEG3 and found that rs7158663 SNP was strongly associated with tumor size and HCC stage. However, we failed to find significant differences between different tumor grades. However, TNM represents a complex staging system with many subclasses consisting of tumor size, vascular invasion, number of tumors, invasion to the portal or hepatic vein, and direct invasion or impact on the visceral peritoneum[54]. Thus, it may be N1 or a higher stage or more advanced according to other factors.

Further studies should focus on the relationship of rs7158663 SNP and HCC progression and survival; however, Zhuo et al[55] hypothesized that MEG3 could be a possible biomarker for predicting the prognosis and survival of HCC patients. They demonstrated that MEG3 was regulated by UHRF1, (a recently identified oncogene) by recruiting DNMT1 [DNA (cytosine-5-)-methyltransferase 1] and regulating p53 expression. Also, Zhang et al[56] found that MEG3 regulates the PTEN/AKT/MMP-2/MMP-9 signaling pathway and contributes to the progression of HCC by targeting miRNA-10a-5p. In addition, Braconi et al[46] demonstrated that methylation-dependent tissue-specific regulation of lncRNA MEG3 by miR-29a may affect HCC progression and highlighted the interplay between two kinds of non-coding RNA, miRNAs and lncRNAs, and epigenetic gene regulation.

However, the specific molecular mechanisms underlying the altered expression levels of lncRNA MEG3 in relation to different genotypes and alleles in HCC patients need to be elucidated. Thus, further studies should focus on the cause and effect of molecular mechanisms. Some potential limitations of the current study should be considered. First, we could not study the effect of environmental and other risk factors. Second, our results focused on a single SNP in MEG3 and a relatively small sample of HCC Egyptian patients. Thus, it is necessary to test a larger patient population and different MEG3 SNPs. Finally, consecutive measurement of MEG3 expression during follow-up periods after receiving treatment should be done to better clarify the role of MEG3 and this could be of value in detecting the possible recurrence of HCC.

Conclusions

Our study showed that the mutant and/or heterozygote genotype of lncRNA MEG3 rs7158663 are risk factors for HCC development in Egyptian patients. Also, the GA+AA and A allele are associated with large tumor size. The A allele is associated with advanced tumor staging. Moreover, the mutant AA genotype is associated with lower serum MEG3 expression levels. Finally, decreased expression level of lncRNA MEG3 as well as the GA and AA genotypes is positively associated with increased risk of HCC in both univariate and multivariate analyses.

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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