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Original paper

Study of the association between a *MICA* gene polymorphism and cholangiocarcinoma in Egyptian patients

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Abstract

Introduction: An inflammatory environment is the common pathway for the development of cholangiocarcinoma (CCA). The natural killer group 2D receptor (NKG2D), an activating receptor for NK cells, is a potent immune axis in the antitumor and antimicrobial immune response through its binding to NKG2D ligands (NKG2DLs). NKG2DLs are normally absent or poorly expressed in most cells; conversely, they are upregulated in stressed cells. We studied the rs2596542 polymorphism located upstream of the *MICA* gene, which encodes an NKG2DL, in patients with CCA as a marker for early disease detection and a possible therapeutic target.

Material and methods: A case-control study was conducted on 40 patients with CCA and 45 healthy individuals (as controls). After routine examination, the rs2596542 polymorphism of the *MICA* gene was investigated using real-time PCR.

Results: We found that a TT homozygous genotype was significantly predominant in patients with CCA (p = 0.039), with the T allele being dominantly distributed in CCA (p = 0.007). High levels of CA19-9 were significantly associated with the TT genotype in the patients. However, we did not detect significant differences in rs2596542C/T genotype and allele distribution between patients with CCA with cirrhosis and those without cirrhosis ($p > 0.05$).

Conclusions: The MICA rs2596542 polymorphism may affect the susceptibility to CCA, but not its progression. The TT genotype could be used as a potential diagnostic marker for CCA and triggering the MICA pathway could be a promising therapeutic target.

Key words: cholangiocarcinoma, Egyptian patients, epidemiology.

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Introduction

Cholangiocarcinoma (CCA) is a biliary epithelial malignancy that occurs at any site along the length of the biliary tree [1]. Its incidence rates vary significantly according to country, possibly because of discrepancies in risk factors and genetic differences [2]. However, globally, the incidence of CCA has been increasing over the past four decades [3].

Most patients with CCA have a poor prognosis, as they are diagnosed at late stages, when the advantages of treatment are very limited [4]. Therefore, novel biomarkers are urgently needed for CCA management and treatment.

Although various risk factors predispose to CCA development, an inflammatory environment is the common pathway [5]. The natural killer group 2D receptor (NKG2D) is an activating receptor for NK cells and some T-cell subsets [6]. NKG2D, through binding to NKG2D ligands (NKG2DLs), is a potent immune axis for the antitumor and antimicrobial immune response [7].

NKG2D ligands are normally absent or only poorly expressed in most cells; in contrast, they are upregulated in stressed cells. Thus, the NKG2D pathway serves as a mechanism for the detection and elimination of stressed cells [8]. Strikingly, NKG2D is encoded by a single gene; in contrast, eight different NKG2DL-encoding genes are present within the human genome [9].

Major histocompatibility complex (MHC) class I polypeptide-related chain A (MICA) is one of the NKG2DLs. The rs2596542C/T SNP is located 4.7 kb upstream of the *MICA* gene on chromosome 6p21, which is the promoter or enhancer region; thus, it is expected to alter the expression of *MICA* [10] and change the binding of stress-inducible transcription factors [11].

We aimed to study the rs2596542 polymorphism in patients with cholangiocarcinoma as a marker for early disease detection and a possible therapeutic target.

Material and methods

The current case-control study included 40 adult (> 18 years of age) patients with CCA. To characterize earlier cases of CCA, patients were divided into CCA patients with cirrhosis and CCA without cirrhosis. They were admitted to the Endoscopy Unit and inpatient departments of the National Liver Institute, Menoufia University. The diagnosis of CCA was confirmed by histopathological correlation and/or a contrast enhancement pattern in triphasic computed tomography (CT) and/or a contrast enhancement pattern in dynamic contrast-enhanced magnetic resonance imaging (DCE MRI). Diagnosis of cirrhosis was based on characteristic clinical stigmata, ultrasonographic (US) criteria and laboratory findings. Forty-five healthy unrelated subjects from the Blood Donation Unit of the National Liver Institute were enlisted as controls.

We excluded patients with hepatocellular carcinoma (HCC), portal vein thrombosis, multi-organ failure, concurrent evidence of sepsis, pregnancy, and significant comorbidities. The study was approved by the ethics committee of the National Liver Institute, University of Menoufia, and informed consent was obtained from all participants.

Routine investigations

All participants underwent thorough history taking, as well as clinical and radiological investigations. Blood samples were collected for the assessment of complete blood count (Sysmex xp-300AM; Sysmex Corporation, Kobe, Japan), liver and kidney functions, α fetoprotein (AFP), and CA19-9 tests (Cobas 6000; Roche Diagnostics, Mannheim, Germany).

DNA extraction and MICA rs2596542 polymorphism genotyping

Genomic DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Catalog number 51104; Qiagen, Hilden, Germany).

The genotypes of the MICA rs2596542C/T polymorphism were determined by real-time polymerase chain reaction (PCR; TaqMan: C_27301153_10, Catalog number: 4351379, Applied Biosystems, Thermo Fisher Brand, Foster City, USA). The reaction was performed with 10 µl of PCR genotyping Master Mix (2X), 0.5 µl of TaqMan assay mix, 5 µl of extracted DNA, and 4.5 µl of nuclease-free water (total reaction volume 20 µl). The probes were labeled using the fluorescent dyes FAM and VIC for recognition of T and C alleles respectively. The PCR mixture without a DNA sample was used as a negative control. PCR included initial denaturation at 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 s, and lastly extension at 60°C for 5 min using the Qiagen Rotor GENE Q real time PCR system (Qiagen GmbH, Hilden, Germany).

Statistics

The results were collected, tabulated, and statistically analyzed using the statistical package SPSS version 20 (Armonk, NY; IBM Corporation). The data analysis was divided into two phases: a descriptive study (number, percentage, mean, and standard deviation) and an analytical study (χ^2 test, Mann-Whitney test, Kruskal-Wallis test, and ANOVA test followed by a post hoc test (Dunn's multiple comparisons test), and Fisher's exact test and odds ratio (OR) and confidence interval (CI) test). Significance was set at $p < 0.05$.

Results

Characteristics of study participants

The present study included 40 patients with CCA and 45 healthy controls. They had a similar age distribution (56.05 ±7 years and 54.6 ±5.3 years, respectively; $p = 0.284$). However, there was a significant male predominance in the CCA group ($p = 0.003$) (Table 1).

Significant differences were detected between the two groups regarding biochemical laboratory parameters. The patients with CCA had a significantly elevated white blood cell count and significantly lower hemoglobin and platelet levels ($p < 0.001$); moreover, liver enzymes (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and γ glutamyl transferase (GGT)) and bilirubin (total and direct) were significantly elevated, indicating the effect

Table 1. Statistical analysis of the demographic parameters and basal values of biochemical laboratory parameters in the cholangiocarcinoma (CCA) and control groups

Parameters	CCA (*n* = 40)	Control (*n* = 45)	*P* value
Age (years)	56.05 ±7	54.6 ±5.3	0.284
Sex (M/F)	29/11	18/27	0.003
Hemoglobin (g/dl)	10.9 ±1.4	13.3 ±1.2	< 0.001
WBCs (10^3/µl)	12.1 ±7	6.2 ±1.1	< 0.001
Platelets (10^3/µl)	200.7 ±90.1	281.8 ±57.1	< 0.001
ALT (U/l)	66.75 ±48.81	18.4 ±5.48	< 0.001
AST (U/l)	137.1 ±222.3	17.04 ±5.47	< 0.001
ALP (U/l)	382.4 ±397.4	62.11 ±9.89	< 0.001
GGT (U/l)	366.8 ±266.9	16 ±4.89	< 0.001
Total bilirubin (mg/dl)	16.94 ±7.54	0.72 ±0.12	< 0.001
Direct bilirubin (mg/dl)	13.58 ±6.28	0.17 ±0.06	< 0.001
Albumin (g/dl)	2.89 ±0.72	4.43 ±0.36	< 0.001
Total protein (g/dl)	6.52 ±1.21	7.45 ±0.29	< 0.001
Urea (mg/dl)	45.26 ±27.43	29 ±6.52	0.001
Creatinine (mg/dl)	1.08 ±0.58	0.84 ±0.10	0.283
AFP (ng/ml)	6.04 ±5.87	3.73 ±2.18	0.082
CA19-9 (U/ml)	2169.6 ±2499.9	17.39 ±6.68	< 0.001

WBCs – white blood cell count, AST – aspartate aminotransferase, ALT – alanine aminotransferase, ALP – alkaline phosphatase, GGT – γ-glutamyl transferase, AFP – α-fetoprotein, CA19-9 – cancer antigen19.9/carbohydrate antigen
Statistically significant at p > 0.05

Table 2. Statistical analysis of demographic parameters and basal values of biochemical laboratory parameters in cholangiocarcinoma (CCA) only and liver cirrhosis (LC) with CCA groups

Parameters	CCA only (*n* = 23)	CCA with LC (*n* = 17)	*P* value
Age (years)	53.6 ±7.4	59.4 ±4.9	0.007
Sex (M/F)	18/5	11/6	0.343
Hemoglobin (g/dl)	10.9 ±1.4	11 ±1.5	0.778
WBCs (10^3/µl)	10.8 ±8.3	13.9 ±4.2	0.165
Platelets (10^3/µl)	215 ±90.5	281.2 ±88.9	0.246
ALT (U/l)	66.87 ±41.45	66.18 ±58.69	0.290
AST (U/l)	122.83 ±217.18	156.41 ±234.38	0.432
ALP (U/l)	416.61 ±503.51	336.18 ±180.18	0.914
GGT (U/l)	391.70 ±224.14	333.0 ±320	0.156
Total bilirubin (mg/dl)	17.53 ±7.12	16.15 ±8.23	0.533
Direct bilirubin (mg/dl)	14.43 ±6.26	12.43 ±6.30	0329
Albumin (g/dl)	3.02 ±0.68	2.71 ±0.74	0.179
Total protein (g/dl)	6.47 ±1.09	6.58 ±1.39	0.795
Urea (mg/dl)	41.87 ±28.20	49.86 ±26.49	0.369
Creatinine (mg/dl)	1.14 ±0.65	1.0 ±0.47	0.356
AFP (ng/ml)	6.2 ±6	5.9 ±5.8	0.894
CA19-9 (U/ml)	2480.6 ±2727.7	1748.9 ±2162.2	0.367

WBCs – white blood cell count, AST – aspartate aminotransferase, ALT – alanine aminotransferase, ALP – alkaline phosphatase, GGT – γ-glutamyl transferase
AFP – α-fetoprotein, CA 19-9 – cancer antigen 19.9/carbohydrate antigen
Statistically significant at p > 0.05

of cholestasis. In contrast, albumin and total proteins were significantly lower, indicating a decreased synthetic power of the liver in relation to prolonged cholestasis. The CA19-9 levels were significantly higher in the patient group (Table 1).

Conversely, there were no significant differences in CCA patient subgroups (with and without cirrhosis) regarding the biochemical analysis. However, CCA patients with cirrhosis were significantly older (Table 2).

Association between the MICA rs2596542 SNP and cholangiocarcinoma

The genotype and allele frequencies of the MICA rs2596542 polymorphism in patients with CCA and healthy controls are reported in Table 3. The genotype distribution in the controls was within the Hardy-Weinberg equilibrium (p-value > 0.05). The CC genotype was significantly more prevalent in the healthy control group (48.9%) compared with the CCA group (32.5%). Conversely, the TT homozygous genotype was significantly predominant in patients with CCA (40%) vs. the control group (15.6%) (p = 0.039). The TT genotype was associated with a significant increase in the risk of CCA compared with the CC genotype (OR = 3.868, 95% CI: 1.260-11.880, p = 0.018). Conversely, the CT genotype was associated with a non-significant increase in the risk of CCA (OR = 1.163, 95% CI: 0.416-3.257, p = 0.773).

There was a significant increase in the T allele (53.8%) in the CCA group vs. the control group (33.3%) (p < 0.05). The T allele caused a highly significant increase in the risk of CCA compared with the C allele (OR = 2.324, 95% CI: 1.250-4.324, p = 0.007). The variant T allele also was significantly associated with CCA risk in the recessive model [CC + CT vs. TT (OR = 3.619, 95% CI: 1.299-10.084, p = 0.014)]. However, the T allele showed a non-significant association with CCA risk in the dominant model [CC vs. CT + TT (OR = 1.987, 95% CI: 0.822-4.803, p = 0.128)].

Conversely, we did not detect a significant difference in the rs2596542C/T genotype and allele distribution among CCA patients with cirrhosis and CCA patients without cirrhosis (p > 0.05) (Table 4).

The study of the demographic and biochemical parameters among patients with CCA with a different genotype distribution revealed an absence of significant differences. However, high levels of CA19-9 were significantly associated with the TT genotype, imply-

Table 3. Genotype distribution and allele frequencies of the MICA SNP (rs2596542) in the cholangiocarcinoma (CCA) and control groups

Parameter	CCA (*n* = 40)		Control (*n* = 45)		χ^2 (p^1)	OR	95% CI	p^2
	n	%	*n*	%				
Genotypes								
CC®	13	32.5	22	48.9	6.490* (0.039*)	1.000		
CT	11	27.5	16	35.6		1.163	0.416-3.257	0.773
TT	16	40.0	7	15.6		3.868	1.260-11.880	0.018*
$^{HW}\chi^2$ ($p0$)	1.800 (0.180)							
Dominant model								
CC®	13	32.5	22	48.9	2.348 (0.125)	1.000		
CT+ TT	27	67.5	23	51.1		1.987	0.822-4.803	0.128
Recessive model								
CC+ CT®	24	60.0	38	84.4	6.411* (0.011*)	1.000		
TT	16	40.0	7	15.6		3.619	1.299-10.084	0.014*
Allele								
C®	37	46.3	60	66.7	7.205* (0.007*)	1.000		
T	43	53.8	30	33.3		2.324	1.250-4.324	0.007*

χ^2, p1 – chi-squared test, OR – odds ratio, CI – confidence interval, LL – lower limit, UL – upper limit, ® reference group, p2 – p value for comparing between the studied groups
$^{HW}\chi^2$ (p0): chi-squared for goodness of fit for Hardy-Weinberg equilibrium (if p < 0.05, not consistent)
** Statistically significant at p < 0.05*

Table 4. Genotype distribution and allele frequencies of the MICA SNP (rs2596542) in the cholangiocarcinoma (CCA) only and CCA with liver cirrhosis (LC) groups

Parameter	Liver cirrhosis				χ^2 (p_1)	OR	95% CI	p^2
	No (*n* = 23)		Yes (*n* = 17)					
	n	%	*n*	%				
Genotype								
CC®	7	30.4	6	35.3	0.274 (0.872)	1.000		
CT	6	26.1	5	29.4		0.972	0.194-4.87	0.973
TT	10	43.5	6	35.3		0.700	0.158-3.099	0.638
Dominant model								
CC	7	30.4	6	35.3	0.105 (0.746)	1.000		
CT + TT	16	69.6	11	64.7		0.802	0.211-3.043	0.746
Recessive model								
CC + CT	13	56.5	11	64.7	0.273 (0.601)	1.000		
TT	10	43.5	6	35.3		0.709	0.195-2.581	0.602
Allele								
C	20	43.5	17	50.0	0.334 (0.563)	1.000		
T	26	56.5	17	50.0		0.769	0.316-1.873	0.563

ing that the TT genotype may be used as a biomarker for the diagnosis of CCA (Table 5).

Discussion

As an inflammation-related cancer, the study of the machineries of the immune system could help identify high-risk groups for the early diagnosis of CCA and could lead to the development of new therapies in the future [12]. In the present study, we investigated the association between the *MICA* rs2596542C/T SNP and the risk of CCA development.

We observed that male gender was significantly increased in the CCA group. Similarly, Abdel Wahab

Table 5. Statistical analysis of the demographic parameters and basal values of biochemical laboratory parameters according to MICA SNP (rs2596542) genotype groups among patients with cholangiocarcinoma (CCA)

Parameters	CC (*n* = 13)	CT (*n* = 11)	TT (*n* = 16)	*P* value
Age (years)	57.46 ±6.10	54.27 ±8.14	56.13 ±7.07	0.552
Sex (M/F)	9/4	9/2	11/5	0.820
Hemoglobin (g/dl)	10.78 ±1.58	11.21 ±1.71	10.86 ±1.14	0.750
WBCs (10^3/µl)	11.52 ±5.26	13.15 ±6.65	11.87 ±8.61	0.592
Platelets (10^3/µl)	189.0 ±92.36	195.18 ±82.48	213.88 ±97.46	0.750
ALT (U/l)	66.31 ±53.14	60.18 ±29.85	71.19 ±57.34	0.996
AST (U/l)	109.92 ±116.87	95.91 ±47.98	187.50 ±333.84	0.419
ALP (U/l)	353.15 ±165.11	510.0 ±710.93	318.50 ±186.79	0.847
GGT (U/l)	388.46 ±284.01	371.55 ±187.91	345.81 ±310.14	0.651
Total bilirubin (mg/dl)	17.6 ±8.52	16.25 ±6.52	16.87 ±7.79	0.827
Direct bilirubin (mg/dl)	13.90 ±6.87	13.14 ±5.08	13.63 ±6.87	0.850
Albumin (g/dl)	3.12 ±0.84	2.91 ±0.73	2.69 ±0.57	0.294
Total protein (g/dl)	6.65 ±1.24	6.47 ±1.15	6.44 ±1.29	0.888
Urea (mg/dl)	41.23 ±26.53	40.36 ±17.01	51.91 ±33.49	0.467
Creatinine (mg/dl)	1.02 ±0.42	0.91 ±0.39	1.24 ±0.76	0.342
AFP (ng/ml)	7.30 ±7.05	5.90 ±6.71	5.11 ±4.19	0.241
CA19-9 (U/ml)	136.19 ±71.68	810.7 2 ±1350.4	4756.01 ±1719	< 0.001

WBCs – white blood cell count, AST – aspartate aminotransferase, ALT – alanine aminotransferase, ALP – alkaline phosphatase, GGT – γ-glutamyl transferase, AFP – α-fetoprotein, CA19-9 – cancer antigen 19-9/carbohydrate antigen
Statistically significant at p < 0.05

et al. [13] reported that the male-to-female gender distribution was 1.7 : 1 among Egyptian patients with CCA, and the review of Banales *et al.* [14] reported that CCA mortality was higher in men than it was in women worldwide.

Furthermore, the mean age of the patients with CCA was 53.6 ±7.4 years. This was consistent with the age distribution reported in a previous study conducted in Egypt by Abdelaal *et al.* [15], who reported that the median age of patients with CCA was 52.5 years, which is lower than the higher age of incidence for CCA in most parts of the world, particularly in Western countries, which is in the seventh decade of life [16]. This indicates that a large multicentric study is needed to identify demographic and risk factors for CCA in Egypt.

The investigation of the rs2596542C/T polymorphism revealed that it may have an impact on the susceptibility to CCA, with the T allele being recognized as a risk allele (0.007) and increased TT genotype frequency being detected among patients with CCA (p = 0.039). However, this polymorphism was not correlated with progression to cirrhosis in patients with CCA.

To the best of our knowledge, this was the first study to evaluate the rs2596542C/T polymorphism in patients with CCA in Egypt. Nevertheless, there were previous attempts to study the effect of the MICA rs2596542 C/T polymorphism in HCC [11, 12, 17, 18]. Marangon *et al.* [19] reported the rs2596542 T allele as a risk allele. These results were contradicted by those of Burza *et al.* [20]. Conversely, Lange *et al.* suggested a protective role for the rs2596542 T allele [21]. These variable results indicate the need for further large studies to detect the impact of different rs2596542C/T alleles on the development of carcinomas in different ethnic groups.

Previously, Tsukagoshi *et al.* evaluated the expression of different NKG2D ligands in CCA tissue specimens. They found that MICA/B was highly expressed (96.3%) in all tissue samples. Interestingly, they found that high expression of MICA/B was significantly correlated with low lymphatic invasion, suggesting that the NKG2D pathway could be a promising target for controlling cancer progression [22].

It is worth noting that the human *MICA* gene consists of six exons. Exon 5 (encoding the transmembrane domain) is present in alleles which harbor a variable number of short tandem repeats, consisting of 4, 5, 6, 7, 8, 9, and 10 GCT repeats, designated as A4, A5, A6, A7, A8, A9, and A10, respectively, in addition to an extra

guanine (G) insertion after 5 GCT repeats (A5.1 allele). The A5.1 allele causes a frameshift, leading to a premature stop codon; hence, in this case, MICA is shorter and more easily cleaved from the cell surface [23, 24].

Kumar *et al*. [11] demonstrated that the T risk allele at rs2596542 is in linkage disequilibrium (LD) with the A9 and A4 alleles, whereas the non-risk C allele is in LD with the A5 and A5.1 alleles. Moreover, Melum *et al*. found that carriers of the MICA 5.1 allele were protected against CCA development [25].

Conversely, the MICA 5.1 allele has been reported to be associated with primary sclerosing cholangitis (PSC) [26]. A possible explanation for this dual role of the MICA 5.1 allele is that genetic variants predisposing to increased activation of the immune system, thus leading to inflammation, could at the same time protect against neoplastic cells through the same activating mechanisms.

Furthermore, MICA shedding triggers effective escape of cancerous cells from NKG2D recognition, leading to the development of cancers [27]. Onyeaghala *et al*. revealed that higher serum levels of soluble MICA were associated with the MICA A5.1 polymorphism, which increased the risk of pancreatic cancer [28]. This is in contrast with the results reported by Kumar *et al*. [11] and Matsuda *et al*. [29], who reported that the risk T allele of rs2596542 was correlated with lower soluble MICA levels in patients with HCC.

Kumar *et al*. suggested that individuals who carry the rs2596542 T risk allele could express low levels of membrane-bound MICA, which leads to poor activation of natural killer cells and $CD8^+$ T cells and, likely, progression to HCC. This indicates that a larger study of patients with CCA correlating the rs2596542 polymorphism with the serum levels of MICA is necessary to identify the possible cause of progression to CCA [11].

Here, we were not able to detect significant differences between the various MICA rs2596542 genotypes regarding biochemical laboratory parameters in patients with CCA. Similar results were reported by Motomura *et al*. [30] and Mohamed *et al*. [12], who studied HCC. This indicates that the MICA rs2596542 polymorphism might not be directly correlated with liver functions.

However, high levels of CA19-9 were significantly correlated with the TT genotype, implying that the TT genotype could be used as a biomarker for the diagnosis of CCA.

Conclusions

The MICA rs2596542 polymorphism modulated the susceptibility to CCA. The TT genotype may be used as a tumor marker for the diagnosis of CCA. Patients with benign inflammation in the biliary tract carrying the risky T allele should be closely followed up. Triggering the MICA pathway could be a promising therapeutic target. However, rs2596542 polymorphism does not affect cholangiocarcinoma progression.

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Disclosure

The authors declare no conflict of interest.

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