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ORIGINAL ARTICLE

Role of MICA rs2596542 and IFN-gamma rs2069727 Polymorphism in HCC Development in Egyptian Patients with HCV-related HCC

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ABSTRACT

Background: *Chronic infection with hepatitis C virus (HCV) is considered as a predisposing factor for liver cirrhosis (LC) and hepatocellular carcinoma (HCC). In several malignancies, MICA and IFN-γ genes were found to be highly expressed.* ***Objectives:*** *to identify the impacts of MICA rs2596542 and IFN-γ rs2069727 polymorphisms on LC and HCC development among Egyptian HCV patients, as well as impacts of gene-gene interaction on the disease incidence.* ***Methodology:*** *Blood samples were taken from 75 subjects with confirmed chronic hepatitis C (CHC), HCV-related LC or HCC on top of HCV infection and 25 healthy controls. Genotyping of MICA rs2596542 and IFN-γ rs2069727 polymorphisms were studied using real time PCR and PCR-Restriction fragment length polymorphism (RFLP) respectively.* ***Results:*** *A significantly elevated MICA rs2596542 TT genotype and T allele was found in HCC group (28%) and LC group (28%) compared to control group (16%). CC genotype and C allele were found to be more prevalent in healthy controls (64%) than HCC group (16%) and LC group (24%). AA genotype of IFN-γ rs2069727 SNP and A allele were significantly elevated in control group, while GG genotype and G allele were significantly elevated in HCC and LC groups. In assessing the hazard of HCC development, wild forms of both genes were higher in controls, and this is the protective forms while the mutant forms were elevated in HCC group.* ***Conclusion:*** *MICA rs2596542 and IFN-γ rs2069727 SNPs had significantly implicated in LC and HCC susceptibility.*

INTRODUCTION

Chronic HCV infection is a risk factor for cirrhosis and chronic liver disease (CLD), that identified as the common significant antecedent to hepatocellular carcinoma (HCC) [1].HCC is a virulent tumor and one of the common abundant cancer-related reasons of mortality worldwide [2]. Thus, the advancement of liver disease in HCV-infected persons will impact their quality of life and impose a substantial fiscal burden on the public healthcare [3].

The human major histocompatibility complex class I chain A related gene (MICA) is identified on chromosome 6 short arm within the MHC-I region [4].

MICA gene is presented in the primary epithelial cells of several normal tissues and in various neoplasms, including HCC, lung, breast, and prostatic cancer [5].

Interestingly, soluble, and membrane-bound MICA molecules display diverse functional properties. MICA molecules must be recognized for the immune system to recognize and diminish virally infected and tumor cells, hence playing a crucial function in immunological surveillance [6].

MICA SNPs are believed to influence antitumor immunity [7]. One of these SNPs, rs2596542, is located in the MICA promoter area and might thus modify the linkage of stress-stimulated transcription factors [8]. Therefore, the rs2596542 SNP may impact MICA gene expression or enhance tumor growth associated routes [9].

Interferon (IFN) is a type of cytokine which has a variety of antiviral, antitumor, and immunological regulatory properties[10]. Many reports have linked SNPs in the IFN- gene's noncoding region to chronic inflammation, autoimmune response, and tumor formation [11]. By altering transcription factor binding sites, SNPs in the IFN- gene may impact the expressions of associated genes, resulting in changes in cytokine concentrations and immunological activities [12].

Although several research have proven the role of MICA and IFN-γ genes in different cancers, there are few studies concerning the impacts of gene-gene interaction on HCC development on top of HCV infection. Therefore, our research objected to identify

MICA rs2596542 and IFN-γ rs2069727 polymorphisms impacts on HCC development among Egyptian subjects with HCV genotype 4, besides impacts of gene-gene interaction on the disease incidence.

METHODOLOGY

This case-control research was performed after obtaining written informed consent from all participants (patients and healthy volunteers). The National Institute Ethical Review Board approved the research protocol IRB number 00378/2022 (Nov.1st 2022). Seventy-five HCV subjects were involved in the period from September 2019 to December 2021. Subjects were equally allocated into three groups, CHC group, LC related CHC (LC group) and HCC- related CHC (HCC group). All cases were affirmed positively for HCV-Ab and negatively for anti-HBV and anti-HIV. Twenty-five healthy blood donors were selected as healthy controls (group d; n = 25). All controls were also negatively affirmed for both HCV Ab and HCV RNA and had no history of liver disease or any other viral diseases.

Exclusion criteria:

Individuals who were infected with other viral hepatitis A, B, E, or human immunodeficiency virus (HIV), pregnant women and autoimmune disease.

Biochemical investigation:

Ten milliliters of fasting venous blood were collected under aseptic precautions (3 ml for genotyping techniques and 7 ml for other tests). Assessment of liver functions tests; ALT, AST, total bilirubin, direct bilirubin, albumen, were estimated utilizing Integra 800 Auto analyzer (Roche-Germany Catalogue number; M, 87432). Alpha fetoprotein (AFP) in subjects' sera was measured utilizing a Beckman CX4 chemistry analyzer (NY; USA). HCV antibodies were measured by DIA.PRO Diagnostic Bioprobes kit (Milano, Italy), based on ELISA. Identification and quantification of HCV-RNA was done utilizing the QIAamp® DSP Virus Spin Kit. Complete blood examination was applied on subjects' plasma.

Genotyping of rs2596542 SNP in the MICA promoter region:

Genomic DNA was extracted from peripheral blood mononuclear cells utilizing DNA isolation kit (QiAamp DNA mini kit: Qiagen, Germany). All individuals' extracted DNA was genotyped for the SNP rs2596542 utilizing the TaqMan Real Time PCR System (Applied Biosystems: Foster City, USA). Two allelic probes were included, one tagged with FAM dye and the other one with fluorescent VIC dye. The PCR was conducted utilizing a TaqMan universal master mix (Applied Biosystems, Foster City, CA, USA) at a 20X probe concentration. The reaction was applied on a 96-well format in a total reaction volume of 25 µL utilizing 20 ng of genomic DNA. The reaction plates were heated for 2 min at 50 °C then for 10 min at 95 °C, proceeded by 40 cycles of 95 °C for 15 s then 60 °C for 1.5 min. Each well's fluorescence strength was red in an assay plate [13]. (Figure 1)

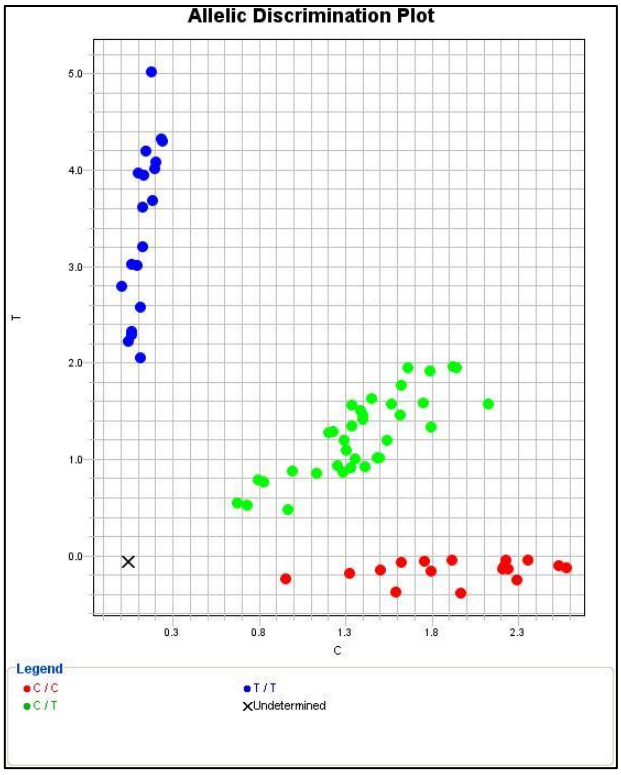

Fig 1: Allelic discrimination plot of MICA genotypes.

Genotyping of SNP (rs 2069727) in IFN-γ gene:

Polymorphism in IFN-γ rs 2069727 was genotyped by a PCR-RFLP assay. The subsequent primers were utilized; forward primer 5'-AGGTTCTGCTATGGAATGTA-3' and reverse primer 5'-AAACTACATTCCATAGCAGA- 3' [14] .

PCR amplification was applied on a total volume of 10 µl involving 10 mM Tris–HCl (pH 8.3), 50 mM KCl, Tween-20 0.01%, 0.2 mM deoxyribonucleotides, 2–4 pmol of each primer, 2.0 mM MgCl2, 0.5 units hot-start Taq DNA polymerase (Right Taq, Euroclone, Milan, Italy). 10 ng of genomic DNA were exposed to 35 cycles of degeneration at 94°C for 30 seconds then elongation (at 72 0C for 30 s), followed by annealing at 60°C for 30 seconds, and last extension was done at 72°C for 5 minutes [14].

RFLP analysis for IFN-γ **genotypes:**

In a total volume of 20 µl, 10 µl of the amplicons were digested utilizing 1 unit of the Hinf restriction endonuclease enzyme at 37°C over-night. The digested fragments were visualized by gel electrophoresis (4%) after staining with ethidium bromide. AA genotype, GG genotype were visible at 159-bp, 223-bp fragments respectively, and AG genotype had 64-bp and 159-bp fragments [14]. (Figure 2)

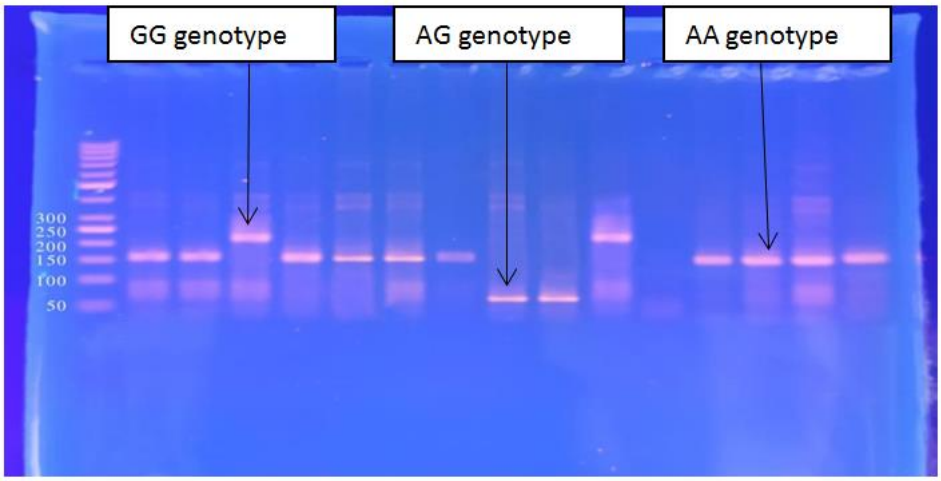

Fig2: The digestion results for rs2069727 polymorphism. The amplified fragment of rs2069727 polymorphism was digested by *Hinf*I. In this setting,AA genotype had 159-bp, GG showed 223-bp fragment, and AG had 64-bp fragments

Statistical Analysis:

Analysis of data was done utilizing SPSS version **22.0 (IBM, Armonk, NY, USA) for Windows.** Categorical data were presented as number and percentages, Chi square test (X^2), or Fisher's exact test (FET) were utilized to analyze them, Odds ratios (OR) and the corresponding 95%CI were estimated. Quantitative data were tested for normality utilizing Shapiro-Wilks test, assuming normality at P>0.05.

RESULTS

Demographic analysis of the studied subjects revealed no significant difference among the four groups.

Table 1 Demographic data of the studied groups.

Variable		CHC (n=25)		LC (n=25)		HCC (n=25)		Control (n=25)		ANOVA	P-value
Age (years)	**Mean±SD**	52.6±4.1		54.1±6.8		55.5±5.9		51.5±3.9		**2.58**	**0.058 (NS)**
	Range	45-59		48-65		50-70		40-56			
		No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**χ^2/ Fisher's test**	**P-value**
Sex	**Male**	17	68.0	15	60.0	19	76.0	15	60	**1.96**	**0.58 (NS)**
	Female	8	32.0	10	40.0	6	24.0	10	40		
Residence	**Urban**	7	28.0	4	16.0	5	20.0	9	36	**3.15**	**0.37 (NS)**
	Rural	18	72.0	21	84.0	20	80.0	16	64		
Smoking	**Non smoker**	17	68.0	16	64.0	15	60.0	19	76	**1.58**	**0.66 (NS)**
	Smoking	8	32.0	9	36.0	10	40.0	6	24		
Hypertension	**Present**	4	16	5	20	2	10	2	8	**1.87**	**0.6 (NS)**
	Absent	21	84	20	80	18	90	23	92		
Diabetes mellitus	**Present**	2	8	6	24	4	20	2	8	**3.95**	**0.27 (NS)**
	Absent	23	92	19	76	16	80	23	92		

Biochemical analysis of the studied participants revealed a significant difference among the studied groups as regards liver function test, CBC profile and AFP.

Higher serum AST and ALT in CHC, LC and HCC groups than controls, while AFP and total bilirubin are higher in HCC group than controls. But albumin levels were reduced significantly in LC and HCC groups than control and CHC ones (P value <0.001).

Table 2 Biochemical analysis of the studied subjects.

Variable	CHC (n=25)	LC (n=25)	HCC (n=25)	Control (n=25)	KW & P-value
AST (IU/L) Mean±SD	51.8± 36.3	57±25.6	34.8±12.9	15.9± 3.02	53.6 & <0.001 (HS)
ALT (IU/L) Mean±SD	53.5±36	37.2±19	35.4±15.3	16± 4.2	44.2 & <0.001 (HS)
Total bilirubin (mg/dl) Mean±SD	0.7± 0.2	2.5±1.6	5.7±4.7	0.5± 0.2	70& <0.001 (HS)
Direct bilirubin (mg/dl) Mean±SD	0.08±0.05	1.7±1.8	3.9±3.8	0.1±0.03	62.1& <0.001 (HS)
Albumin (g/dl) Mean±SD	3.9±0.3	2.8±0.5	2.2±0.4	4±0.2	75.9& <0.001 (HS)
AFP Mean±SD	2.1±0.9	2.5±1	249.8±309.1	1.6±0.5	59.3& <0.001 (HS)
Hb (gm/dl) Mean±SD	11.6± 0.96	10.3± 1.4	10.4± 1.32	12.93± 0.83	**27.7 &<0.001 (HS)**
RBCs (x10^3)	3.6±.55	3.3±.38	3.4±.57	5.2±.7	**77.1 & <0.001 (HS)**
WBCs (X103/mm^3) Mean±SD	9.3± 6.37	9.8± 5.02	13.2± 5.30	5.7±114	**†26.7& <0.001 (HS)**
PLTs (X103/mm^3) Mean±SD	162.8± 35.8	105.6± 48.4	111.2± 54.6	240.0± 57.4	**†53.8 (<0.001, HS)**

Results of MICA SNP rs2596542 and IFN-γ rs2069727 genotypes among control and (CHC, LC and HCC) groups exhibited a significant difference among the four groups as regards rs2596542C/T (*P* = 0. 022). The CC genotype and C allele of MICA SNP rs2596542 were prominent in control group, while TT genotype and T allele were prominent in HCC and LC groups (*P* = 0.025). Genotypic and allelic distribution of IFN-γ rs2069727 SNP revealed AA genotype and A allele were significantly elevated in controls, while GG genotype and G allele were significantly elevated in HCC and LC groups as presented in table 3.

Table 3: Distribution of MICA rs2596542 and INF--γ rs2069727 genotypes and alleles among the studied groups

Genotype and Alleles		Groups								X^2(P)	P-value
		CHC (n=25)		LC (n=25)		HCC (n=25)		Controls (n=25)			
		No.	%	No	%	No.	%	No.	%		
MICA rs2596542 Genotypes	**CC**	11	44	6	24	4	16	16	64	15.4	(0.018, S)
	CT	8	32	12	48	14	56	5	20		
	TT	6	24	7	28	7	28	4	16		
MICA rs2596542 Alleles	**C**	30	60	24	48	22	44	37	74	11.1	(0.011, S)
	T	20	40	26	52	28	56	13	26		
IFN-gamma rs2069727 genotype	**AA**	11	44	6	24	4	16	14	56	12.9	(0.045, S)
	AG	7	28	8	32	8	32	7	28		
	GG	7	28	11	44	13	52	4	16		
IFN-gamma rs2069727 alleles	**A**	29	58	20	40	16	32	35	70	17.8	(<0.001, HS)
	G	21	42	30	60	34	68	15	30		

MICA rs2596542 CC genotype was taken as a reference; we found a significant relation to rs2596542 CT and rs2596542 TT genotypes among HCC versus controls (*P* = 0. 002, *P* = 0. 023) and HCC versus CHC cases (*P* = 0. 032, *P* = 0. 15), while no significant difference among HCC versus LC cases *(P* = 0. 46, *P* = 0. 62). Also, there was a significant difference among LC versus controls (*P* = 0. 009, *P* = 0. 042). Regarding the alleles, the C allele was a reference. Allele T significantly detected in HCC and LC versus control and CHC groups. (Table 4)

Table 4: Association between the MICA SNP rs2596542 with HCC and LC susceptibility

MICA rs2596542		HCC vs. Controls		HCC vs. CHC		HCC vs. LC		LC vs. Controls		LC vs. CHC	
		OR (95% CI)	P	OR (95% CI)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
Gene	CC	Ref		Ref		Ref		Ref		Ref	
	CT	11.2 (2.5-50.1)	0.002 (S)	4.8 (1.1-20.2)	0.03 2 (S)	1.75 (0.4-7.7)	0.46 (NS)	6.4 (1.6-26)	0.009 (S)	2.75 (0.7-10.5)	0.14 (NS)
	TT	7.0 (1.3-36.3)	0.02 (S)	3.1 (0.6-15.5)	0.15 (NS)	1.5 (0.3-7.4)	0.62 (NS)	4.7 (1.09-21.9)	0.042 (S)	2.1 (0.49-9.4)	0.31 (NS)
Allele	C	Ref		Ref		Ref		Ref		Ref	
	T	3.6 (1.6-8.4)	0.003 (S)	1.9 (0.86-4.2)	0.11 (NS)	1.17 (0.5-2.6)	0.69 (NS)	3.1 (1.3-7.1)	0.008 (S)	1.6 (0.7-3.6)	0.23 (NS)

Ref = reference genotype or allele OR = odd ratio CI = confidence interval

INF-**γ rs2069727** AA genotype was taken as a reference, we found a significant relation of **rs2069727** AG and **rs2069727** GG genotypes among HCC versus controls (*P* = 0. 07, *P* = 0. 003) and HCC versus the CHC cases (*P* = 0.14, *P* = 0. 02), while no significant difference among HCC versus LC cases *(P* = 0. 46, *P* = 0. 62). Also, there was a significant difference among LC versus control (*P* = 0. 01). Regarding the alleles, the A allele was a reference. We found that allele G significantly detected in HCC and LC versus control and CHC.

Table 5: Association between the INF- -γ rs2069727 with HCC and LC susceptibility

INF- -γ rs2069727		HCC vs. Controls		HCC vs. CHC		HCC vs. LC		LC vs. Controls		LC vs. CHC	
		OR (95% CI)	P	OR (95% CI)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
Gene	AA	Ref		Ref		Ref		Ref		Ref	
	AG	4.0 (0.9-18)	0.07 (S)	3.1 (0.7-14.5)	0.14 (NS)	1.5 (0.3-7.4)	0.62 (NS)	2.7 (0.66-10.8)	0.16 (NS)	2.1 (0.5-8.7)	0.31 (NS)
	GG	11.4 (2.3-55.1)	0.003 (S)	5.1 (1.2-22.2)	0.02 (S)	1.77 (0.4-7.9)	0.45 (NS)	4.6 (1.4-28.5)	0.015 (S)	2.9 (0.73-11.4)	0.13 (NS)
Allele	A	Ref		Ref		Ref		Ref		Ref	
	G	4.95 (2.1-11.6)	0.001 (HS)	2.93 (1.3-6.6)	0.01 (S)	1.4 (0.6-3.2	0.41 (NS)	3.5 (1.5-8)	0.003 (S)	2.07 (0.9-4.6)	0.07 (NS)

Ref = reference genotype or allele OR = odd ratio CI = confidence interval

Wild form of both MICA and IFN-**γ** genotypes is high in controls and considered reference for mutant form which was high in HCC cases with a high significant difference (p value=0.001).

Table 6 Interaction between polymorphisms of MICA and IFN- γ genes with HCC in comparison with controls

MICA-IFN interaction		Groups				OR (95%CI)	P
		HCC (n=25)		Controls (n=25)			
		No	%	No	%		
+	+	2	8	11	44	ref	---
+	-	2	8	5	20	2.2 (0.23-20.4)	0.49 (NS)
-	+	1	4	3	12	1.83 (0.12-27.8)	0.66 (NS)
-	-	20	80	6	24	18.3 (3.1-106.7)	0.0012 (HS)

+ wild form - mutant form

Relation between MICA genotypes and some biochemical parameters as no significant difference between AFP, ALT and AST and different MICA genotypes (P value > 0.05). Also, there was no significance between AFP, ALT, and AST and IFN- **γ** genotypes (p value >0.05).

Table 7: Levels of some biochemical markers associated with MICA And INF--γ genotypic frequencies in LC and HCC groups.

	AFP (ng/dl)	**ALT (IU/ml)**	**AST(IU/ml)**	**AST/ALT Ratio**	**P-value**	LSD
	Median (Min.- Max.)	Median (Min.- Max.)	Median (Min.- Max.)	Median (Min.- Max.)		
MICA genotypes						
CC	1.6(1.3-7.5)	28(24.5-43.5)	41(30.5-87)	1.4(1.2-2)	0.32(NS)	P1: 0.244 P2: 0.386 P3: 0.603
CT	4.1(3.1-120)	35(27.3-51.3)	44.5(31.7-64)	1.27(1.16-1.29)	0.24(NS)	P1: 0.144 P2: 0.486 P3: 0.803
TT	44.5(2.3-443)	32(20.3-41)	34.5(23.5-43)	1.07(1.05-1.09	0.09(NS)	P1: 0.244 P2: 0.186 P3: 0.603
INF--γ genotypes						
AA	1.7(1.4-19.8)	29(25.3-40.8)	45.5(31.8-86.5)	1.56(1.2-2.12)	0.45(NS)	P4: 0.589 P5: 0.564 P6: 0.935
AG	4(2.4-97)	33(25.8-36)	42(25.8-67)	1.27(1-1.8)	0.38(NS)	P4: 0.902 P5: 0.637 P6: 0.679
GG	4.5(3.5-210)	35(24-59)	36(32-66)	1.02(1.01-1.1)	0.12(NS)	P4: 0.144 P5: 0.286 P6: 0.703

P1:CC versCT P2:CC versTT P3:CT vers TT
P4:AA versAG P5:AA vers GG P6:AG vers GG

DISCUSSION

HCC is a leading reason of cancer-related death and morbidity globally [15]. MICA molecule expression is stimulated by a variety of stressors, including viral infections [16].

In our research, there was a high significant elevation in serum ALT, AST, total and direct bilirubin in HCC subjects and LC in relation to controls and this was in line with Baghdady et al [17].

In our research, there was a significant difference among cases and control groups as regards hematological finding as there was anemia in cirrhotic patient and HCC cases. Schuppan and Afdhal, [18] reported that anemia was one of the laboratories finding in liver cirrhosis which may be attributed to folate deficiency, hypersplenism, and gastrointestinal blood loss (e.g., via esophageal varices). In the same study it was documented that hypersplenism may also lead to thrombocytopenia and leukopenia and this was found in our study.

Our research showed that, MICA rs2596542 CC genotype and C allele were more common in the controls than in HCC and LC cases, suggesting that it reduces the risk of HCC, while rs2596542 TT, CT genotypes and T allele were risk factors for LC and HCC vulnerability in chronic HCV cases and this agreed with Jiang et al [19].

MICA genotype was among HCC, LC cases the controls was significantly different (P value = 0. 01) and this in line with Lange et al [20]. There was a relation among MICA gene polymorphism and CLD (LC and HCC) in cases with HCV genotype 4. Serum MICA elevated among liver disease development. According to a prior research, HCV-infected individuals with a greater membrane-bound MICA concentration may elicit a stronger immune response [21]. mMICA is subsequently converted to sMICA by metalloproteinases that are typically overexpressed in cancerous tissues. The debilitating impact of sMICA on NK cells promotes tumor growth. Consequently, sMICA concentrations in HCV cases rose [22].

Therefore, there is a relationship among MICA gene polymorphism and HCC in HCV cases. Our research is comparable to that of Mohamed et al. [23], who found the T allele enhanced the hazard of HCC development in HCV cases.

The increased rs2596542 CC genotype in controls over LC and HCC cases shows the CC genotype protects from advancement of HCV-related liver cancer. On the contrary, Aguilar-Olivos et al.[24] discovered the

minor T allele of rs2596542 seemed to protect against the development of HCC.

No significant relationship was discovered between rs2596542 SNP variations and clinical indicators including ALT, AST and AFP which in line with Motomura et al. [25].

Human IFN- polymorphisms have been linked with a diversity of malignancies, autoimmune illnesses, and infectious diseases as tuberculosis, hepatitis B, and leishmaniasis in several recent research conducted in China and overseas [26].

This research revealed that, rs2069727 AA genotype and A allele was most commonly in the controls than HCC and LC ones suggesting it leads to lower HCC hazard, whereas rs2069727 GG,AG genotypes and G allele was a hazard of HCC and LC vulnerability in chronic HCV and this agrees with **Li et al [14].**

Our research outcomes revealed the gene-gene interaction between IFN- and MICA polymorphisms has a significant role in HCC prevalence and development. Consequently, we should focus further on the impacts of IFN-γ and MICA genes on HCC.

CONCLUSIONS

This research provides comprehensive information on the clinical state, MICA SNP rs2596542C/T genotype, and IFN-γ rs2069727 polymorphism. The presence of MICA genotype variations (TT/CT) was associated with an elevated hazard of CLD development. Accordingly, T allele led to an elevated hazard of HCC progress in HCV cases, and focus was on the possible predictive function of MICA genotype in liver disease development.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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