Egypt. J. Chem. **Vol. 66**, No.9.pp. 479 - 490 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

TLR2 Gene Polymorphism and MicroRNA 301 gene Expression Level as Signatures for Hepatocellular Carcinoma in Egyptian Patients

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Abstract

HCC is the most prevalent gastrointestinal malignant tumor worldwide. Genetic determinants of HCC occurrence have been always an interest for most hepatologists. The aim of this research to discover if TLR2 gene (rs3804099) polymorphism and MicroRNA301 gene expression can affect HCC incidence in Egyptians.

Methodology: 247 participants were divided to 3 groups. Group 1:85 adult HCC patients on top of HCV. Group 2: 82 chronic HCV patients. They were followed up for one year, 80 supposedly healthy subjects who were matched in age and sex with patients (Group 3). All participantswere subjected to clinical evaluation and history-taking, laboratory investigations and molecular testing (TLR2 SNP analysis and microRNA301 gene expression by qPCR).

Results: Our study found that HCC patients were mainly males(60%),age was 53.67 ± 7.62 years. TLR2 (rs3804099) CT, CC genotypes were significantly prominent in HCC patients (37.6% and 45.6%), CT prevails inhepatitis C patients (48.8%), and CT was the main genotype of healthy controls. Significant difference regarding genotypes (P=0.007) between HCC, control and (P=0.004) regarding (CT+CC) vs TT. Individuals carrying genotype CT were significantly associated with decreased risk of HCC (OR 1.95% CI, P= 0.204), while those of CC showed a higher HCC risk (OR 3.048 95% CI (1.269–7.320), P = 0.013).

A highly significant variation in MicroRNA301 level regarding Barcelona stages (P <0.001) and TNM classification with increased level in advanced stages, increased tumor size mainly with CC genotype [T4=27 (84.4%), T3 =5 (15.6%)]. We concluded that TLR2 SNP CC and CT variants carry the highest risk of HCC development, while microRNA301 expression can be considered diagnostic and prognostic marker in HCC Egyptian patients.

Keywords: Hepatocellular Carcinoma, hepatitis C virus, MicroRNA301, TLR2SNP.

1. Introduction

The most frequent type of liver tumor, representing about 90% of cases, is hepatocellular carcinoma. (HCC) is a serious global problem; it is the fourth most prevalent cancer in Egypt [1].

Unfortunately, it has a high mortality rate and few available treatments **[2]**. In individuals with cirrhosis, it is the leading cause of death. It occurs in1-6% of people **[3, 4]**. An international public health issue is the hepatitis C virus. The largest global HCV incidence is found in Egypt **[5]**.

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Received: 2022-12-26; revised date 2023-01-08; accepted date 2023-01-12
DOI: 10.21608/ejchem.2023.183053.7379
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The outcome of HCC depends on the stage of the tumor, with treatment options providing a 5-years survival rate of more than 70% for early-stage patients against a median survival of 1-1.5 years for symptomatic late-stage patients received systemic treatment **[3-6]**.

For innate immunological and inflammatory responses, type l trans-membrane proteins known as toll-like receptors (TLRs) are in charge **[7-8]**. The first human TLR engaged in host defense to be described was TLR2, which recognizes variations of microbial components **[8-9]**. There has been a great deal of research and reporting on the connection between TLR2 polymorphisms and the development of malignancies **[10]**. TLR2 genetic variants have been linked to an increased risk of cancer formation in the gall bladder, cervical cancer, non-Hodgkin lymphoma, and endometrial cancer **[11]**.

Significant evidence supports the role of TLR signaling in the development of hepatocarcinogenesis **[12-13]**. As proxies for the significance of TLR signaling in the development and initiation of HCC, there is a correlation between TLR2 polymorphisms and HCC via different transcriptional factors like microRNAs [**12]**.

Small non-coding RNA molecules known as micro RNAs (miRNAs) have 20–30 nucleotides and can either impose a translational blockade or cause mRNA degradation to reduce the production of mRNA at the post-transcriptional phase **[14]**. MicroRNAs up regulation has been observed in a variety of cancers **[14-15]**, where they play important roles in controlling gene expression during cell cycle and differentiation. The majority, if not all ,characteristics of malignant transformation, such as persistent proliferative signaling ,resistance to cell death, immortality, angiogenesis, invasion, and metastasis, have been linked to deregulated miRNAs in cancer cells **[13-14]**. Pancreatic, hepatocellular, and small cell lung malignancies have been shown to exhibit unregulated MicroRNA301 **[16- 17]**. MicroRNAs are discovered to have regulatory effects in HCC and have been shown to functionally contribute to tumor growth **[14, 16]**.

Significant improvements in our understanding of the functions of innate immunity and genome-wide association studies (GWAS) have shown complex connections between environmental variables and toll-like receptor gene polymorphisms. The link between the ability of TLRs polymorphisms to impact immunity and the occurrence of various genetic effects is similarly complicated. We still don't know how TLRS polymorphisms affect how receptors behave, how TLR signaling works, or how they relate to other genetic indicators such microRNAs and malignancies **[18]**. So, the aim of our study was designed to evaluate the relation between TLR2 (rs3804099) SNP, circulating microRNA 301 and chronic HCV related HCC in Egyptians.

2. Materials and methods

2.1. Patients

247 participants were included and divided to 3 groups: Group 1 of 85 adult HCC patients with HCV collected from the HCC clinics, National Liver Institute (NLI) at Menoufia University. HCC patients were diagnosed by detecting hepatic focal lesion (s) on ultrasound examination confirmed by arterial hyper-vascular focal lesions with rapid washout by tri-phasic spiral CT, MRI, and elevated serum AFP >200 ng/ml

Group 2 of 82 chronic HCV patients collected from HCV clinics, this study took place from January 2018 to June 2019 at NLI, Menoufia University, Egypt. Patients were followed up for a whole year. Group 3 of 80 healthy subjects who were matched in age and sex with patients.

Study received NLI ethical committee's approval. All patients and the control gave their explicit consents.

2.2. Exclusion criteria

History of chronic or acute inflammatory conditions within the last three months, malignancy or autoimmune illness, evidence of a debilitating liver condition, testing positive for HIV or HBsAg, or have other liver condition. Neutropenia (neutrophil count < $2000/mm^3$), thrombocytopenia (PLT< $75000/mm^3$), moderate to severe anaemia (Haemoglobin < 10 g/dl), and a considerable past medical history of cardiovascular and neuropsychiatric conditions.

2.3. All participants were subjected to:

2.3.1. Clinical evaluation and history-taking

2.3.2. Laboratory Investigations

Biochemical studies were conducted on (SYNCHRONCX9ALX, Beckman, CA, USA). Haematology tests were performed on automated Sysmex KX-21(Sysmex Corporation, Kobe, Japan), and alpha fetoprotein (AFP) and HCV RNA were performed on an Architect i1000SR, provided by Abbott, USA

Molecular testing

2.3.2.1 TLR2 SNP testing

Genomic DNA was isolated from whole blood using a spin column method (Gene-jet whole blood genomic-DNA extraction Minikit) in accordance with instructions of the manufacturer (Thermo Fisher Scientific, Lithuania). DNA variations, including the following, were distinguished using TaqMan Assay analysis:

- Homozygous: samples are those that just contain allele 1or 2.
- Heterozygous: samples are those that contain both alleles 1and 2.

0.5 µl of genotyping assay 40x (primers and probe) (ThermoFisher Scientific, MA, USA), 10µl of genotyping Master Mix, 3.5 µl of DNase-free water, and 6µl of genomic DNA template were added to a total volume of 20 µl. In place of DNA, 6µl of DNase free water was used for the reaction's negative control.

The real-time fast 7500 system (Applied Biosystems, Life Technologies CA, USA) was used for the PCR and genotyping. The cycle parameters were as follows:

The first denaturation process (holding stage) is performed at 95°C for 10 minutes. Then 35 cycles of denaturation at 95°C for 15 seconds and annealing, extension for 1 minute at 60°C

2.3.2.2 MicroRNA301 extraction and Reverse transcription

RNA was extracted using the manufacturer's instructions by micRNeasy Mini Kit (Qiagen, Hilden, Germany) from 200 µL of fresh blood samples taken from participants. Nanodrop 2000 spectrophotometry used to measure the amount of extracted RNA (ThermoScientific, Wilmington, Delaware, USA).

For cDNA preparation: Reverse transcription (RT) primer (3µL), 10 RT buffer (1.5µL), reverse Transcriptase (1µL), RNase inhibitor (0.19µL), deoxynucleotide (dNTP) mix (0.15 µL), nuclease-free water (4.16 µL) (using TaqMan MicroRNA Reverse Transcription Kit Applied Biosystems, Foster City), then mixed with (5 µL) of RNA sample .Thermal cycler conditions : 16 °C for 30 minutes, 42 °C for 30 minutes, and 85 °C for 5 minutes.

For the expression of the microRNA301 gene, TaqMan Universal-Master Mix II Kit was used with reaction volume of 20 µL including:

- 4µL of RNA ase free water, 10 µL of TaqMan universal master mix, 1µL of TaqMan mic RNA assay and 5µLof cDNA.
- Cycling conditions: an initial denaturation of 95 °C for 10 min, then 40 cycles of denaturation at 95°C, annealing and extension at 60 °C for 60 s.
- The microRNA301 and U6 snRNA primers (hsa-microRNA301 and U6 snRNA, respectively; Assay IDs (000528 and 001973) (Thermo Fisher Scientific).

Comparing the evaluated gene expression to the reference-control gene: relative expression $=2^{-\Delta\Delta CT}$, where CT is the threshold cycle for every sample and $\Delta\Delta CT= \Delta CT$ (tested sample) − ΔCt (control sample).

2.4. Statistical analysis

With the aid of the IBM SPSS software package version 20.0, data were fed into the computer and evaluated. (IBM Corp, Armonk, NY). Categorical data were shown as percentages and numbers. To investigate the relationship between the categorical variables, the chi-square test was used. When more than 20% of the cells have an expected count of less than 5, the Monte Carlo correction test was instead used. The Kolmogorov-Smirnov test was used to determine the normality of continuous data. Range (minimum and maximum), mean, standard deviation, and median were used to express quantitative data. The three groups under study were compared using a one-way ANOVA, and pairwise comparisons were then made using the Post Hoc test (Tukey). However, for non-normally distributed quantitative variables, the Mann Whitney test was used to compare two groups, the Kruskal Wallis test was used to compare several groups, and the Post Hoc test (also known as the Dunn's multiple comparisons test) was used to compare two groups side by side. The Spearman coefficient was employed to determine the correlation between quantitative variables with irregular distributions. The factor that affected HCC the most was found using logistic regression. To determine the equilibrium of the examined sample's population, Hardy-Weinberg equation was used. The diagnostic performance of the markers was assessed using the Receiver Operating Characteristics Curve (ROC).

An area greater than 50% indicates an acceptable level of performance, while an area around 100% indicates the test's optimal performance. The relationship with overall survival was determined using Kaplan-Meier survival curve. At the 5% level, significance of the results was determined.

3. Results

3.1. Sample size calculation

Unmatched case control study, the sample size was calculated using the Epi-info web site (https://www.cdc.gov Epi-info unmatched case control).

3.2. Clinical and demographic features

Table 1 lists the clinical and demographic features of all participants. Age, sex, AST, and ALT did not change significantly across study groups (P >0.05), however there were significant variations in HB, albumin, bilirubin, platelets count, and INR (P< 0.001) (Table 1). MicroRNA301 revealed a highly significant difference (P < 0.001) among the groups. Between HCC and HCV groups, AFP demonstrated a highly significant difference (P < 0.001). MicroRNA 301 expression levels in HCC patients (Mean ± SD) (14.59± 5.0) were significantly greater than those in chronic HCV patients (7.74± 2.20) (P<0.001), and they were also significantly higher than those in the control group (0.95± 0.04) (P<0.001) (Table1).

3.3. Distribution of TLR2 genotypes in the studied groups

The distribution of TLR2 genotypes in the control group did not differ significantly according to Hardy-Weinberg equilibrium (P = 0.243, x^2=1.361). CT genotype frequency was most common in the three studied groups (45.9% in HCC, (48.8%inHCV), and (42.5%) in the control group. In HCC, CC was 32(37.6%), CT was 39 (45.9%), and TT was 14 (16.5%), while in HCV, CC was 18 (22%), CT was 40 (48.8%), and TT was 24 (29.3%). In control group CC was 17 (21.3%), CT was 34 (42.5%), and TT was 29 (36.3%). Regarding TLR2 genotypes, there were appreciable differences between HCC and HCV (P=0.039). Regarding TLR2 genotypes in HCC and control showed marked significant difference (P=0.007). There was a highly significant difference between HCC and control regarding (CT+CC) vs TT (P=0.004) as well as substantial difference between HCC and HCV regarding (CT+CC) vs TT (P=0.049). In all groups, T allele predominated (Table 2).

3.4. ROC curve analysis

ROC curve analysis to distinguish between HCC and HCV by AFP at cut off value > 40.1(ng/ml) AUC=0.846, sensitivity was 60%, specificity was 96.34%, PPV was 94.4%, and NPV was 69.9% (P<0.001). (Figure 1). MicroRNA 301 at a cut-off value greater than 9.11, with sensitivity 85.88%, specificity 70.73%, PPV of 75.3%, and NPV of 82.9% (P < 0.001) (Figure 1). This study showed 70% overall survival rate after 12 months of follow up, increase death rate mainly in those with advanced TNM stage and increased MicroRNA301 levels (Figure 2).

3.5. Relation between TLR genotypes, microRNA 301 and different parameters

With higher levels of microRNA301 in advanced stages, increased tumor size mostly with CC genotype, T4=27 (84.4%) T3 =5 (15.6%), lymphnodes N1=11(34.4%), N2= 6 (18.8%), and metastasis M1= 9 (28.1%), there was a highly significant change (P < 0.001) in MicroRNA301 level with respect to Barcelona stages, and TNM classification. TT genotype was high in early stages of different classifications (Table 3a).

Regarding tumor size, there was a highly significant increase (P < 0.001) in MicroRNA301 levels (Table 3b).

The level of microRNA301 varied significantly among the various TLR2 genotypes (P=0.030), with the difference between CC and CT genotypes showing the greatest variation (P<0.009) (Table 3c).

3.6. Regression analysis

Using univariate regression analysis for risk factors affecting HCC development revealed that there was a highly significant risk (P<0.001) in HB at (95% C.I) OR 0.658 (0.546 – 0.794), platelets 0.985 (0.980–0.990), T.bilirubin 26.146 (7.575–90.239), D.bilirubin 21.003 (3.977 –110.930), Albumin 0.111 (0.047–0.261), INR 788.17 (42.584– 14588), AFP 1.029 (1.013–1.045), microRNA301 (P<0.001), OR1.941 (1.561–2.414) and CC genotype of TLR2 had a significantly higher risk of developing HCC (P=0.013) OR 3.048 (1.26–7.32).

Individuals with genotype CT had a considerably lower chance of developing HCC (P = 0.204) (at 95% CI, OR 1.671 (0.756 – 3.694) and by multivariate analysis, microRNA301 was highly risky (P< 0.001) at (95% CI) OR (2.294 (1.501-3.506)) compared to AFP which was (P = 0.002) at OR (1.036 (1.013-1.059)) (Table 4).

Table1

Comparison among the three studied groups according to different parameters:

	HCC(n=85)	HCV(n=82)	Control (n=80)	P	HCC vs HCV	HCC vsControl	HCV vsControl
Gender							
Male	51 (60%)	37 (45.1%)	40 (50%)	χ^2=3.858			
Female	34 (40%)	45 (54.9%)	40(50%)	(0.145)	>0.05	>0.05	>0.05
Age(years)							
Mean± SD.	53.67±7.62	51.56±8.99	52.17±13.81	F=0.908	>0.05	>0.05	>0.05
Median(Min.– Max.)	54 (23 – 70)	52(24–79)	60 (23 –68)	(0.405)			
HB							
Mean± SD.	12.52±2.15	14.02±1.65	13.22± 1.30	F=15.44*	*	*	*
Median (Min. – Max.)	13(8.1–15.4)	13.95(9.3–17.4)	13.15(9.6–16.1)	(<0.001*)	<0.001	0.028	0.011
Platelets							
Mean± SD.	140.9±77.06	230.3± 74.32	227.82 ± 46.36	H=70.36*	*	*	
Median (Min. – Max.)	119 (40– 365)	235(71 –438)	226(140–341)	(<0.001*)	<0.001	<0.001*	0.875
AST							
Mean± SD.	56.19± 46.15	45.82± 32.69	38.99±17.99	H=4.437	>0.05	>0.05	>0.05
Median (Min. – Max.)	40 (10–258)	34 (6 – 180)	35(12–95)	(0.109)			
ALT							
Mean± SD.	45.27± 36.49	50.02± 44.31	44.86±22.39	H=2.654	>0.05	>0.05	>0.05
Median (Min.– Max.)	35 (8 – 195)	32.50(12–257)	40(14–130)	(0.265)			
S.Albumin							
Mean± SD.	3.75±0.58	4.19± 0.29	4.37±0.35	F=45.43*	<0.001*	<0.001*	0.025*
Median (Min. – Max.)	3.7 (2.3 –4.8)	4.15(3.8–5)	4.3 (3.8–5.2)	(<0.001*)			
Bilirubin T							
Mean± SD.	1.02± 0.49	0.61±0.27	0.93±0.2	H=70.79*	*		*
Median (Min. – Max.)	0.9 (0.2 – 2.5)	0.55(0.3 – 1.5)	1(0.5–1.6)	(<0.001*)	<0.001	0.403	<0.001
Bilirubin D							
Mean± SD.	0.4 ±0.34	0.23±0.14	0.45± 0.16	H=53.46*	*	*	*
Median (Min. – Max.)	0.2 (0.1 – 1.6)	0.2(0.1–0.9)	0.5(0.1–0.7)	(<0.001*)	<0.001	<0.001	<0.001
INR							
Mean± SD.	1.2 ±0.19	1.07±0.09	1.04± 0.06	F=36.03*	*	*	
Median(Min.– Max.)	1.1 (1–1.8)	1.03(1–1.39)	1(0.96–1.2)	(<0.001*)	<0.001	<0.001	0.174
AFP							
Mean± SD.	1385± 5066	9.81± 25.76	2.4±1.77	U=1072*	*	*	
Median (Min. – Max.)	61 (0.8– 26181)	3(0.9–182)	2(0.8-4.4)	(<0.001*)	<0.001	<0.001	< 0.01*
MicroR-301							
Mean± SD.	14.59±5.0	7.74 ±2.20	0.95±0.04	H=200.9*	*	*	*
Median(Min. – Max.)	13.51(6.35–24.8)	7.67(3.45–16.1)	0.96(0.89–1.04)	(<0.001*)	<0.001	<0.001	<0.001

SD: Standard deviation, U: Mann Whitney test χ^2: Chi square test,

H: H for Kruskal Wallis test, pairwise comparison bet. each 2 groups were done using Post Hoc Test (Dunn's for multiple comparisons test)

F:Ffor ANOVA test, pairwise comparison bet. each 2 groups were done using Post Hoc Test

p:pvalue for comparing between the three studied groups

*: Statistically significant at p≤ 0.05

Table 2

Comparison among the three studied groups according to TLR2 genotypes

TLR Genotypes	HCC (n =85)	HCV (n =82)	Control (n =80)	HCCvs. HCV	HCCvs. Control	HCV vs. Control
Genotype						
CC	32(37.6%)	18 (22%)	17(21.3%)			
CT	39(45.9%)	40(48.8%)	34(42.5%)	0.039*	0.007*	0.618
TT	14(16.5%)	24(29.3%)	29(36.3%)			
(CT+CC) vs TT	71/14	58/24	51/29	0.049*	0.004*	0.344
$^{HWE}\chi^2$	0.131	0.030	1.361			
p_0	0.717	0.862	0.243			
Allele						
C	84(49.4%)	76(46.3%)	68 (42.5%)	0.574	0.208	0.487
T	86(50.6%)	88(53.7%)	92(57.5%)			

HWE: Chi square for goodness (If P < 0.05 - not consistent with HWE)
p_0 :p value for HWE
P :p value for Ch isquare test for comparing between each two groups
*: Statistically significantatp ≤0.05

Table(3a)

Relation between TLR genotypes, microRNA301 and different parameters in HCC group (n=85)

	TLRgenotypes			microRNA301		
	CC(n=32)	CT(n= 39)	TT(n= 14)	N	Mean± SD.	Median (Min.–Max.)
Barcelona class						
A	9(28.1%)	13(33.3%)	3(21.4%)	25	11.65± 2.96	11.91(6.35–16.42)
B	16(50.0%)	19(48.7%)	7(50.0%)	42	15.59± 4.42	15.61(9.64–24.79)
C	6(18.8%)	4(10.3%)	2(14.3%)	12	19.04± 5.38	21.13(7.99–24.78)
D	0(0.0%)	1(2.6%)	0(0.0%)	1#	22.97#	
O	1(3.1%)	2(5.1%)	2(14.3%)	5	8.55±2.26	7.89(6.53– 12.32)
Test of Sig.(p)	χ^2=5.029,MCp=0.812				H=26.023*,p<0.001*	
Child Class						
A5	14(43.8%)	16(41.0%)	3(21.4%)	33	13.96± 4.57	12.90(6.53–22.98)
A6	10(31.3%)	14(35.9%)	5(35.7%)	29	14.0± 5.16	12.54(6.35–24.55)
B7	8(25.0%)	4(10.3%)	5(35.7%)	17	17.57± 5.14	17.87(10.22– 24.79)
B8	0(0.0%)	4(10.3%)	1(7.1%)	5	12.86± 3.79	14.10(7.99–17.77)
B9	0(0.0%)	1(2.6%)	0(0.0%)	1#	10.45#	
Test of Sig.(p)	χ^2=10.343,MCp=0.193				H=6.499,p=0.090	
T stage						
T2	0(0.0%)	0(0.0%)	3(21.4%)	3	11.14± 6.84	7.89(6.53– 18.99)
T3	5(15.6%)	15(38.5%)	1(7.1%)	21	11.05± 4.16	10.45(6.35–24.79)
T4	27(84.4%)	24(61.5%)	10(71.4%)	61	15.98± 4.55	16.11(9.64–24.78)
Test of Sig.(p)	χ^2=14.945*,MCp=0.001*				H=18.162*(<0.001*)	
N stage						
N0	15(46.9%)	31(79.5%)	9(64.3%)	55	11.51± 2.70	11.22(6.35–17.77)
N1	11(34.4%)	4(10.3%)	3(21.4%)	18	19.21± 2.59	18.56(15.39– 24.79)
N2	6(18.8%)	4(10.3%)	2(14.3%)	12	21.76± 2.55	22.55(17.07– 24.78)
Test of Sig.(p)	χ^2=8.671,MCp=0.056				H=56.214*(<0.001*)	
M stage						
M0	23(71.9%)	35(89.7%)	11(78.6%)	69	12.77± 3.52	12.33(6.35–19.98)
M1	9(28.1%)	4(10.3%)	3(21.4%)	16	22.44± 1.73	22.81(18.99– 24.79)
Test of Sig.(p)	χ^2=3.747,p=0.154				U=3.0* (<0.001*)	

SD: Standard deviation U: Mann Whitney test H: H for Kruskal Wallis test
χ^2:Chi squaretest MC:Monte Carlo
p:pvalue for comparing between the different categories
#: Excluded from the comparison due to small number of case (n=1)
*: Statistically significantat p ≤ 0.05

Table (3b)

Correlation between MicroRNA301and tumor size in HCC group (n=85)

	r_s	P
microRNA301 vs. tumor size (mm)	0.608	<0.001*

r_s:Spearman coefficient

*: Statistically significant at $p \leq 0.05$

Table (3c)

Correlation between microRNA301, TLR genotypes in HCC group (n =85)

	TLRgenotypes			H	p
	CC (n=32)	CT (n= 39)	TT (n= 14)		
microRNA301					
Mean ±SD.	16.45 ± 5.39	13.24± 4.11	14.09 ± 5.36	6.982*	0.030*
Median (Min.–Max.)	16.63(6.87–24.79)	12.33(7.08–22.98)	13.50(6.35–22.99)		
Sig.bet.genotypes					p_1=0.009*
					p_2=0.189
					p_3=0.508

SD: Standard deviation

H: H for Kruskal Wallis test, pairwise comparison bet.each 2 groups were done using Post Hoc Test (Dunn's for multiple comparisons test)

p: pvalue for comparing between the different categories

p_1:pvalue for comparing CC and CT

p_2: p value for comparing CC and TT

p_3: p value for comparing CT and TT

*: Statistically significant at $p \leq 0.05$

Table (4)

Univariate and multivariate logistic regression analysis for the parameters affecting HCC (n=85) vs.HCV (n=82)

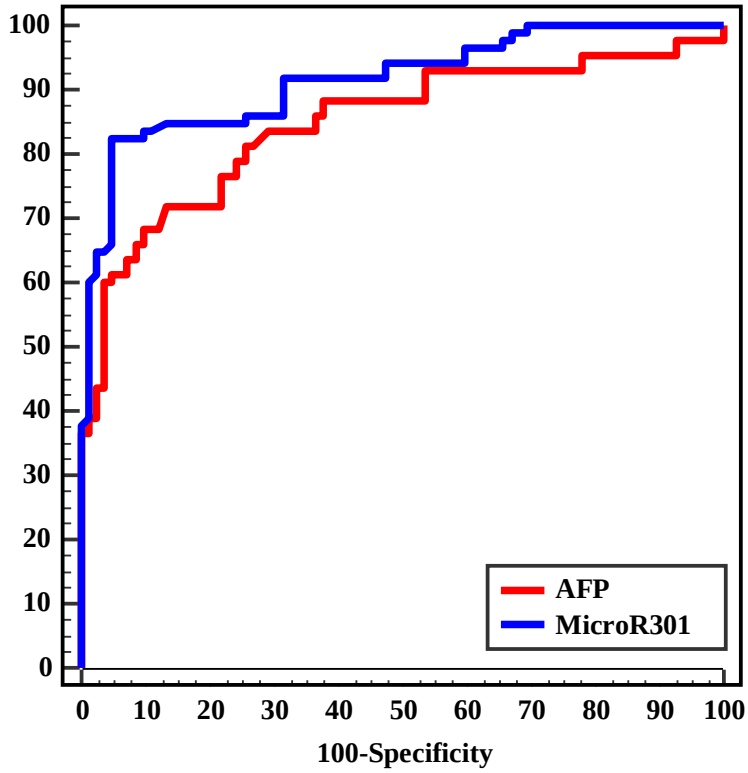
	Univariate		#Multivariate	
	Pvalue	OR (95% CI)	Pvalue	OR (95% CI)
Male	0.055	1.824(0.987– 3.372)		
Age(years)	0.106	1.032(0.993– 1.071)		
HB	<0.001*	0.658(0.546–0.794)	0.010*	0.454(0.250– 0.825)
Platelets	<0.001*	0.985(0.980–0.990)	0.007*	0.975(0.957– 0.993)
AST	0.107	1.007(0.999– 1.015)		
ALT	0.449	0.997(0.989– 1.005)		
S.Albumin	<0.001*	0.111(0.047–0.261)	0.187	4.316(0.492– 37.843)
Bilirubin T	<0.001*	26.146 (7.575– 90.239)	0.200	19.017 (0.211– 1717.26)
Bilirubin D	<0.001*	21.003 (3.977– 110.930)	0.988	0.952(0.001– 653.896)
INR	<0.001*	788.17(42.584 – 14588)	0.642	0.217(0.000– 136.847)
AFP	<0.001*	1.029(1.013–1.045)	0.002*	1.036(1.013– 1.059)
MicroR-301	<0.001*	1.941(1.561–2.414)	<0.001*	2.294(1.501– 3.506)
TLR				
GenotypesTT®		1.000		
TC	0.204	1.671(0.756–3.694)	0.914	0.896(0.121– 6.611)
CC	0.013*	3.048(1.269–7.320)	0.965	0.949(0.095– 9.478)

OR: Odd`sratio

C.I: Confidence interval LL: Lower limit UL: Upper Limit

#: All variables with p<0.05 was included in the multivariate

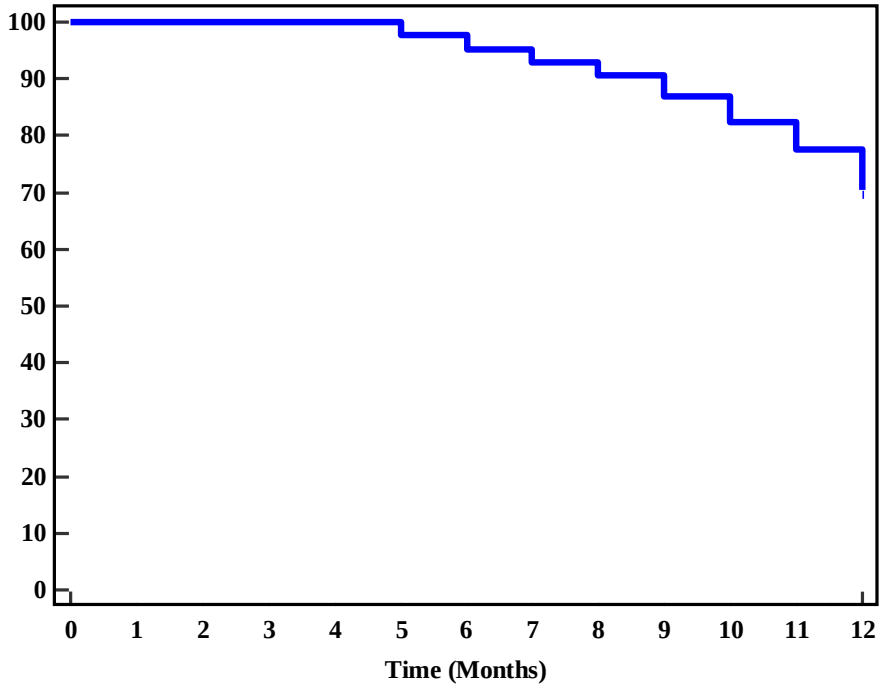
*: Statistically significantat $p \leq 0.05$



	AUC	P	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
AFP (ng/ml)	0.846	<0.001*	0.907–0.785	>40.1	60.0	96.34	94.4	69.9
miRNA301	0.914	<0.001*	0.958–0.871	>9.11	85.88	70.73	75.3	82.9

P value CI: Confidence Interval
NPV: Negative predictive value PPV: Positive predictive value
*: Statistically significantat $p \leq 0.05$

Figure1: ROC curve for AFP and microRNA30 1to discriminate HCC (n=85) from HCV patients (n=82)



	Mean (months)	% Study End
Overall Survival	11.24	70.6

Figure 2: Kaplan-Meier survival curve in HCC

4. Discussion

Since about a quarter of CHC patients proceed to liver cirrhosis and nearly 3% of HCV cirrhotic patients acquire HCC each year, CHC is a potentially fatal condition **[19].** In the etiology of hepatic disease and the infection with HCV, TLR genotyping is crucial. Hepatitis C infection risk factors and its complications may be clarified by SNP polymorphisms **[20].**

Immune system control, the prevention of infectious disease, and the treatment of autoimmune diseases all depend on TLR2. The malfunctioning of this receptor can have a variety of outcomes, despite conflicting reports regarding the clinical relevance having been published, it's obvious that it is highly related to infection, inflammation and carcinogenesis **[20–21]**, also, it is obvious that MicroRNA301 has significant biological effects in many inflammatory and infectious diseases as well as cancer including HCC **[14, 22]**. To better understand how the clinical outcome of hepatitis C and the development of HCC are related to the TLR2 gene SNP (rs3804099) and miRNA3013 expression, this study was carried out.

According to this research, a positive connection between TLR2 SNP (rs3804099) and the risk of HCC has been found with its heterozygous genotypes. HCC was found to be considerably lower in those with the wild-type homozygous genotype (TT), when compared to heterozygous CT and mutant CC genotypes. These findings led to the hypothesis that TLR2 gene variation (TT) may have a significant protective role in the development of HCC.

According to these outcomes, **Junjie et al.** found that CC variant of TLR2 rs3804099 was three times more risky than other genotypes for developing HCC, CC had a considerably higher chance of developing HCC; CC was intimately linked to the disease. (OR 2.743, 95% CI 1.915–3.930, P= 0.000). TT genotype had a considerably lower chance of developing HCC (OR 0.524, 95% CI 0.394-0.697, P = 0.000) **[23]**.

Additionally, this SNP's genotypes have a significant impact on how susceptible HCC is to development **[23]**. Reduced TLR2 signaling may lessen the effectiveness of the immune response, which could result in increased viral copy number and incidence HCC **[24]**. Also **Xu et al**. declared that the risk of HCC connected to HCV was highly correlated with the rs3804099 C allele (P=0.013) **[20]**.

Similarly ,the association between HCC, HCV and TLR2 rs3804099 was examined by **Huo et al.** Significant differences between HCC and HCV were shown by CT variant (P=0.0001, OR=2.00) and allele T (P=0.019, OR=1.17). The results were in line with their earlier findings (P=0.001, OR=1.81; P = 0.014, OR=1.26; P=0.009, OR=1.33) **[25].** They discovered that rs3804099 CT genotype was substantially related with the occurrence of hepatocellular carcinoma and may alter the degree of TLR expression **[25].**

The risk of HCC connected to HCV was highly associated with C allele of rs3804099, as demonstrated by **Neamatallah etal**. **[26]**.

HCV selectively inhibits TLR signaling that controls HCV replication while activating TLR pathways that cause chronic inflammatory state results in permanent liver damage. HCV's inhibition of TLR signaling causes inflammation, and become chronic, all of which eventually led to fibrosis and cirrhosis. Additionally, activation of NS3 and the TLR2 HCVcore protein enhance liver inflammation and damage **[27].** It has been hypothesized that HCV inhibits the TLR signaling that controls HCV replication while concurrently promoting inflammatory and hepatotoxic pathways. The chronicity and viral dissemination of HCV are attributed to its induction of TLR signaling suppression [**26–27].** The interaction of ligands with TLR2 results in tumor activation and, in later stages, promotes the development of cancer **[28].**

The involvement of TLR2 rs3804099 in the development of cancer has been demonstrated by research. The following processes explain how it functions as a pro-oncogene: TLR2 expression is influenced by messenger RNA processing, stability, structure [**23].**

The current study clarified that the level of microRNA301 varied significantly among the various TLR2 genotypes (P=0.030), with the CC and CT genotypes showing the greatest variation (P< 0.009) (the riskier variants). TLR2 SNP variants may change the activation of transcription factors like NF-k B and the release of important inflammatory mediators and microRNA [29]. Growing data suggests that miR-301 plays a role as driving several human malignancies. The expression pattern, functional importance, and underlying molecular mechanism of miR-301 in HCC are promising fields of study. [16]

According to this study, plasma microRNA301 levels can distinguish between HCC patients and those with chronic HCV with 95% confidence interval of (0.87.1-0.96) was significant. (P<0.001).With a cut off value of >9.1, there is 85.88% sensitivity and 70.73% specificity and AUC 0.914.

Furthermore, MicroRNA 301 is up-regulated in HCC and HCV patients, with levels noticeably rising in late TNM stages and with bigger tumor sizes.

Similarly, **El-Hamouly** et al, demonstrated that miRNA301 concentrations could distinguish between HCC and chronic HCV with an (AUC) of 0.89 (95% CI 0.88-0.96). With a threshold value of 9.91, the sensitivity and specificity were 78.57% and 89.58%, respectively. **[30]**. Similar to this, **Zhou** et al research revealed that HCC patients had higher levels of miRNA301 expression than HCV patients did **[31]**. In addition, **Guo et al.** discovered that patients with HCC had higher microRNA301 levels (P=0.0038) **[32]**. A correlation between large-sized HCC, advanced tumor- node- metastasis (TNM) stages, and increased miRNA-301 expression was shown by Guo et al . **[32]**. According to **Dong et al. ,** results also confirmed that miR-301 was highly over expressed in HCC compared to normal hepatic tissues and explained that by microRNA301 stimulates cell death and prevents hepatocellular cancer cell proliferation **[33]**. MiRNA301 was significantly overexpressed in metastatic and large HCC **[34]**. Additionally, HCC patients with high miRNA301 levels had a worse prognosis and a higher mortality rate than the other group (P= 0.0039). MicroRNA-301 was considered as a potential prognostic HCC marker. **[34]**

In a study by **Hu et al**, they found that microRNA-301 was up regulated in HCC tissues and cell lines, and that high expression had a poor prognosis. They also indicated that miR-301 overexpression increased the capability of proliferation, invasion, in HCC cell lines, and conversely, decreasing its expression induced the reverse **[35].** This study showed that AFP at cut off value > 40.1 ng/dL, (95% CI 0.75 to 0.92) was significant (P<0.001), AUC was 0.846, the specificity was 96.34% and the sensitivity was 60%. These results are consistent with those of **El-Hamouly et al.,** who found that AFP had cut off level 29.25 ng/dL, (AUC) was 84%, sensitivity of 73.81%, and specificity of 85.42% **[30]**.

5. Conclusion

TLR2 SNP genotypes (CC and CT) may be considered risk factor for HCC development; where as circulating MicroRNA-301 might be considered as a promising non-invasive diagnostic and prognostic biomarker especially with risky TLR2 SNP variants. Additional research involving more diverse populations and larger sample size is still required.

6. Declaration of conflicting interests

The authors declared that no conflict of interest with respect to the research, authorship, and publication of this article

7. Formatting of funding sources

The authors declare that no funds or grants were received during the preparation of this manuscript.

8. Acknowledgment

The authors thank Clinical Biochemistry and Molecular Diagnostics department National Liver Institute, and all its members, for supporting this work.

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