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Vascular Invasion in Hepatocellular Carcinoma and Matrix Metalloproteinase-14 Gene Polymorphisms

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Abstract

Background*: Matrix metalloproteinase (MMP) 14 is a cell surface proteinase that considered a key protein of activity against extracellular matrix components and associated with tumor invasion, metastasis and angiogenesis. MMP14 is overexpressed in HCC, expression of MMP14 is correlated with advanced tumor stageas MMP14 gene is overexpressed in highly invasive hepatocellular carcinoma.The purpose of this study was to examine whether the MMP14 gene polymorphisms are associated with Vascular invasion.*

Methods*:A case control study was carried in Tropical Medicine Department in Zagazig University Hospitals and Medical Biochemistry Department Faculty of Medicine in Zagazig University, The study included 540 subject, classified into 3 groups HCC, Cirrhotic, control (180 in each group)MMP-14 gene polymorphism analysis in the promoter of the MMP-14 gene(+7096) was detected by polymerase chain reaction-restriction fragment length polymorphism*

Results*: It was found that the most frequent age category affected by HCC was 51–60 years, 83.3% were males, 88.9% of HCC were HCV,Vascular invasion was found in 44.4% of HCC,it was found that 86.2% of HCC patients with vascular invasion had TC genotype.*

Conclusion*:Our study results demonstrated that TC polymorphism of MMP 14 (+7096) is associated with increased risk of vascular invasionin HCC*

Keywords*: HCC- MMP14-vascular invasion*

I. Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths in the world, accounting for 9% of total deaths from cancer **(Akinyemiju *et al.*, 2017).**

HCC is one of the few human cancers with an identified etiological risk factor, chronic viral infections of hepatitis B virus (HBV) and hepatitis C virus (HCV) are well documented risk factors for HCC ,In Egypt with

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the significantly high HCV epidemiology, it is likely that HCC will continue to rise within the next few years. HCV rather than HBV was linked to the development of **(Abd-Elsalam *et al.*, 2018).**

Invasion and metastasis are characteristic features of HCC and the major cause of treatment failure in patients with HCC so despite improvements in treatment modalities during the past few decades, the prognosis of HCC is still very poor because of frequent intrahepatic metastasis and tumor recurrence **(Jing-Song Chen *et al.*, 2009).**

MMP-14 was the first characterized metalloproteinase in MMP family and naturally anchored to the cell membrane in an activated form. MMP-14, also known as the membrane-type MMP (MT1-MMP), have been shown to correlate with a variety of physiological functions and tumor-related behaviors such as migration, invasion, metastasis, basement membrane remodeling, and angiogenesis**(Kaimal *et al.*, 2013).**

Expression of various MMPs including MMP-14 were implicated in regulating HCC tumor progression , also MMP 14 overexpression is associated with increased tumor size and stage, extra-hepatic metastases, portal vein thrombosis, and decreased survival **(Liu *et al.*, 2014).** Also HCC cells migration and Invasion are inhibited by Targeting MMP14 **(Tao Li *et al.*, 2014).**

Genetic factors, including single nucleotide polymorphisms (SNPs) are important in mediating an individual's susceptibility to many types of cancer **(Shastry, 2006).**

Different studies was designed to examine the association of *MMP-14* gene polymorphisms with the clinicopathological development of different cancers including ovarian cancers**(Vos *et al.*, 2019).** and HCC**(Tzy-Yen Chen *et al.*, 2011).**

This study was done to assess frequency of MMP 14 gene polymorphism (+7096) in HCC patients with vascular invasion

II. Methods

This study was conducted in Tropical Medicine Department in Zagazig University Hospitals and Medical Biochemistry Department Faculty of Medicine in Zagazig University in the period between January 2019 and September 2019 with approval of ethical committee and ethical consent was taken from persons included in the study.

- Study design : case control
- Population : The study included 540 person, classified into 3 groups (180 in each group):
- **Group A:**

Included (180) patients with liver cirrhosis associated HCC (diagnosed by typical criteria of triphasic CT according to European Association for the Study of the Liver (EASL) with or without elevation of serum alpha feto protein (AFP) **(Easl and Eortc, 2012).**

- **Group B:**

Included (180) Cirrhotic patients with no evidence of hepatic focal lesions in ultrasound evaluation .The diagnosis of liver cirrhosis were established on the basis of clinical, laboratory and ultrasound analysis.

- **Group C:**

Included (180) healthy persons with normal liver functions, no evidence of cirrhosis and no hepatic focal lesion.

Inclusion criteria:

1. Adults > 18 years old.
2. patients with HCC, diagnosed by triphasic CT with or without elevated AFP included in group (A)
3. Cirrhotic patients with no evidence of hepatic focal masses in ultrasound included in group (B).
4. Healthy control in group (C).

Exclusion criteria:

1. Patients who had a history or recently diagnosed any other tumors.

All groups were subjected to the following:

1. **Careful history taking with special emphasis on:**

- Age, gender, and special habits of medical importance.
- Presence of predisposing factors for HCC as liver cirrhosis and viral hepatitis.
- The complaint, history of the present illness, and history suggestive of malignant or chronic liver disease.

2. **Complete general examination:**

Level of consciousness**:**Hepatic encephalopathy was graded according to West Haven Criteria

3. **Local examination:**

Full abdominal examination

Ascites was graded according to the grading proposed by the international ascites club

4. **Routine laboratory investigations**:

- Complete blood count ,Liver function test ,Coagulation profile ,Kidney function tests ,Viral markers and Alpha-feto protein (α-FP)

5. **Radiology include :**

Abdominal ultrasonography and Triphasic CT: For assessement of hepatic focal lesion, patency of portal vein and lymph node metastasis.

6. **MMP-14 gene polymorphism analysis in the promoter of the MMP-14 gene(+7096) was detected by polymerase chain reaction-restriction fragment length polymorphism(PCR-RFLP)**

A- Sampling :

Collection of blood samples was performed by taking 2ml of peripheral venous blood from each subject under complete aseptic conditions and collected with dipotassium EDTA (1mg/mL) for DNA extraction and analysis of gene polymorphism, all samples were frozen at-20°c until use.

B- DNA extraction :

1. About 400 µl of lysis solution and 20 µl of proteinase K solution were added to 200 µl of whole blood and mixed thoroughly by pipetting to obtain a uniform suspension.

2. The samples were incubated at 56 °c, while vortexing occasionally until the cells are completely lysed (10 min).

3. About 200 µl of ethanol (96-100%) were added and mixed by pipetting.

4. The prepared lysate was transferred to Gene JET Genomic DNA purification (thermo scientific, Lithuania) column inserted in a collection tube. The column was Centrifuged for 1 min at 6000 x g. Discard the collection tube containing the flow-through solution. Place the Gene JET Genomic DNA purification column into a new collection tube.

5. About 500 µl of wash buffer I were added (with ethanol added). Centrifuged for 1 min at 8000x g. the flow-through was discarded and the purification column was placed into collection tube.

6. About 500 µl of wash buffer II was added (with ethanol added) to Gene JET Genomic DNA purification column. Centrifuged for 3 min at maximum speed (≥ 12000 x g).
Optional. If residual solution is seen in the purification column, empty the collection tube and re-spin the column for 1 min at maximum speed.
Discard the collection tube containing flow-through solution and transfer the Gene JET Genomic DNA purification column to a sterile 1.5 ml micocentrifuge tube.

7. About 50 µl of Elution Buffer was added to the center of Gene JET Genomic DNA purification column membrane to elute genomic DNA. Incubated for 2 min at room temperature and centrifuged for 1 min at 8000x g.

8. The purification column discarded: the purified DNA was used immediately in downstream applications or store at -20 °c.

C- Polymerase Chain Reaction for amplification of MMP14 gene (442bps) :

The forward and reverse primers used for amplification of MMP14 gene were designed as 5`GTAGTCTACACCCACGCCTG-3´ and 5`-GAC AAACATCTCCCCTCGGA-3´ (for MMP14+7096, 442 bps).

The PCR was performed in a 20µL volume containing 5 µL DNA template, 10 µL of 2× PCR buffer (Invitrogen, Carlsbad, CA), 0.5 U of Taq DNA polymerase (Invitrogen, Carslbad, CA), and 1 µL of each primer

(MDBioInc, Taipei, Taiwan). The PCR cycling conditions were 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 64°C, and 2 min at 72°C, with a final step at 72°C for 20 min.

D- Digestion of MMP14 PCR product by *HphI*restriction enzyme *:*

Ten-microliter aliquot of PCR product was subjected to digestion at 37°C for 4 h in a 15μL reaction buffer containing 1.5 μ¨L of 10× buffer and 5 U of *HphI* (New England Biolabs, Beverly, MA).

Digested products were separated on a 2% agarose gel and then stained with ethidium bromide. For MMP14 +7096, T allele yielded 302- and 140-bp products, and C alleles yielded a 442-bp product.

III. Results

Table (1) Comparison of demographic data and medical history among the studied groups:

Variable	HCC group (N=180)		Cirrhotic group (N=180)		Control group (N=180)		F test	P value
Age: (years) Mean ± SD	55.8 ± 4.07		55.3 ± 5.68		45.8 ± 7.89		**15.4**	**<0.001 (HS)**
	No	**%**	**No**	**%**	**No**	**%**	**χ^2**	**P**
Sex:								
Male	150	83.3	120	66.7	120	66.7	1.66	0.436
Female	30	16.7	60	33.3	60	33.3		(NS)
Smoking:								
Absent:	80	44.4	80	44.4	90	50	0.149	0.928
Present:	100	55.6	100	55.6	90	50		(NS)
DM:								
Absent:	80	44.4	50	27.8	120	66.7	5.51	0.06
Present:	100	55.6	130	72.2	60	33.3		(NS)

F test: ANOVA. χ2: chi-square test

HS: highly significant difference (p<0.001). NS: non-significant difference (p>0.05).

This table shows that there was non-significant difference between the studied groups as regarding sex, however the difference between them was highly significant as regarding age as older age was found among those with HCC and cirrhosis.

Table (2) Comparison of child class and ascites among hepatic studied groups:

Variable	HCC group (N=180)		Cirrhotic group (N=180)		χ^2	P value
	No	%	No	%		
Child class:						
A:	80	44.4	0	0	**36.24**	**<0.001**
B:	30	16.7	6o	33.3		**(HS)**
C:	70	38.9	12o	66.7		
Cause of cirrhosis:						
HCV:						
HBV:	160	88.9	150	83.3	0.630	0.500
	20	11.1	30	16.7		(NS)

χ2: Chi-square test. HS: highly significant difference (p<0.001).

This table shows that there were highly statistically significant differences between the studied groups as regarding child score.

Table (3) Radiological characters among HCC studied group:

Variable	HCC group (N=180)	
	No	%
Focal lesions number:		
1:	80	44.4
2-3:	20	11.1

>3:	80	44.4
Size of focal lesion:		
<3 cm:	50	27.8
3-5 cm:	50	27.8
>5 cm:	80	44.4
Metastatic lesions:		
Absent:	140	77.8
Present:	40	22.2
vascular invasion:		
Absent:	100	55.6
Present:	80	44.4
Site of focal lesions:		
Left lobe:	20	11.1
Right lobe:	100	55.6
Both lobes:	60	33.3

Table (4) Studying of genotype and alleles in between the control and HCC groups:

Variable	**Control group (n=180)**		**HCC group (n=180)**		**OR (95% CI)**	**P**
	No.	**%**	**No.**	**%**		
Genotype:						
TT:	60	33.3	30	16.7	4.5 (0.37-54.1)	**0.007**
TC:	30	16.7	140	77.8	**42 (3.76-469)**	**(S)**
CC:	90	50	10	5.6	Reference	

Alleles:						
T:	150	41.7	200	55.6	1.75 (0.68-4.45)	0.239
C:	210	58.3	160	44.4	Reference	(NS)

OR: odds ratio. CI: confidence interval.

This table shows that there was statistically significant difference between HCC patients and the control group as regarding genotypes.

It was noticed that TC genotype was found to be significantly higher among HCC patients when compared to the control group (77.8% versus 16.7% respectively).

Having TC genotype has almost a higher risk forty-two times (OR=42) than having CC genotype to be HCC patient.

Table (5) Studying of* vascular invasion*in different genotypes among the HCC patient's group:

Variable	**TT group (n=30)**		**TC group (n=140)**		**CC group (n=10)**		χ^2	**P**
	No	**%**	**No**	**%**	**No**	**%**		
Vascular invasion :							**7.63**	
Absent:	20	66.7	71	50.7	9	90		**0.022**
Present:	10	33.3	69	49.3	1	10		**(S)**

This table shows that there was statistically significant difference between different genotypes regarding vascular invasion

IV. Discussion

Despite chronic infection with HBV or HCV are the most well established risk factors for HCC during their life time only a fraction of infected patients develop HCC and that suggest gene polymorphism and protein expression plays a key role in determining susceptibility and occurrence of HCC numerous candidate gene studies have reported associations between SNPs and the presence of HCC**(Nahon and Zucman-Rossi, 2012).**

Matrix metalloproteinase (MMP)-14 is one of the pericellular collagenases to degrade extracellular matrix (ECM), which is involved to the modulation of susceptibility or clinicopathological features of cancer. The contributions of MMP-14 on the susceptibility or clinicopathological features in HCC have been well

documented, and the expression of MMP-14 in HCC also has been observed. Different studies was designed to examine the association of *MMP-14* gene polymorphisms with the susceptibility and clinicopathological development of HCC **(Tzy-Yen Chen *et al.*, 2011).**

Age is a surrogate of duration of infection in many patients thereby predicting a risk of HCC. In our study age was high significant between 3 groups These results in agreement with **Shaker *et al.* (2013)**who found that the most frequent age category affected by HCC was 51–60 years, but disagree with**El-Serag, (2007)**who reported the distribution of patients with HCC has shifted towards relatively younger ages, this can be explained by the HCV epidemic in Egypt in contrast with NAFLD in USA.

In every geographical area, men are more affected by HCC than women, due to the differences in the exposure to environmental factors known to be carcinogenic to the liver **(Venook *et al.*, 2010).**

There was no significant difference in gender between 3 groups. These results were in accord with **Khorshed *et al.* (2015)** who found that there was no significant difference between study groups as regard the gender, The present study showed that male-to-female ratio of 5 : 1, previous studies by **Nordenstedt *et al.* (2010)**show male to female ratios usually averaging between 2:1 and 4:1 .

Our study show highly statistically significant differences between the studied groups as regarding child score most of HCC group was Child A followed by child C then B this result match with **(Nafee *et al.*, 2012).**who found that most of HCC group was Child A, but disagreeing with the result of**(Ziada *et al.*, 2016).** who reported that 91.3% of HCC cases were Child B or C.

In our study 88.9% of HCC patients were on top of HCV infection and 11.1% on top of HBV infection this result consistent with **(Holah *et al.*, 2015).**who reported that 91% of HCC patients are on top of HCV.

In our study 44.4% of patients have tumor size larger than 5 cm and the majority of tumor size was less than 5cm with 55.6% of the HCC group same result was reported by **(Abd-Elsalam *et al.*, 2018).**who found that 44.93% of patients presented with tumor size above 5 cm

In our study, right lobe was the site of HCC in 55.6 of cases, followed by bilobular in 33.3% and in left lobe in 11.1%. Similar results were obtained by **(El-Zayadi *et al.*, 2005).** , Also **(Morsy and Emi, 2018).**found that HCC most commonly involved the right lobe of liver (48%), followed by bilobular involvement (32%) and the left lobe was involved in (19%).

In our study vascular invasion was seen in 44.4% of cases near result by **(Nagaich *et al.*, 2016).** who reported in previous study that vascular invasion was seen in 38.7% cases, while **(Ghafar *et al.*, 2019).**reported that vascular invasion was seen in 13.7% only.

Globally the prognosis for patients with extra hepatic metastasis of HCC known to be poor, extra hepatic metastasis ranges between 15% and 42% **(Uka *et al.*, 2007).**

In our study metastatic lesion was reported in 22.2% of HCC patients , this result match with **(Elmoghazy *et al.*, 2019).**who report that 26% of the patients had extra hepatic metastasis.

Our study shows that TC genotype is significantly higher among HCC patients when compared to the control group ,Having TC genotype has almost a higher risk forty-two times (OR=42) than having CC genotype to develop HCC this also match with **(Tzy-Yen Chen *et al.*, 2011).**

Our study shows that there were statistically significant differences between different genotypes of HCC patients as regarding vascular invasion**.** vascular invasion was higher among HCC patients TC genotype when compared with TT and CC genotype. It is possible that the MMP (+7096) gene polymorphism play in susceptibility tovascular invasion in HCC patients

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