Matrix Metalloproteinase-9 Gene Polymorphism in Patients with Hepatocellular Carcinoma

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ABSTRACT

Background: The focus of the study was to investigate the gene polymorphism of MMP-9 in hepatocellular cancer patients. Hepatocellular carcinoma is one of the most prevalent cancers. Matrix metalloproteinases are enzymes that degrade extracellular matrix proteins and are essential for cell migration during cancer invasion. The frequency of MMP-9 1562 C/T (3918242) was determined in HCC patients and the level of MMP-9 protein and its variation in HCC progression were evaluated in this study. **Method:** The study was conducted in a cohort of 100 samples (50 patients with HCC with cirrhosis and 50 patients with hepatitis C virus infection as controls). MMP-9 1562 C/T was determined by PCR-RFLP and MMP-9 protein level were assessed by ELISA. **Result:** In HCC patients P = 0.001, indicated a significant difference in the TT genotype between the patient group when compared to HCV group and the T allele had a high statistical significance among cancer patients (p = 0.001). Comparing MMP-9 protein levels between the control group and cancer patients identified a significant variation in MMP-9 protein level (p = 0.02). **Conclusions**: According to the data, the development of hepatocellular carcinoma was positively correlated with MMP-9. Hepatocellular carcinoma prognosis and prediction may be greatly affected by MMP-9.

Key Words:

Hepatocellular carcinoma, Matrix metalloproteinase-9, Restriction Fragment Length Polymorphism

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1. INTRODUCTION

The most frequent liver tumor is Hepatocellular carcinoma (HCC), a form of cancer which is seen mainly in people with chronic liver disease or cirrhosis **[1].** One of the most frequent primary liver cancers and a major global cause of cancer-related deaths is HCC. In Egypt it considers the fourth

common cancer, increasing the incidence of complications of HCV consider the most important risk factor in progressing of HCC in Egypt **[2].**

HCC is one of the most frequent and severe cancers. HCC may not often have clear early symptoms and most patients are identified after they have progressed to an advanced stage. In the treatment of liver cancer, various therapy techniques have recently made some progress. However, due to the high recurrence and metastasis of HCC, the patient's prognosis remains unfavorable. As a result, developing reliable prognostic markers is critical to improving HCC patients' prognosis **[3].**

Matrix metalloproteinase-9 (MMP-9) is secreted as zymogens or inactive. When granulocytes differentiate in the bone marrow, MMP-9 is regularly produced. There are MMP inhibitors work by attaching to the zinc (Zn+2) ion at the catalytic site and blocking the action of matrix metalloproteinases (MMPs), which can be utilized as an anticancer drug [4]. Increased MMP-9 observed in the tumor portion around the capsule in patients with HCC. Tumor duration, capsule status, tumor stage and recurrence risk of HCC, all linked to MMP-9 gene polymorphism [5].

2.   MATERIALS AND METHODS Subjects

This study included 100 subjects would be classified into two groups, 50 patients they were diagnosed hepatocellular carcinoma with cirrhosis and 50 patients with HCV infection as control group considering the HCV as one of the most typical causes of HCC and identifying the genotypes in C virus patients is crucial. Between November 2019 and August 2020, patients from the Oncology Center El-Mansoura University were chosen.

Samples collection and analysis

Venous blood from patients was collected for analysis of gene polymorphism of MMP-9 1562 C/T (rs3918242) by PCR- RFLP method. Another sample of 5 venous blood was taken (2 ml for CBC and 3 ml for biochemical tests including liver functions Alanine aminotransferase (ALT)، Aspartate Aminotransferase (AST)، Total Bilirubin and Albumin, kidney functions (uric acid, and creatinine)، Alpha-Fetoprotein (AFP) and (HCV antibody). MMP-9 protein was done using enzyme-linked immune sorbent assay (ELISA) technique, kit supplied by Bioassay Technology Laboratory. Shanghai. China. (Cat. No: E0936Hu) using (ELISA plate analyzer, Rebonik, India, whole blood samples which were subjected to DNA extraction using QIAamp DNA Blood Mini Kit #51104 supplied by (Centurion, UK).

For amplification, reverse and forward primers were used:
Forward 5'-GCC TGG CAC ATA GTA GGC CC-3'
Reverse 5'-CTT CCT AGC CAG CCG GCA TC-3'

DNA has been replicated. Then the PCR protocol was run for MMP-9 polymorphism site at 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute, and 72°C for 5 minutes for final extension, then at 4°C. After that, a restriction enzyme (SphI) was used to digest the PCR result at 37°C for one hour. PCR products were run onto a gel. The existence of an allele-specific band was then visible on the gel as described by **Barakat et al., [13]**.

Statistical Analysis:

Statistical Analysis completed utilizing SPSS Version 22.0. Since none of the continuous variables in this study had a normal distribution, their values were all given as medians. We utilized non-parametric tests. Spearman correlation was assessed and coefficient. Receiver operating characteristic (ROC) curves, area under the curve (AUC), and severity was evaluated using adjusted odds ratios and 95% CI were calculated by odd ratio calculator. Chi-square ($\chi 2$) test and P-values to indicate a statistically significant result. The calculation of the Hardy-Weinberg equilibrium using **http://.oege.org/software/hwe-mr-calc.shtmlwebsite**.

3. RESULTS

Table 1 show the patient distribution, (39) men and (11) women cancer patients ranging in age from 45 to 71 years., there were 24 patients had a family history and 26 had none, positive HCV were in 30 patients and 20 were negative. The HCV control group were (23) men and (27) women, were ranging in age between 30 to 66 years.

Table2 showed no clear difference among disease and HCV samples in uric acid, albumin and platelets, but there was a significant difference in ALT, AST, T. Bilirubin (p =0.001), creatinine (p 0.03), (AFP) (p 0.001), WBCs (p 0.02) and hemoglobin (p 0.01), MMP-9 protein demonstrated a significant (p 0.02). **Fig. 1A** shows Total Bilirubin, Albumin, Uric acid, and Creatinine. **Fig. 1B** shows (AFP), (ALT), and (AST). **Fig. 1C** shows hematological data of White blood cells, Hemoglobin, and Platelets.

According to data in Fig. **2A**, the optimal serum MMP-9 cut-off value for patients was 869 ng/l, AUC=.789, with a 94% sensitivity and a 70% specificity. Using ROC curves, levels of the MMP-9 protein were determined in disease samples, according to **Fig. 2B** it was determined how efficient MMP-9 in serum is as a diagnostic marker. The MMP-9 protein level in the investigation revealed that it was directly related to AFP (p 0.001, r 0.444), T. Bilirubin (p 0.009, r 0.300), there is a high association between MMP-9 genotypes and levels of MMP-9 protein p 0.001 and r 0.417 as demonstrated in **Table 3**.

The genotypes of the MMP-9 demonstrated that the CC frequency in disease samples (8%) but in HCV group (24%) as shown in **Table 4** and **Fig. 3A**, TT homozygous genotype was more common in disease samples (19%) than in the HCV group (4%). CT in HCC samples (23%) compared to HCV group (22%). differences that are statistically significant among the disease group and the control group were detected based on the TT genotype, p 0.001. There are statistically strongly significant differences between the control group (n=30) and the cancer samples (n=61) for the T allele frequencies, p 0.001.

The genotype distribution of the variant was determined using Hardy-Weinberg equilibrium (HWE), which showed that there were no significant differences in cancer group, P 0.972. The HCV group showed no apparent differences (p 0.945). **Fig. 3B** presents the genotype models for MMP-9. Codominant model TT genotype of the MMP-9 gene demonstrated a major connection with severity of HCC, (OR) = 7.04, p 0.001. The codominant model CT genotype, (OR) = 1.08, p 0.84, and the codominant CC genotype OR = 0.20, p 0.001. This data is shown in **Table 5**. According to **Table 6**, dominant model (CT+TT), OR = 4.84, p 0.001. and TT recessive model OR = 4.29.

PCR-RFLP analysis showed the presence of TT homozygotes at (188 and 247 bp), CT heterozygotes at (435, 247, and 188 bp), and CC homozygotes at (435bp). A representative RFLP analysis of the MMP-9 SNPs variation is shown in **Figure 4 (A)** shows the digestion of 10 control samples; CC genotype is present in all of the ensuing samples (2, 3, 5, 7, and 10). Whereas lanes 1, 6, 8, and 9 show the CT genotype, lane 4 has the TT genotype. **Figure 4 (B)** shows the digestion of 10 HCC samples, lanes 2 and 7 show the mutant TT genotype, Lanes 4, 5, 6, 8, and 9 show the mutant CT genotype, and Lanes 1, 3, and 10 show the mutant CC genotype.

4. DISCUSSION

It is involved in processes as inflammation, apoptosis, and immunity. MMP-9 is localized on chromosome 20 q11.2-13.1. MMP-9 is a key player in the destruction of basement membrane and extracellular matrix, widely expressed in the tissues and cells of our body **[6].** Among other physiological and pathological processes, several gene polymorphism sites exist in MMP-9 **[7].**

Among them is the single nucleotide polymorphism 1562 C/T (rs 3918242). T allele owners had higher plasma protein levels and MMP-9 activity than C allele owners, which is associated with a higher probability of developing a number of disorders. Hence, it is possible to evaluate the efficacy and prognosis of clinically customized treatment and to evaluate it by employing the C/T polymorphism in MMP-9 as a potential biological detection marker. **[8].** MMP-9 gene is a major oncogene that promotes HCC development **[9].**

The study determined the behavior of the MMP-9 gene, which were supported by the suggested blood MMP-9 protein level. The findings of this investigation provided evidence that MMP-9 are markedly greater in HCC.

In this study, the median age (in years) was 61, the median values were ALT (37.5), AST (38.5), T. Bilirubin (0.9), Creatinine (0.9), Albumin (3.5), Uric acid (5.2), and AFP (162.5), and there were 39% male and 11% female cancer patients It was previously observed in a study by **El Samanoudy et al., [10]** revealed that the mean age was 52.6, the ratio of females and males was 21.2% and 78.8%, respectively. The mean values of ALT (40.3), AST (38.1), T. bilirubin (20.0), and AFP were reported (20.7).

Hou et al., [11] Demonstrated that the patients and control group differed significantly in terms of MMP-9 genotype TT (OR = 2.33, 95% confidence interval (CI) = 1.14-4.8) and a higher frequency of MMP-9 in the TT genotype and the T allele (P= 0.05).

According to **Wang et al.,[12]**, MMP-9 was strongly linked to HCC metastasis. **Ding et al.,[3]** indicated that MMP-9 was an independent marker for predicting HCC survival.

This study showed that there were significant variations for the TT genotype among the disease group and the HCV group (p 0.001). For the allelic frequencies of the T allele, there were statistically significant differences among control group (n=30) and cancer samples (n=61), p 0.001. The genotype distribution of the variables was used and the results revealed no significant variation in hepatocellular cancer (P 0.972). The MMP-9 dominant model (CT+TT) was statistically related to the hazard of hepatocellular carcinoma (OR) = 4.84, p = 0.001, and TT recessive model OR= 4.29, p =0.01.

According to this study there were statistically significant differences between the disease group and the HCV group for TT genotype (p = 0.001). There were statistically strongly significant differences between the control group (n=30) and the cancer patients (n=61) for the allelic frequencies of the T allele, p 0.001. Hardy-Weinberg equilibrium (HWE) was assessed on the genotype distribution of the variant, and the results showed no significant variation in hepatocellular carcinoma (P= 0.972). There was no apparent difference in HCV group (p=0.945), dominant model (CT+TT) of the MMP-9 was statistically related with risk of hepatocellular carcinoma (OR) = 4.84, p = 0.001, and TT recessive model OR= 4. 29, < 0.01 as reported in **Barakat et al.,[13]**, the frequency of mutant genotype TT was found to be greater in cancer group (35%), compared to the control group (17%). CT, which were shown to be higher (51%) than the control group (37%). The minor TT genotype reported a significant difference among HCC group and the control group (p= 0.004). There are statistically significant variations among the T allele frequencies of the cancer patients (n=121) and the control group (n=71) with high significance (p=0.001).

5. CONCLUSION

According to research, hepatocellular carcinoma risk may be correlated with the screening genotype of MMP-9. MMP-9 genotyping may be used as an indicator for how HCC may progress.
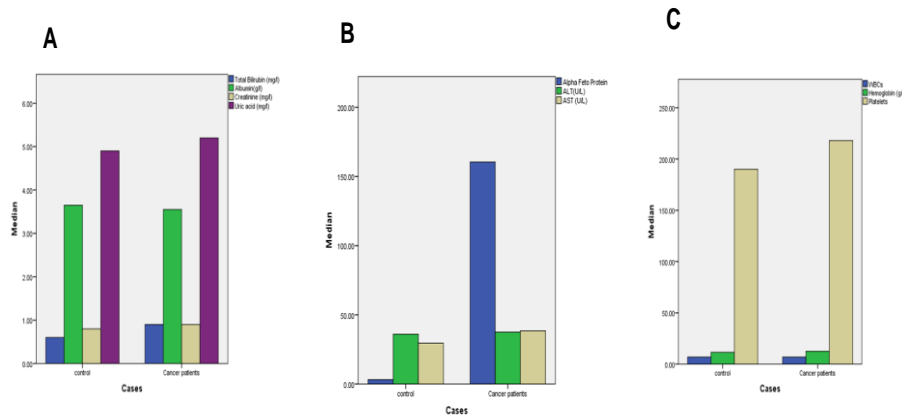

Figure 1. shows the percentage of various parameters in HCC patients compared to the HCV group (A) Total bilirubin, albumin, uric acid, and creatinine level. (B) Tumor marker AFP, ALT and AST activity. (C) Hematological analysis of CBC.
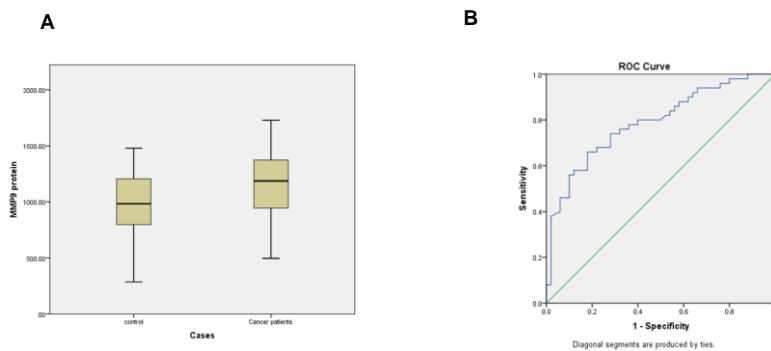

Figure 2. (A) A boxplot comparing the serum MMP-9 protein levels of disease patients and the HCV group. The interquartile range is illustrated by the box. (B) Hepatocellular carcinoma MMP-9 protein receiver operating characteristics curve (ROC).
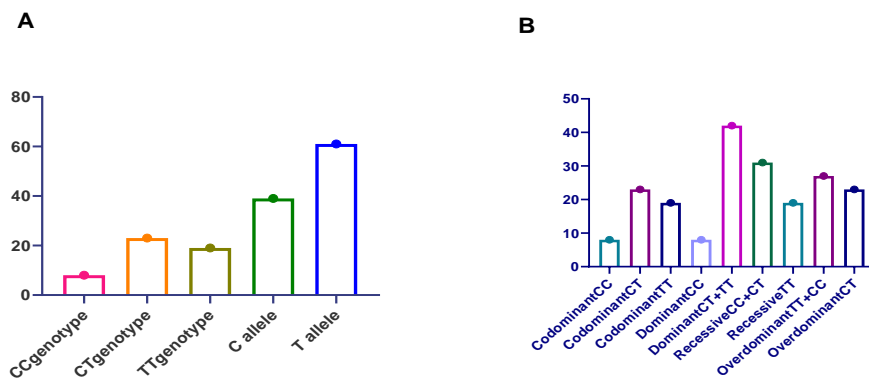
Figure3. (A) The frequency of genotypes and alleles (B) MMP-9 models in disease patients
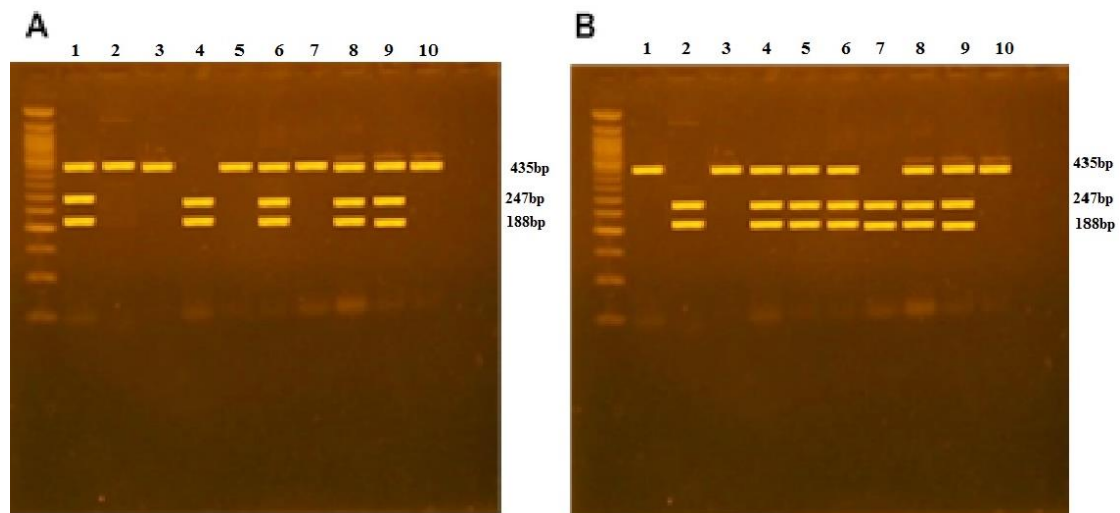
Figure 4. Micrograph represent RFLP study of the SNP variation. (A) The digestion of 10 HCV samples that were digested using the SphI restriction enzyme. The samples in lanes 2, 3, 5, 7, and 10 display the CC genotype, 4 displays the TT genotype. while lanes 1, 6, 8, and 9 displays the CT genotype. (B) Displays the digestion for 10 cancer samples, 1, 3, and 10 shows CC genotype. Lanes 2 and, 7 shows TT genotype. Ianes 4, 5, 6, 8, and 9 shows CT genotype.

Table1: Characteristics of the HCC patients and HCV control group

Clinical parameters	Cancer patients	Control group
Age Median (max- min)	**61 (75-40)**	**53(65-33)**
Sex		
Male	**39**	**23**
Female	**11**	**27**
Family history		
Absent	**26**	**-**
Present	**27**	**-**
Hepatitis C (HCV)		
Absent	**30**	**-**
present	**20**	**100**

The data are shown as the median (max-min)

Table2: The parameters of HCC patients compared HCV control group.

Tests	HCC patients (N=50) Median(max-min)	HCV group (N=50) Median (max-min)	p
ALT(U/L)	**37 (56-25)**	**36(57-19)**	**0.001***
AST(U/L)	**38 (60-20)**	**52(75- 33)**	**0.001***
T. Bil. (mg/l)	**0.9 (1.9-0.4)**	**0.6(1.3-0.3)**	**0.001***
Alb. (g/l)	**3.55(4.8-1.98)**	**3.65(4.9-2.6)**	**0.78**
Creat. (mg/l)	**0.9 (1.4-0.4)**	**0.8(1.40-0.10)**	**0.032***
Uric acid(mg/l)	**5.2 (8.5-2.3)**	**4.9(7.60-2.7)**	**0.214**
AFP (ng/ml)	**162.5(210- 87)**	**3(5.4-1.1)**	**0.001***
WBC (x10^3 / µL)	**7 (12-1.6)**	**6.9(10.60-1.9)**	**0.025***
Hemoglobin(g/l)	**12.5(15.40 -7.5)**	**11.6(14.6-5.2)**	**0.017***
Platelets (x10^3/ µL)	**218 (425-35)**	**191.5(286-16.78)**	**0.47**
MMP9(ng/dl)	**1728(1186-496)**	**983.5(1478-286)**	**0.02***

* Significant at P 0.05 when compared to the control.

Table3: Correlation MMP-9 protein marker, parameters and MMP-9 genotypes in HCC patients

parameters	MMP-9 protein
AFP	**r = 0.444, p 0.001**
T. Bil	**r = 0.300, p 0.009**
MMP-9 genotypes	**r = 0.417, p 0.01**

Spearman's rho and *P0.05, which indicate statistical significance, were used to perform correlations.

Table4: The percent of gene polymorphisms in MMP-9 in HCC patients Compared with HCV group

	Cases	frequency
HCV group	CC	24
	CT	22
	TT	4
	Total	50
Cancer patients	CC	8
	CT	23
	TT	19
	Total	50

Table5: The genotypic frequencies of MMP-9 gene polymorphism and MMP-9 for HCC compared with HCV group

Genetic polymorphism of MMP-9	HCC patients	HCV group	OR (95% CI)	P
CC	8	24	0.20 (0.0808 ,0.5271)	P = 0.001[*]
CT	23	22	1.0842 (0.4930, 2.384)	P = 0.840
TT	19	4	7.0484 (2.1865,22.7208)	P = 0.001[*]
HWE	$X2=0.056$ $P=0.972$	$X2=0.113$ $P=0.945$		
Allelic frequency	N (%) 100	N (%) 100		
C	39	70	0.274 (0.152, 0.492)	< 0.001[*]
T	61	30		

Comparison of HCC patients vs control group was performed by *P < 0.05 is statistically significant; CI: confidence interval; OR: odd ratio and *X2*: chi square.

Table6: The distribution of genotypes and models of MMP-9 for hepatocellular carcinoma compared with control group

Model	Genotypes of MMP-9	Cancer patients N (50)	Control group N (50)	OR	95% CI	P
codominant	CC	8	24	0.19	(0.096,0.038)	< 0.001[*]
	CT	23	22	1.77	(1.007,3.11)	0.04[*]
	TT	19	4	2.63	(1.353,5.107)	0.004[*]
Dominant	CC	8	24	0.20	(0.0808,0.527)	0.001[*]
	CT+TT	42	26	4.84	(1.897,12.378)	
Recessive	CC+CT	31	28	0.233	(0.070,0.7686)	0.01[*]
	TT	19	4	4.290	(1.30,14.14)	
Over dominant	TT+CC	27	28	0.922	(0.419,2.028)	.840
	CT	23	22	1.084	(0.493,.2.38)	

Comparison of HCC patients vs control group was performed by *P < 0.05 is statistically significant; CI: confidence interval; OR: odd ratio and *X2*.

6. Recommendations

- Investigation the functional impact of the identified variants on other gene variants that are linked to hepatocellular carcinoma.
- Analysis of association of other SNPs of MMP-9 genes with Hepatocellular carcinoma.

- The highest specificity and sensitivity in identifying the early stages of hepatocellular carcinoma are included in the screening and follow-up.

7. Conflict of interest

There are no competing interests.

8. Fund

No specific grant was given to this research by funding organizations in the public, private, or nonprofit sectors.

9. Data availability

The Mansoura Cancer Center and the Faculty of Medicine at Mansoura University produced the majority of the research data. Dr. Nancy Mahsoub can provide further information upon request.

10. References

[1] C. Gadiparthi, E. R. Yoo, V. S. Are, P. Charilaou, D. Kim, G. Cholankeril, C. Pitchumoni and A. Ahmed, "Hepatocellular carcinoma is leading in cancer-related disease burden among hospitalized baby boomers," *Annals of hepatology*, 18(5):679-84, Sep. 2019, https://doi.org/10.1016/j.aohep.2019.04.014.

[2] W.M. Rashed, M.A. Kandeil, M.O. Mahmoud and S. Ezzat, "Hepatocellular Carcinoma (HCC) in Egypt," A comprehensive overview. *Journal of the Egyptian National Cancer Institute*, 32(1):1-1, Dec. 2020, https://doi.org/10.1186/s43046-020-0016-x.

[3] H. Ding, H. Hu, F. Tian and H. Liang, "A dual immune signature of CD8+ T cells and MMP9 improves the survival of patients with hepatocellular carcinoma," *Bioscience reports*, 41(3), Mar. 2021, https://doi.org/10.1042/bsr20204219.

[4] S.A. Amin, N. Adhikari and T. Jha, "Is dual inhibition of metalloenzymes HDAC-8 and MMP-2 a potential pharmacological target to combat hematological malignancies," *Pharmacological Research*, 122:8-19, Aug. 2017, https://doi.org/10.1016/j.phrs.2017.05.002.

[5] M. Tahmasebi Birgani and V. Carloni," Tumor microenvironment, a paradigm in hepatocellular carcinoma progression and therapy," *International journal of molecular sciences*,18(2):405, Feb. 2017, https://doi.org/10.3390/ijms18020405.

[6] K. A. Szychowski, A. K. Wójtowicz and J. Gmiński, "Impact of elastin-derived peptide VGVAPG on matrix metalloprotease-2 and-9 and the tissue inhibitor of metalloproteinase-1, -2, -3 and-4 mRNA expression in mouse cortical glial cells in vitro," *Neurotoxicity research*, 35(1):100-10, Jan. 2019, https://doi.org/10.1007/s12640-018-9935-x.

[7] H. Huang, "Matrix metalloproteinase-9 (MMP-9) as a cancer biomarker and MMP-9 biosensors: recent advances," *Sensors* 27;18(10):3249, Sep. 2018, https://doi.org/10.3390/s18103249.

[8] T. Yang, S. Cheng, S. Li and Y. Ji, "Relationship between Matrix Metalloproteinase-9 1562C/T Polymorphism and Liver Diseases: A Meta-Analysis," (2021), https://doi.org/10.21203/rs.3.rs-255552/v1.

[9] L. Lu, Q Zhang, K. Wu, X. Chen, Y. Zheng, C. Zhu and J. Wu, "Hepatitis C virus NS3 protein enhances cancer cell invasion by activating matrix metalloproteinase-9 and cyclooxygenase-2 through ERK/p38/NF-κB signal cascade," *Cancer Letters*, 356(2):470-8, Jan. 2015, https://doi.org/10.1016/j.canlet.2014.09.027.

[10] A. El Samanoudy, R. Monir, A. Badawy, L. Ibrahim, K. Farag, S. El Baz, D. Alenizi and A. Alenezy, "Matrix metalloproteinase-9 gene polymorphism in hepatocellular carcinoma patients with hepatitis B and C viruses," *Genet Mol Res*, 13(3):8025-34, Sep. 2014, https://doi.org/10.4238/2014.september.29.15.

[11] Y. Hou, S. Wang, M. Gao, J. Chang, J. Sun, L. Qin, A. Li, F. Lv, J. Lou, Y. Zhang and Y. Zhao," Interferon-Induced Transmembrane Protein 3 Expression Upregulation Is Involved in Progression of Hepatocellular Carcinoma," *BioMed Research International*, 2021, Mar. 2021, https://doi.org/10.1155/2021/5612138.

[12] G. Wang, J. Liu, Y. Cai, J. Chen, W. Xie, X. Kong, W. Huang, H. Guo, X. Zhao, Y. Lu and L. Niu, "Loss of Barx1 promotes hepatocellular carcinoma metastasis through up-regulating MGAT5 and MMP9   expressionand indicates poor prognosis," *Oncotarget*, 8(42): 71867, Sep.2017, https://doi.org/10.18632/oncotarget.18288.

[13] L.A. Barakat, A.R. Elsergany, M.H. Ghattas, N. Mahsoub and R.M. Bondok, "Relationship between Interferon-Induced Transmembrane protein 3 and Matrix Metalloproteinase-9 gene polymorphisms in patients with Hepatocellular carcinoma", *Clinics and Research in Hepatology and Gastroenterology*, 102110, 2023, https://doi.org/10.1016/j.clinre.2023.102110