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Plasminogen activator inhibitor (*PAI-1*) gene polymorphism (4G/5G) and hepatocellular carcinoma in Egyptian patients

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Abstract

Background: Plasminogen activator inhibitor-1 (PAI-1), which is a part of urokinase plasminogen activation (uPA) system, had been reported to have a crucial role in the development of different types of cancers. The *PAI-1* gene, located on chromosome 7, contains nine exons and eight introns. This gene is highly polymorphic, and its most common polymorphism (4G/5G) affects PAI-1 biosynthesis and consequently its circulating level.

Aim: The current study investigated the distribution of genotypes and the allelic frequency of the PAI-1 4G/5G polymorphism in hepatocellular carcinoma (HCC) compared to chronic HCV patients living in Egypt. Additionally, the effect of the *PAI-1* 4G/5G polymorphism on serum PAI-1 levels was assessed.

Methods: The study was carried on 50 HCC and 47 chronic HCV patients using real-time polymerase chain reaction.

Results: The genotypic distributions of the 4G/5G polymorphism (5G/5G, 4G/4G, 4G/5G, and 4G/4G + 4G/5G) and the frequency of alleles (5G and 4G) were not statistically significantly different between both study groups ($p > 0.05$). In addition, serum levels of PAI-1did not show any significant difference between HCC patients and HCV patients regarding all different genotypes of the 5G/4G polymorphism at $p > 0.05$ neither between the different genotypes of the 5G/4G polymorphism in the same group at $p > 0.05$.

Conclusion: Our study suggests that the *PAI-1* 4G/5G polymorphism may not be considered as one of the underlying genetic causes of hepatocarcinogenesis in chronically HCV-infected patients living in Egypt.

Keywords: Plasminogen activator inhibitor-1, Hepatocellular carcinoma, Genetic polymorphism

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and aggressive cancer all over the world as it has been the second cancer-related death worldwide (Suh et al., 2018). Human hepatocarcinogenesis is a multifactorial disease. Among the underlying steps involved in its pathogenesis are at least four molecular pathways that regulate either cell proliferation or cell death which include the irregular expression of β-catenin (resulting from β-catenin gene mutations) (Ozturk, 1999), upregulation of many growth factors (insulin-like growth factor (IGF), insulin receptor substrate 1, hepatocyte growth factor (HGF), and transforming growth factor β (TGF-β)) (Moradpour & Blum, 2005), and increased expression of angiogenic factors such as angiopoietin-2 (Mitsuhashi et al., 2003) and vascular endothelial growth factor (VEGF) (Mitsuhashi et al., 2003), in addition to several mutations affecting the transcription factors that control the cell cycle such as phospho-retinoblastoma (pRb), P53, and TGF-β mutations, leading to uncontrolled mitosis and cancer (Abdel-Hamid, 2009).

In Egypt, HCC had been reported to develop in about 4.7% of chronic liver disease (CLD) patients (El-Zayadi et al., 2001). The incidence of HCC is currently increasing, which highlights the relative importance of hepatitis

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C virus (HCV) and hepatitis B virus (HBV) infection as major risk factors (EL Zayadi et al., 2005) manifested as a doubling in the incidence rate in the past 10 years (Anwar et al., 2008). One of the major risk factors for the progression to liver cirrhosis and HCC is HBV infection (Ohata et al., 2004). The relative risk of developing HCC in HBV carriers may be 100–200-fold higher than that for non-carriers (Xiong et al., 2003).

In addition, the rising trend of HCC has been associated with increased prevalence of HCV infection (El-Serag, 2002). In Egypt, the prevalence of HCV infection among general population has been estimated to be around 14% (seropositivity) (EL Zayadi et al., 2005). HCV most probably has an indirect role in cancer development by inducing fibrosis and cirrhosis. On the other hand, since HCV is an RNA virus, and it is thought to be unable to integrate its genome into the host genome, it may play a direct role in hepatic carcinogenesis through the interaction between HCV proteins and host proteins (El-Nady et al., 2003). However, it seems that cirrhosis is the common pathway by which several risk factors exert their carcinogenic effect (Fattovich, 1998).

Multiple molecular epidemiological researches have been performed to study the association between the *PAI-1* polymorphisms and risks for different types of tumors, including breast cancer, colorectal carcinoma, endometrial cancer, ovarian cancer, oral cancer, and HCC, in diverse populations (Xu et al., 2012). In several tumor types including HCC, poor prognosis is associated with high levels of PAI-1 (Fox et al., 2001).

The *PAI-1* gene which encodes the PAI-1 protein which is considered as the main regulatory element in thrombolysis is located on 7q21.3–q22 and containing eight introns and nine exons (Ny et al., 1986). Evidences suggest the stunning role of the PAI-1 protein in the development of fibrosis through the disruption of the normal balance between the deposition of the extracellular matrix (ECM) by myofibroblasts and its clearance by matrix metalloproteinases (MMPs), hence accelerating the profibrotic process. In addition, the endless proliferation of myofibroblasts may occur in fibrotic states due to the lack of gene under expression caused by impeded control of different mediators such as the transforming growth factor beta 1 (TGF-β1) and connective tissue growth factor (CTGF) or epigenetic effects that may affect apoptosis (Eriksson et al., 2018).

Gene variability can influence the level of PAI-1. Among the variants of the *PAI-1* gene is the guanosine insertion/deletion (4G/5G) polymorphism which had been utterly involved in several studies. The 4G allele of 4G/5G insertion/deletion polymorphism which is present in the promoter region, 675 bp upstream from the transcription start sequence of the *PAI-1* gene, is responsible for high levels of plasma PAI-1 (Kohler & Grant, 2000). Several studies (Anvari et al., 2001; Grancha et al., 1999) had shown that the 4G allele has greater activity than the 5G allele and that higher frequencies of the 4G allele were associated with elevated plasma levels of PAI-1.

In the current study, we investigated the genotype distribution and the frequency of alleles of the *PAI-1* 4G/5G polymorphism in patients with HCC compared to HCV patients living in East of Egypt in addition to the effect of the *PAI-1* 4G/5G polymorphism on the circulating PAI-1 levels.

Methods

The study was carried on 50 HCC patients with chronic HCV infection and negative for HBsAg (36 males (76.6%) and 14 females (23.4%)) and 47 chronic HCV-infected patients (HBsAg negative) without development of HCC, which served as a control group for this study (36 males (72.0%) and 10 females (28.0%)). All subjects were recruited from Suez Canal University Hospital. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Sampling

Peripheral blood samples were from both groups. EDTA whole blood sample was used for DNA extraction for subsequent genetic investigations, and serum was used for assessment of serum PAI-1 level.

Genomic DNA extraction

Genomic DNA was extracted from the peripheral blood leucocytes of EDTA anticoagulant blood (Miller et al., 1988) using QIAamp DNA Blood Mini Kit (Cat # 51106; Qiagen, UK). DNA samples were quantitated at 260 nm using the NanoDrop® (ND)-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA).

Genotyping of *PAI-1* promoter 4G/5G polymorphism

TaqMan allelic discrimination systems were designed and used for genotyping of the *PAI-1* 675 4G /5G SNP using the following primers: (sense) 5′-TCT TTC CCT CAT CCC TGC C-3′ and (anti-sense) 5′-CCA ACC TCA GCC AGA CAA GG-3′. The probes for PAI-1-675 4G>5G were 4C-probe—(TET) ACACGGCTGACTCC CCACGT (TAMRA), and 5C-probe—(FAM) ACGGCT GACTCCCCCACGT (TAMRA).

PCR was performed in Rotor-Gene 6000 (Qiagen) and included 20–40 ng genomic DNA, 12.5 μL of ready for use master mix, ABsolute ™ QPCR Mix (Thermo Fisher Scientific, Inc, ABgene®, UK) 15 pmol of each primer, and 5 pmol of each probe in a final volume of 25 μL. Thermal cycling conditions were one initial hold (95 °C for 15 min) followed by a 40-cycle two-step program (95 °C for 15 s and 60 °C for 1 min). The fluorescent

contribution of each dye was determined directly after the completion of PCR. The ready for use master mix from absolute™ QPCR Mix (Thermo Fisher Scientific, Inc, ABgene®, UK) was composed of Taq DNA polymerase (recombianant) in reaction buffer (0.1 unit/μL), antibodies to Taq DNA Polymerase, concentration adjusted for the effective inhibition of DNA polymerase activity at 37 °C, 32 mM (NH4)2SO4, 130 mM MgCl2 TrisHCl, pH 8.8 at 25 °C, 0.02% Tween-20, 5.5 Mm MgCl2, and dNTPs (dATP, dCTP, dGTP, dTTP) 0.4 mM of each.

Non-template controls were included in each run. The results were represented by two curves as the yellow channel represents C probe labeled by (VIC) dye; this is the wild type. The green channel represents T probe labeled by (FAM) dye; this is the mutant allele. If the sample was positive to both FAM and VIC dyes, it is heterozygous for the C and T alleles (CT in tPA −7, 351C/T), while the sample that was positive to FAM dye is homozygous for the T allele (TT in tPA −7,351C/T). If the sample was positive to VIC dye, it is homozygous for the C allele (CC in tPA -7,351C/T). Each study participant is classified into one of the three possible genotypes: 4G/4G, 4G/5G, or 5G/5G.

Serum PAI-1 level

Serum PAI-1 level was measured using a commercial Elisa assay (ZYMUTEST PAI-1 Antigen # RK012A, HYPHEN BioMed, France) according to the manufacturer's instructions. A standard curve was drawn by plotting the mean absorbance value measured for each standard versus the corresponding concentration. The absorbance obtained for each sample was directly proportional to the quantity of PAI-1 present in the sample.

Statistical data analysis

Statistical data analysis was carried out using the "Statistical Package for the Social Sciences (SPSS) for windows" software version 22.0 (SPSS Armonk, NY: IBM Corp, USA). Data are presented as mean ± SD. The allelic frequencies and genotype distribution were estimated by gene counting. Differences between the means of the two continuous variables were evaluated by Student's *t* test. Differences between non-continuous variables, allele frequency, genotype distribution, and Hardy-Weinberg equilibrium were tested by χ^2 analysis and Fisher's exact test. The odds ratio (OR) for HCC and their 95% confidence interval (CI) associated with each minor allele were also calculated. Statistical significance was at $p < 0.05$.

Results

The main baseline characteristics of both study groups are shown in Table 1.

Table 1 General characteristics of HCC and HCV cases

		HCC patients	Control	*p* value
Age (years)	Mean ± SD	55.7 ± 6.7	42.6 ± 7.4	< 0.001*[a]
	Range	40–76	25–57	
Sex	Male	36 (76.6%)	36 (72.0%)	0.605[b]
	Female	11 (23.4%)	14 (28.0%)	

HCC hepatocellular carcinoma, *HCV* hepatitis C virus
[a]Student's *t* test
[b]Chi-square test
*Statistically significant at $p < 0.05$

Hemoglobin, platelet count, and serum albumin levels were significantly lower in the patient group than in the HCV group ($p < 0.05$; Table 2), while ALT, AST, total bilirubin, white blood cell count (WBC), prothrombin time (PT), and alpha fetoprotein (AFP) were significantly higher in the HCC group than in the chronic HCV group ($p > 0.05$; Table 2).

We determined the genotypic distribution of the *PAI-1* 4G/5G polymorphism in patients with HCC and HCV, and observed that their distributions in the study sample were consistent with those expected for samples in the Hardy-Weinberg equilibrium (the Hardy-Weinberg equilibrium for genotype frequencies in the patient group: chi-squared value = 1.347935668, chi-squared test *p* value = 0.245639; the Hardy-Weinberg equilibrium for genotype frequencies in the control group: chi-squared value = 0.02221172, chi-squared test *p* value = 0.881525).

Genotype distributions of the 4G/5G polymorphism (5G/5G, 4G/4G, 4G/5G, and 4G/4G + 4G/5G) and the frequency of alleles (5G and 4G) were not statistically significantly different between the HCC patient group and the HCV group (Table 3).

The mean (± SD) level of circulating PAI-1 was 25.64 ± 10.91 ng/mL in HCC patients (Table 4), with no statistically significant difference when compared to that of

Table 2 Biochemical parameters of HCC and HCV cases

Biochemical parameters	HCC patients	Control	*p* value
ALT (IU)	55.8 ± 49.4	50.4 ± 27.7	0.660
AST (IU)	106.4 ± 93.0	50.9 ± 26.3	< 0.001*
T. bilirubin (mg/dL)	4.5 ± 4.9	0.72 ± 0.4	< 0.001*
HB (g/dL)	9.7 ± 1.7	13.8 ± 1.4	< 0.001*
WBC ($\times 10^3$)	9.2 ± 5.4	6.5 ± 1.7	0.009*
Platelets ($\times 10^3$)	134.3 ± 99.5	242.5 ± 65.4	< 0.001*
PT (seconds)	23.0 ± 5.7	12.6 ± 0.8	< 0.001*
Albumin (g/dL)	2.2 ± 0.4	4.0 ± 0.6	< 0.001*
AFP (ng/mL)	1978.9 ± 3866.6	2.6 ± 2.7	< 0.001*

HCC hepatocellular carcinoma, *HCV* hepatitis C virus, *ALT* alanine transaminase, *AST* aspartate transaminase, *HB* hemoglobin, *WBC* white blood cell, *PT* prothrombin time, *AFT* alphafetoprotein
[a]Mann-Whitney test
*Statistically significant at $p < 0.05$

Table 3 The genotype distribution and the frequency of alleles of the PAI-1 4G/5G polymorphism in HCC and control groups

	HCC patients (n = 50)		Control (n = 47)		OR (95% confidence interval)	p value
	No.	%	No.	%		
Genotype						
5G/5G	12	24.0	12	25.5	1 (reference)	Reference
4G/4G	9	18.0	11	23.4	0.82 (0.25–2.69)	0.978
4G/5G	29	58.0	24	51.1	1.21 (0.46–3.17)	0.890
4G/4G + 4G/5G	38	76.0	35	74.5	1.08 (0.43–2.73)	0.952
Alleles						
5G	47	47	48	51.1	1 (reference)	
4G	53	53.0	46	48.9	1.17 (0.67–2.07)	0.673

Chi-square test, *OR* odds ratio
If $p < 0.05$ = not consistent with HWE

the HCV group (24.06 ± 10.99 ng/mL) at $p > 0.05$ (t test). Serum level of PAI-1 did not show any significant difference between HCC patients and HCV groups regarding all the different genotypes of the 5G/4G polymorphism at $p > 0.05$ (t test) (Fig. 1) neither between the different genotypes of the 5G/4G polymorphism in the same group at $p > 0.05$ (Kruskal-Wallis test).

Discussion

The promoter 4G/5G polymorphism is the most well-characterized *PAI-1* polymorphism, but the exact underlying mechanism encountered in the risk of cancer development is not yet well understood (Xu et al., 2012).

Both 4G and 5G alleles bind a transcriptional activator, but 5G binds in addition a repressor one, so the presence of the 4G/4G homozygotes increases the transcription and consequently increases the level of circulating PAI-1, while the 5G/5G homozygote is associated with low level of circulating PAI-1 (Eriksson, 1995).

In addition, 4G/5G polymorphism is located within the functional binding site for transcriptional factors mediating the action of two *PAI-1* expression inducers which are the transforming growth factor β (TGF-β) and tumor necrosis factor α (TNF-α) (Xu et al., 2012).

Table 4 Serum levels of PAI-1 in HCC patients and controls

Genotype	Serum PAI, ng/mL (mean ± SD)				p value[a]
	n	HCC patients	n	Control	
5G/5G	12	24.75 ± 11.53	12	20.17 ± 11.46	0.260
4G/4G	9	25.89 ± 11.83	11	22.09 ± 11.95	0.446
4G/5G	29	25.93 ± 10.92	24	26.92 ± 9.92	0.508
Total	50	25.64 ± 10.91	47	24.06 ± 10.99	0.480[c]
p value[b]		0.911		0.264	

PAI-1 platelet activating inhibitor-1
[a]Mann-Whitney test
[b]Kruskal-Wallis test
[c]Student's t test

In this study, we showed that there is no statistically significant difference regarding the distribution of genotypes between HCC patients compared to HCV patients, neither between the different genotypes of the 5G/4G polymorphism in the same group at $p > 0.05$. In addition, no statistically significant difference was found between these two groups regarding the allele frequency of the *PAI-1* 4G/5G polymorphism nor the serum level of the circulating PAI-1 protein.

Moreover, our results were consistent with those reported by El-EdeL et al. (2017) in a study conducted on 49 Egyptian HCC patients which clarified the distribution of the different genotypes of *PAI-1* 4G/5G polymorphism as 49% (4G/5G genotype), 34.7% (5G/5G), and 16.3% (4G/4G), denying the presence of an association between the PAI-1 4G/5G polymorphism and HCC risk, despite the presence of a significant link between 4G/5G genotype carriers (62.5%) and the rate of thrombosis among HCC patients (p = 0.04) (El-EdeL et al., 2017).

In contrast to our study, Divella et al. (2012) found, in a study done on the Italian population, that the frequency of the 4G/4G genotype (OR = 3.25; p = 0.02), the presence of the 4G allele (4G/4G and 5G/4G genotypes; OR = 1.97; p = 0.09), and the frequency of the 4G allele (OR = 2.16; p = 0.006) were statistically significantly higher in patients than in healthy controls. The frequency of the 4G allele was also statistically significantly higher in patients with HBV and HCV co-infection, than in those with no viral infection (alcoholic and cryptogenetic cirrhosis) and then those with HCV viral infection alone. The mean (± SD) level of circulating PAI-1 was 40.11 ± 26.56 ng/mL in all patients, which was statistically significantly higher when compared to that of the control group (5.75 ± 0.98 ng/mL) at $p < 0.001$ (t test). Furthermore, in HBV/HCV-co-infected patients, the level of PAI-1 was statistically significantly higher, compared to those of patients where there was

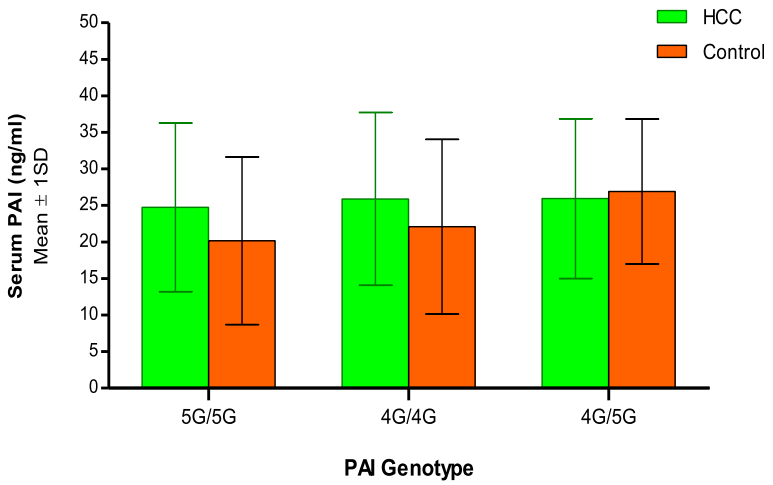

Fig. 1 The distribution of PAI-1 levels between patients, HCC, and control groups

no viral infection: 60.34 ± 26.9 ng/mL versus 28.12 ± 13.13 ng/mL, respectively ($p < 0.001$) (Divella et al., 2012).

Correspondingly, Espino et al. (2011) showed the absence of any differences which existed in the *PAI-1* 4G/5G genotype frequencies ($p = 0.12$), nor in PAI-1 plasma levels among obese patients with liver fibrosis (10.64 ± 4.35) versus those with no evidence of liver fibrosis (10.61 ± 5.2; $p = 0.985$) (Espino et al., 2011).

However, Weng et al. (2010) reported that the HCC risks of males and females with *PAI-1* 5G/5G genotype were 6.06-fold (95% CI = 1.39–26.36) and 0.04-fold (95% CI = 0.003–0.69), respectively, as compared with those with *PAI-1* 4G/4G genotype, in a study done on Taiwanese population (Weng et al., 2010).

However, a vast debate still exists regarding the expression of *PAI-1* gene as well as its role in HCC tumor development and progression; future researches are still under a compulsion taking into consideration the underlying ethnic backgrounds and/or the environmental as well as the genetic and the epigenetic factors of the studied population, the sample size, the different cancer staging, and the different recruitment strategies for control individuals.

Conclusion

Our data suggests that the *PAI-1* 4G/5G polymorphism may not be a part of the genetic makeup of the hepatocarcinogenesis process in the chronic HCV patients in eastern Egypt.

Abbreviations

CLD: Chronic liver disease; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HGF: Hepatocyte growth factor; PAI-1: Plasminogen activator inhibitor-1; pRb: Phospho-retinoblastoma; SNP: Single nucleotide polymorphism; TGF-β: Transforming growth factor β; VEGF: Vascular endothelial growth factor; VLDL: Very-low-density lipoprotein

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Authors' contributions

MM, RK, and DB carried out the molecular genetic studies and drafted the manuscript. MM, MA, and AH carried out the immunoassay. MT participated in the sequence alignment. NN, FA, MA, and MM participated in the design of the study and patient recruitment. RK, DB, FA, and AH performed the statistical analysis. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The study was approved by the Medical Ethics Committee, Faculty of Medicine, Suez Canal University. An informed written consent was obtained from study participants.

Consent for publication

A written informed consent for publication was obtained from all the participants in the study.

Competing interests

The authors declare that they have no competing interests.

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