Influence of Plasminogen Activator Inhibitor-1 (*SERPINE1*) 4G/5G Polymorphism on Circulating SERPINE-1 Antigen Expression in HCC Associated with Viral Infection

ROSA DIVELLA[1], ANTONIO MAZZOCCA[2], COSIMO GADALETA[3], GIOVANNI SIMONE[4], ANGELO PARADISO[5], MICHELE QUARANTA[1] and ANTONELLA DANIELE[1]

[1]Clinical Pathology Laboratory, Department of Experimental Oncology, Giovanni Paolo II National Cancer Institute, Bari, Italy;
[2]Department of Emergency and Organ Transplantation, Section of Internal Medicine, Allergology and Clinical Immunology, University of Bari Medical School, Bari, Italy;
[3]Unit of Interventional Radiology, Department of Critical Area and Surgery, Giovanni Paolo II National Cancer Institute, Bari, Italy;
[4]Department of Pathology Giovanni Paolo II National Cancer Institute, Bari, Italy;
[5]Scientific Directorate of National Cancer Institute Giovanni Paolo II, Bari, Italy

Abstract. *Hepatocarcinogenesis is heavily influenced by chronic hepatitis B (HBV) and C (HCV) infection. Elevated levels of plasminogen activator inhibitor-1 (SERPINE1/PAI-1) have been reported in patients with hepatocellular carcinoma (HCC) associated with viral infection. The gene encoding SERPINE1 is highly polymorphic and the frequently associated 4/5 guanosine (4G/5G) polymorphism in the gene promoter may influence its expression. Here, we investigated the distribution of genotypes and the frequency of alleles of the 4G/5G polymorphism in patients with HCC, the influence of the 4G/5G polymorphism on plasma SERPINE1 levels and its association with viral infection. A total of 75 patients with HCC were enrolled: 32 (42.6%) were HBV_+/HCV_+, 11 (14.6%) were only HCV_+, and 32 (42.6%) were negative for both viruses. A control group of healthy donors was also enrolled (n=50). SERPINE1 plasma concentrations were determined by ELISA and the detection of the promoter 4G/5G polymorphism was performed by an allele-specific PCR analysis. We found that the frequency of both the 4G/4G genotype (p=0.02) and the 4G allele (p=0.006) were significantly higher in patients with HCC compared to the control group, and particularly higher in patients with HCC co-infected with HBV_+/HCV_+ than in those with no viral infection. We also found that patients with the 4G/4G genotype had significantly higher plasma SERPINE1 protein levels when compared with patients with the 4G/5G or 5G/5G genotype (p<0.001). Differences in frequency of 4G allele and genetic variability of 4G/5G SERPINE1 polymorphism with a higher level of SERPINE1 protein in patients with HCC with HBV_+/HCV_+ than those without infection, suggest the presence of two distinct pathogenic mechanisms in hepatocarcinogenesis, depending on the etiology.*

Correspondence to: Rosa Divella, Ph.D., Department of Experimantal Oncology, Clinical Pathology Laboratory National Cancer Institute Giovanni Paolo II, Viale Orazio Flacco, 65, 70100, Bari, Italy. Tel/Fax: +39 805555292, e-mail: rosadive@inwind.it

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Hepatocarcinogenesis is a multistep process in which a series of molecular alterations are accumulated. Chromosomal gains and losses, epigenetic changes, genetic mutations in critical genes (*e.g. p53, Rb* and *β-catenin*) and alterations in gene expression, including up- or down-regulation of certain genes (*e.g. β-catenin, p16, p21* and *E-cadherin*), often affect genes involved in cell cycle control and tumor progression (1). Different risk factors, primarily viral hepatitis, are correlated with hepatocellular carcinoma (HCC), a disease that in most cases arises in a dysplastic cirrhotic liver associated with viral infection (2). Hepatitis B (HBV) and C virus (HCV) are implicated in the development of HCC through an indirect mechanism inducing chronic inflammation, necrosis and nodular hepatic regeneration, in which viral proteins are involved or in the case of HBV, by creating insertional mutations by viral DNA integration into the hepatocyte genome (3, 4). In several tumor types including HCC, elevated levels of plasminogen activator inhibitor-1 (serpin peptidase

inhibitor, type 1; SERPINE1) are associated with a poor prognosis (5). The SERPINE1 protein is a multifaceted proteolytic factor that not only functions as an inhibitor of the serine protease but also plays an important role in signal transduction, cell adhesion, and migration (6). High tissue levels of SERPINE1 have also been consistently reported to predict poor prognosis in several types of human cancers (7, 8). Lastly, elevated SERPINE1 is associated with tumor aggressiveness and poor patient outcome (9). Possible mechanisms by which SERPINE1 contributes to cancer dissemination include prevention of excessive degradation of the extracellular matrix, modulation of cell adhesion, and stimulation of angiogenesis and cell proliferation (10-13). *In vitro* studies have shown that SERPINE1 levels can be altered by cytokines, growth factors, and hormones, but the genetic and environmental determinants of SERPINE1 expression are not fully understood (14, 15). Gene variability may also contribute to the level of SERPINE1 biosynthesis (16). The human *SERPINE1* gene is located on chromosome 7. A guanosine insertion/deletion polymorphism in the promoter region of *SERPINE1* gene at the –675 bp position, named 4G/5G, has been described (17). Recent studies suggest that the protein encoded by the 4G allele has higher activity than the one of the 5G allele because the 5G allele contains an additional binding site for a DNA-binding protein that acts as a transcriptional repressor (18, 19). Studies carried out in different populations have consistently shown that individuals, homozygous for the 4G allele, have significantly higher plasma SERPINE1 levels than those homozygous for the 5G allele (20). In this study, we investigated the distribution of genotypes and the frequency of alleles of the 4G/5G polymorphism in patients with HCC, the influence of the SERPINE1 4G/5G polymorphism on plasma SERPINE1 levels and its association with viral infection.

Materials and Methods

Patients. From June 2007 to December 2010, 75 patients, 56 males (74%) and 19 females (26%), aged from 45 to 87 years (median, 73 years) with liver cancer were enrolled in this study, at the Giovanni Paolo II National Cancer Institute (NCI) of Bari, Italy. Among the 75 patients, 32 (42.6%) had HCC with both HBV- and HCV-positive antibody test, 11 (14.6%) tested positively only for the HCV virus, and 32 (42.6%) tested negatively for both viruses. In our series, HBV infection was always associated with the presence of HCV. A control group was enrolled among donors (n=50), which was found to be healthy from laboratory data and imaging techniques. Their median age was 50 years (range, 40-70 years). Five milliliters of peripheral blood were collected from each participant in a vacutainer system with lithium-heparin. Whole-blood samples were collected for DNA extraction from each participant before any invasive procedures or therapy. Plasma from both patients and healthy donors was immediately separated from the cellular fraction by centrifugation at 1,500 ×*g* for 10 min and was frozen at –20˚C. A written consent was obtained from all patients prior to enrolment in the study, and the Ethical Committee of the NCI approved the protocol which was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

SERPINE1 ELISA assay. Plasma SERPINE1 concentrations were determined with an ELISA assay method (Imunobind Plasma PAI-1 ELISA; American Diagnostica GmbH, Pfungstadt, Germany) according to the manufacturer's recommendations. The absorbance of the solution produced was measured at 490 nm. The absorbance is directly proportional to the amount of SERPINE1 present in the sample. A standard curve was constructed by plotting the mean absorbance value measured for each standard *versus* its corresponding concentration.

SERPINE1 promoter 4G/5G polymorphism. Genomic DNA was extracted from whole blood in EDTA using QIAamp DNA blood mini kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations. DNA was dissolved in (TE) buffer and was quantified by measurement of optical density at 260 nm. DNA amplification for molecular detection of *SERPINE1* promoter 4G/5G polymorphism was performed by an allele-specific (PCR) analysis using the following specific primers: insertion 5G allele 5'-GTC TGG ACA CGT GGG GG-3', deletion 4G allele 5'-GTC TGG ACA CGT GGG GA-3' each in a separate PCR reaction together with the common downstream primer 5'-TGC AGC CAG CCA CGT GAT TGT CTA G-3' and a control upstream primer 5'- AAG CTT TTA CCA TGC TAA CCC CTG GT-3' to verify the occurrence of DNA amplification in the absence of the allele on the genomic DNA. The PCR reaction was carried out in a final volume of 25 μl containing 10 ng of DNA, 13 pmol of specific primers, 1 mM dNTPs and 1 U *Taq* polymerase together with 2.5 μl of 10×*Taq* buffer. The PCR cycle conditions were 94˚C for 60 s, 54˚C for 30 s then 72˚C for 40 s, repeated for 35 cycles. The amplified DNA fragments were separated by a 5% polyacrylamide gel electrophoresis. Each study participant was classified into one of the three possible genotypes: 4G/4G, 4G/5G or 5G/5G.

Statistical analysis. The allelic and genotypic frequencies were estimated by the Chi-square test between patients and controls. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated from the logistic model. The differences between the means of the PAI-1 plasma levels of the patients group were determined using the Mann-Whitney *U*-test, the unpaired Student's *t*-test and ANOVA. *p*-Values <0.05 were considered to be statistically significant. All statistical analyses were performed by the Number Cruncher Statistical System-Power Analysis and Sample Size Software 2007 (NCSS-PASS, 329 North 1000 East Kaysville, Utah. USA).

Results

To study the distribution of genotypes and the frequency of alleles of the 4G/5G polymorphism in patients with HCC, we analyzed genomic DNA extracted from whole-blood in EDTA by allele-specific PCR analysis. We found that the frequency of the *SERPINE1* 4G/4G genotype (OR=3.25; p=0.02), the presence of the *SERPINE1* 4G allele (4G/4G and 5G/4G genotypes); OR=1.97; p=0.09) and the frequency

Table I. *(SERPINE1) 4G/5G polymorphism and allelic frequency in patients with hepatocellular carcinoma and in controls.*

	Patients (N=75)		Control group (N=50)		OR (95% CI)	*p*-Value
	N	Frequency	N	Frequency		
Genotype						
5G/5G	28	0.37	27	0.54	1 (reference)	0.04
4G/4G	27	0.36	8	0.16	3.25 (1.26-8.41)	
4G/5G	20	0.26	15	0.3	1.28 (0.55-3.07)	
Chi-square=6.299, *p*=0.043						
Genotype						
5G/5G	28	0.37	27	0.54	1 (reference)	0.09
4G/4G+4G/5G	47	0.62	23	0.46	1.97 (0.95-4.07)	
Chi-square=2.73, *p*=0.09						
Allele						
5G	76	0.58	69	0.69	1 (reference)	0.006
4G	74	0.41	31	0.31	2.16 (1.27-3.68)	

OR: Odds ratio; CI: confidence interval.

of the *SERPINE1* 4G allele (OR=2.16; p=0.006) were significantly higher in patients than in controls (Table I). The frequency of the SERPINE1 4G allele was also significantly higher in patients with HBV and HCV co-infection, than in those with no viral infection (alcoholic and cryptogenetic cirrhoses), and than those with HCV viral infection alone (Figure 1). Table II shows the distributions of allelic polymorphisms and allelic frequencies found in the three groups of patients in relation to viral presence. In particular, we observed a significantly higher frequency of the 4G/4G genotype (OR=84; p<0.001), an increased presence of 4G allele (OR=11.6; p=0.001) and a higher frequency of 4G allele (O.R.=15.4; p<0.001) in the group of patients co-infected with both viruses (N=32), when compared to patients negative for viral infection (N=32). The mean (±SD) level of circulating SERPINE1 was 40.11±26.56 ng/ml in all patients, 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 SERPINE1 were significantly higher, compared to those of patients where there was no viral infection: 60.34±26.9 ng/ml *versus* 28.12±13.13 ng/ml, respectively (p<0.001, ANOVA) (Figure 2). We also studied whether the *SERPINE1* 4G/5G polymorphism modulates plasma levels of SERPINE1. Patients with the 4G/4G genotype (N=32) had a significantly higher plasma level of SERPINE1 protein (62.4±25.4 ng/ml) when compared to patients (N=24) with the 4G/5G genotype (25.6±12.35 ng/ml), and to patients (N=19) with the 5G/5G genotype (30.9±15.3 ng/ml), at p<0.001 (ANOVA) (Figure 3).

Discussion

To our knowledge, for the first time, we studied the influence of *SERPINE1* 4G/5G polymorphism on the expression of plasma SERPINE1 protein, in patients with HCC with and without viral infection. In this study, we showed that the expression of circulating plasma SERPINE1 is higher in the patient compared to the control group, suggesting a direct role of this protein in liver carcinogenesis. In line with our findings, recent genomic profiling studies have revealed that many inflammation-related genes are involved in virus-related hepatocarcinogenesis hypothesizing the central role of inflammation in the development of HCC (21). HBV and HCV infection results in chronic inflammation within the liver and a high regenerative potential. Significantly, a high level of SERPINE1 was observed when viral co-infection, with both HBV and HCV were present. This confirms that the presence of viral infection increases the risk for HCC. In our previous study, we hypothesized that the increased level of SERPINE1 particularly in HCC patients with HBV_+/HCV_+ co-infection, could be due to: i) an increase of the release of SERPINE1 from hepatocytes; ii) a reduction of SERPINE1 clearance from the circulation; iii) tumoral production of SERPINE1 and a paracrine loop of SERPINE1 secreted by normal parenchyma; iv) a reduction of hepatic receptor activity for SERPINE1 in HCC-HCV-related tumors (22). Finally, the data reported in this study support the hypothesis that genetic variability may contribute to differences in the level of SERPINE1 biosynthesis. Both the frequency of the 4G allele and the 4G/4G *SERPINE1* genotype were found to be significantly higher in patients with

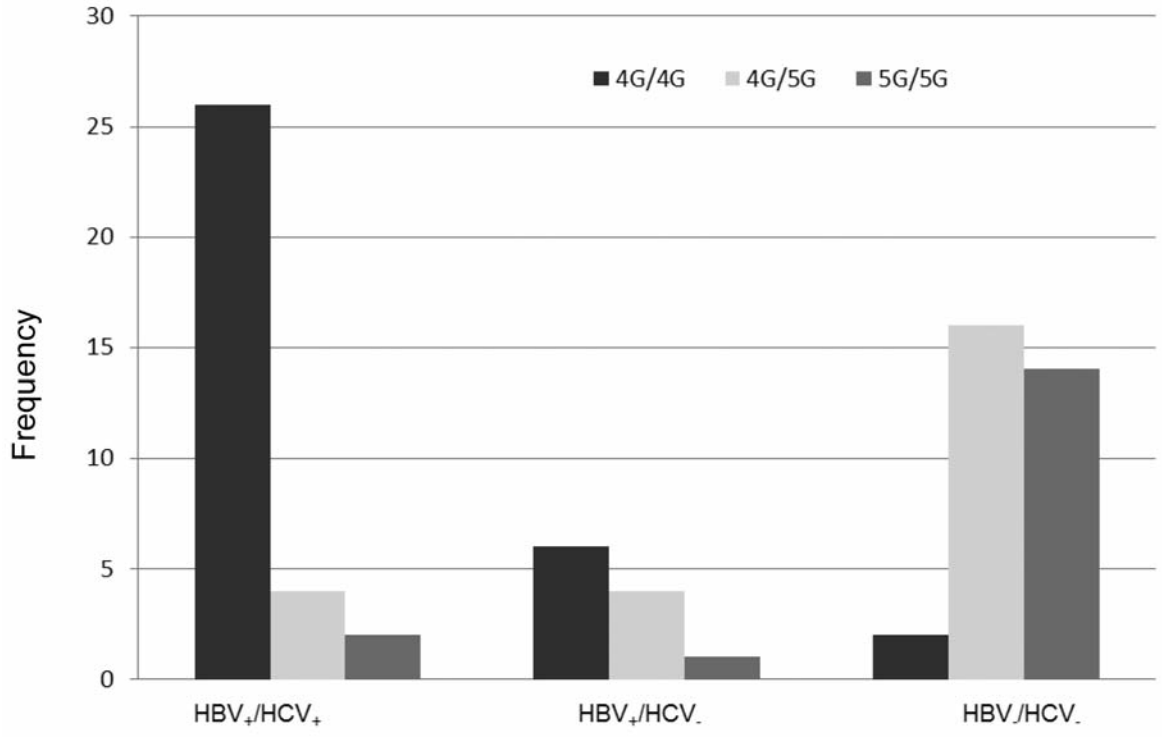

Figure 1. *Distribution of (SERPINE1) 4G/5G genotype in accordance to viral infection in patients with hepatocellular carcinoma.*

HCC than in controls. In addition, the frequency of the 4G allele and the 4G/4G *SERPINE1* genotype were significantly higher in patients with HCC associated with viral infection than in patients without viral infection. Studies carried out in different populations have consistently shown that individuals homozygous for the 4G allele have significantly higher plasma SERPINE1 levels than those homozygous for the 5G allele (23, 24). The 4G/5G polymorphism of the *SERPINE1* gene has been extensively studied for associations with cardiovascular disease, however, little research has been conducted regarding its association with cancer (25-26). Our hypothesis is that, as the presence of the 4G allele results in a higher *SERPINE1* transcription response to cytokines or growth factors than does the 5G allele, the 4G/5G polymorphism may influence circulating SERPINE1 protein levels in patients with HCC through the action of cytokines released by tumor cells. The co-presence of 4G allele and viral infection may also exert an unfavorable influence on tumor behavior. Furthermore, the most valuable perception from genome-wide expression profiles of HCC is that HCC represents several distinct subtypes of liver cancer defined by distinct gene expression profiles (27, 28). By using molecular biology techniques, Stahl and co-workers confirmed that HCC comprises of at least two subtypes, which may be distinguished by their expression of β-catenin (29). Similarly, HCC associated with chronic HBV or HCV infection

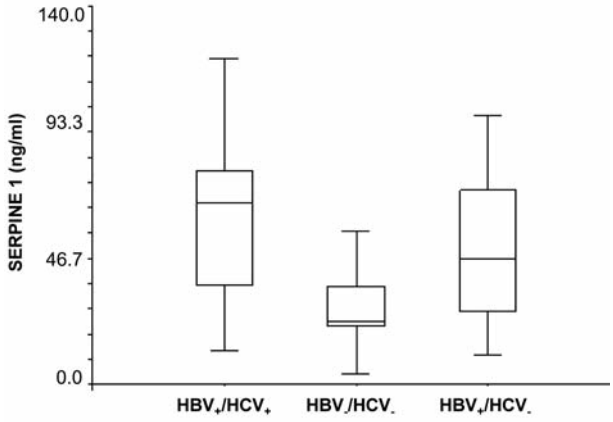

Figure 2. *Box-plot showing the distribution of plasma SERPINE1 levels in patients with hepatocellular carcinoma. The line within the rectangle (box) represents the median value while the whiskers represent the minimum and maximum value.*

is driven by different pathophysiological mechanisms (30). In this context, these conclusions are supported by our study, showing the difference in frequency of the 4G allele and genetic variability of 4G/5G *SERPINE1* polymorphism with a higher level of SERPINE1 protein in HCV/HBV-negative than

Table II. *(SERPINE1) 4G/5G polymorphism and allelic frequency in relation to viral infection.*

	HBV_+/HCV_+ (N=32)		HBV_+/HCV_- (N=11)		HBV_/HCV_ (N=32)		OR (95% CI) *vs*. HBV_-/HCV_+	*p*-Value	OR (95% CI) *vs*. HBV_/HCV_	*p*-Value
	N	Freq.	N	Freq.	N	Freq.				
Genotype										
5G/5G	2	0.06	1	0.09	14	0.43	1 (reference)		1 (reference)	
4G/4G	26	0.75	6	0.54	2	0.06	6 (0.8-44)	0.1	84 (10.6-664.2)	<0.001
4G/5G	4	0.12	4	0.36	16	0.5	1.5 (0.15-14.4)	0.8	1.75 (0.27-11)	0.8
Genotype										
5G/5G	2	0.06	1	0.09	14	0.43	1 (reference)		1 (reference)	
4G/4G+4G/5G	30	0.93	10	0.9	18	0.56	1.5 (0.1-18.3)	0.7	11.6 (2.3-57.3)	0.001
Allele										
5G	8	0.1	6	0.27	44	0.68	1 (reference)		1 (reference)	
4G	56	0.87	16	0.72	20	0.31	2.6 (0.79-8.67)	0.2	15.4 (6.1-38.2)	<0.001

HBV/HCV: Hepatitis B and hepatitis c virus; OR: odds ratio; CI: confidence interval.

HCV/HBV-positive patients with HCC. In conclusion, our data support the hypothesis that in the onset of HCC, there are two distinct pathogenetic mechanisms depending on the etiology involved, suggesting that the *SERPINE1* 4G/4G genotype may be associated with the risk of HCC in patients exposed to viral infection.

Competing Interests

The Author(s) declare that they have no competing interests.

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