PAI-1, t-PA and Circulating hTERT DNA as Related to Virus Infection in Liver Carcinogenesis

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Abstract. *Background: Liver carcinogenesis seems to be heavely influenced by hepatitis B and C viral (HBV, HCV) infection. The aim of our study was to improve the detection of hepatocellular carcinoma (HCC) by measuring alfa-fetoprotein (AFP) in addition to other molecular markers by estimating the plasma levels of human catalytic fraction of reverse telomerase (hTERT) DNA, plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA) in 75 patients with liver desease. Patients and Methods: A control group was enrolled (N=30). PAI-1 and t-PA levels were detected with enzyme-linked immunoassorbent assay (ELISA), DNA hTERT was performed with real time polymerase chain reaction (RT-PCR). Results: PAI-1, t-PA and hTERT DNA levels were much higher than in controls. PAI-1 and t-PA levels were higher in the presence of both viruses compared to their absence, $p<0.001$. Moreover, hTERT was significantly higher in the presence of both viruses, $p<0.05$ and in the presence of HCV alone, $p<0.05$. No decrease or increase of AFP was noted in these patients. Conclusion: Our data suggest the reliability of PAI-1, t-PA and hTERT in detecting HCC, in particular when the carcinogenesis is affected by virus infection.*

Hepatocellular carcinoma (HCC) is one of the human neoplasms etiologically linked to viral factors. Chronic infection with the hepatitis B virus (HBV) and the hepatitis C virus (HCV) has been implicated in about 80% of HCC cases wordwide (1, 2). HBV and HCV can be implicated in the development of HCC in an indirect way through induction of inflammation, necrosis and chronic hepatocellular regeneration, directly by means of viral proteins, or, in the case of HBV, by creating insertional mutations by integration into the genome of the hepatocyte (3, 4). A number of HBV integrations have been shown to occur into or adjacent to genes that have important roles in oncogenesis. These genes include the retinoic acid receptor beta, cyclin A2, SERCA 1, and nuclear matrix protein p84 (5-7). Interestingly, it was found that the HBV genome can be integrated into the *hTERT* promoter region also. The HBV enhancer sequence located *circa* 1.6 kb upstream of the *hTERT* transcription start site, was responsible for the activation of the *hTERT* trascription in HCC (8, 9). In contrast to HBV, HCV is an RNA virus that lacks a reverse-transcriptase enzyme and cannot integrate into the host genome. Thus the molecular pathogenetic mechanisms by which HCV contributes to cell transformation remain unclear (10, 11). In several tumor types, elevated levels of tissue plasminogen activator (t-PA) or its inhibitor plasminogen activator inhibitor-1 (PAI-1) are associated with poorer prognosis in different tumors including HCC (12, 13). Control of t-PA in the circulation is secured by two main mechanisms. Firstly t-PA is inhibited directly and, secondly, it is cleared from the circulation by the liver. In both processes PAI-1, has an important role. PAI-1 also participates in clearance of t-PA by the liver (14). The PAI-1 protein is a multifaceted proteolytic factor. It not only functions as an inhibitor of the protease t-PA, but also plays an important role in signal transduction, cell adherence and cell migration. Thus, an apparent paradox considering its name, although it inhibits t-PA during blood coagulation, it actually promotes invasion and metastasis (15). In many malignancies, including HCC, elevated PAI-1

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is associated with tumor aggressiveness and poor patient outcome (16). Although diagnostic modalities such as ultrasonography and conventional tumor markers are important for detection of HCC, they are not still sensitive enough to detect HCC at an early stage. The prognostic significance of circulating DNA in plasma or serum and its genetic alterations in HCC are other important trends (17, 18). The primary marker for HCC is alfa-fetoprotein (AFP), a single polypeptide chain glycoprotein. Generally AFP shows acceptable sensitivity; however, AFP is not secreted in all cases of HCC and may be at normal levels in as many as 40% of patients with early HCC (19, 20).

The aim of this study was to verify a possible role for the detection of HCC by measuring molecular markers involved in the process of cancer invasion and metastasis by estimating the plasma concentration of circulating *hTERT* DNA and PAI-1 and tPA in peripheral blood of HCC patients. In particular, the role of HCV and HBV infection was evaluated with relation to *hTERT* circulating DNA.

Patients and Methods

Patients. From June 2005 to December 2006, 75 patients, 56 male (74.66%) and 19 female (25.33%), aged from 45 to 87 years (median, 73 years) with liver disease were enrolled in this study at the Istituto Tumori Giovanni Paolo II of Bari. Among the 75 patients, 54 (72%) had hepatocellular carcinoma (HCC), 16 (21.3%) were with liver metastasis from other tumors and 5 (6.7%) had non-tumoral chronic liver disease, HCV+. In regard to the 54 HCC patients, 19 (35.18%) had antibody positivity for HCV and HBV, 8 (14.81%) were positive only for HCV virus and 27 (50%) were negative for both viruses. In our series, HBV infection was always associated with HCV presence. For all patients the clinicopathological characteristics (gender, age, etiology, underlying liver disease, total bilirubin, albumin, alanine, presence of metastasis) were evaluated. A control group found to be healthy from laboratory data and imaging techniques was enrolled among donors (n=30). Their median age was 50 years (range, 40-70 years). Five milliliters peripheral blood were collected from each participant in a vacutainer system with lithium-heparin. Plasma from both patients and healthy donors was immediately separated from the cellular fraction by centrifugation at 2,500 r.p.m. for 10 min at 4˚C and frozen at –80˚C.

PAI-1 and t-PA ELISA assay. PAI-1 and t-PA plasma concentration were determined with an ELISA assay (Imunobind Plasma PAI-1/t-PA 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 PAI-1/t-PA present in the sample. A standard curve was constructed by plotting the mean absorbance value measured for each standard *versus* its corresponding concentration. PAI-1 and t-PA plasma concentration of controls are those reported in the kit data-sheets.

Extraction of DNA from plasma and quantification of hTERT. DNA was extracted from 200 μl of plasma using commercial kits based on affinity columns (QIAamp Blood Mini Kit; Qiagen, Hilden, Germany) following the manufacturer's recommendations. The quantification of hTERT was performed using an RT-PCR (real-time polymerase chain reaction)-based method. The primers and the probes were designed by Applied Biosystems (Foster City, CA, USA) to specifically amplify the gene of interest, hTERT (The Quantifiler® Human DNA Quantification Kit). The Quantifiler® kit contains all the necessary reagents for the amplification, detection and quantification of a human-specific DNA target (*hTERT*, location 5p15.33, amplicon length 62 bases pairs): Quantifiler® Human Primer Mix, Quantifiler Human DNA Standard, Quantifiler PCR Reaction Mix (AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, and optimized buffer components). The reaction was carried out in 96-well plates with a total volume of 25 μl/well containing the following reagents: 12.5 μl of PCR Master Mix, 10.5 μl of Primer Mix and 2 μl of purified DNA from each sample. The reaction was carried out using the GeneAmp 7000 Sequence Detection System (Applied Biosystems) with the following conditions: the mixture of samples and reactants for the PCR reaction was pre-heated to 50˚C for 2 min and then at 95˚C for 15 s and 60˚C for 1 min. This was followed by 40 cycles of: 95˚C for 15 s and 60˚C for 1 min. A standard curve with Quantifiler® Human DNA Standard ranging from 0.023 ng/μl to 50 ng/μl (0.023, 0.068, 0.21, 0.62, 1.85, 5.56, 16.7, 50.0 ng/μl) was used for every plate. Every patient sample and DNA standard were carried in duplicate and only curves with a coefficient of correlation between 0.999 and 0.995 and a slope between 3.25 and 3.35 were accepted.

AFP assay. AFP was tested by using commercially available immunometric assay (Architect AFP assay, Abbot Laboratories, North Chicago, IL, USA). The cut-off value of AFP for HCC was set at 20 ng/ml, the most commonly set value.

Statistical analysis. Data were analysed by unpaired *t*-test, Mann-Whitney *U*-test and Kruskal-Wallis test, as well as analysis of variance (ANOVA) by SPSS software for window 9.0.1. Numerical data were expressed as mean±standard deviation (SD). A p-value$\leq$0.05 was considered statistically significant.

Table I. *Association between PAI, t-PA and hTERT plasma concentrations and clinical characteristics of patients.*

		PAI-1		t-PA		hTERT	
	N (%)	Mean±sd (ng/ml)	*P**	Mean±sd (ng/ml)	*P**	Mean±sd (ng/μl)	*P***
Control group	30	5.75±0.98[a]		4.2±9[a]		0.03±0.09	0.07
Patients	75	40.11±26.65		37.04±30.21		2.26±7.44	
Diagnosis							
HCC	54 (72)	42.67±25.88		40.85±30.36		2.91±8.68	
HCV+	5 (6.7)	36.62±28.14	n.s.[b]	37.67±42.18	n.s.[b]	0.64±0.58	0.05[b]
LM	16 (21.3)	32.23±28.73	n.s.[b]	24.50±22.59	0.05[b]	0.54±1.04	0.05[b]
Gender							
Male	56 (74.66)	40.08±27.34	n.s.	38.35±31.56	n.s.	0.80±1.81	0.001
Female	19 (25.33)	40.19±25.21		32.95±26.19		6.75 ±13.82	
AFP							
<20 ng/ml	46 (61.33)	41.49±29.03	n.s.	36.57±31.75	n.s.	1.15±1.72	0.1
>20 ng/ml	29 (38.66)	37.91±22.67		37.81±28.19		4.01±11.67	
Tumor differentiation							
Well (G1)	28 (50.9)	44.23±27.93	n.s.[c]	43.11±30.7	n.s.[c]	1.65±3.10	0.1[c]
Moderate (G2)	17 (30.9)	38.76±24.18		35.55±30.0		3.16±8.33	
Poor (G3)	10 (18.18)	46.67±22.95		40.11±31.55		6.19±16.56	

**P*-values were calculated with unpaired *t*-test; ***P*-values were calculated with Mann-Whitney *U*, Kruskal-Wallis test; n.s.: not significant. [a]PAI-1 and t-PA plasma concentration of controls are those reported in the kit data-sheets; [b]compared with HCC diagnosis; [c]compared with poor tumor differentiation; HCC: hepatocellular carcinoma; HCV+: non-tumoral chronic liver disease; LM: liver metastasis.

Results

PAI-1 and t-PA plasma concentration. The mean values of the two markers were: 40.11±26.56 ng/ml for PAI-1 and 37.04±30.27 ng/ml for t-PA, much higher with respect to the normal values reported by the manufacturer. Levels of PAI-1 and t-PA plasma concentration were studied in relation to patient clinical and pathological characteristics (Table I). PAI-1 and t-PA did not relate to gender, age, tumor differentiation or AFP values (unpaired *t*-test). No significant association was noted between the plasma concentration of PAI-1 and that of t-PA (unpaired *t*-test).

Circulating hTERT DNA. The mean value±SD of circulating *hTERT* DNA levels in the plasma of patients (2.26±7.44 ng/μl) was higher than in control individuals (0.03±0.09 ng/μl), $p=0.07$ (Mann-Whitney U, Kruskal-Wallis test).

The *hTERT* DNA levels were 1.65±3.10, 3.16±8.33 and 6.19±16.56 ng/μl in well-, moderately and poorly differentiated HCCs, respectively, although these data were not significantly different. Interestingly, high *hTERT* levels were associated with poorly differentiated tumors, in particular when viruses were present ($p=0.02$; 0.45±0.25 ng/μl in HBV–/HCV– *vs.* 28.63±26.37 ng/μl in HBV+/HCV+); moreover *hTERT* was significantly lower in patients with liver metastasis (0.54±1.04 ng/μl) than in patients with primary HCC (2.91±8.68 ng/μl) ($p=0.01$; Mann-Whitney *U*, Kruskal-Wallis test). DNA *hTERT* circulating levels in plasma of patients was significantly higher in females (6.75±13.82 ng/μl) than in males (0.80±1.81 ng/μl) ($p=0.001$; Table I).

PAI-1, t-PA and hTERT association with HCC and viral infection. PAI-1 levels were higher in the presence of both viruses compared to when there was no viral infection: (60.34±26.9 ng/ml in patients HCV+/HBV+ compared to 28.12±13.13 ng/ml in patients HCV–/HBV–; $p<0.001$, ANOVA). In addition, t-PA seemed to be influenced by virus presence (58.64±32.55 ng/ml in HCV+/HBV+ patients *vs.* 28.13±19.9 ng/ml in uninfected patients; $p=0.002$, ANOVA).

Finally, *hTERT* was significantly higher in the presence of both HCV and HBV 4.37±11.93 ng/μl, ($p<0.05$, ANOVA) and in the presence of only HCV (6.14±12.13 ng/μl; $p<0.05$, ANOVA) with respect to patients without infection (0.93±2.56 ng/μl). (Table II).

Serum AFP levels. The AFP level above 20 ng/ml was found in 29 out of 75 (38.66%) patients. For this group of patients was noted higher level of *hTERT* DNA when compared to

Table II. *PAI-1, t-PA, hTERT DNA and AFP concentration association with HCC viral infection.*

Concentration mean±sd	HCV+/HBV+ (n=19)	HCV+/HBV– (n=8)	HCV–/HBV– (n=27)	*P*-value
PAI-1 (ng/ml)	60.34±26.9	50.39±28.8	28.12±13.13	<0.001
t-PA (ng/ml)	58.64±32.55	41.35±35.87	28.13±19.9	0.002
hTERT (ng/μl)	4.37±11.93	6.14±12.13	0.93±2.56	0.05
AFP (ng/ml)	34.2±36.81	23.9±19.46	32.06±35.43	0.7

patients with AFP level <20 ng/ml (4.01±11.67 ng/μl *vs.* 1.15±1.72 ng/μl) although these data were not significantly different. (Table I). Moreover, no association was noted with HCC viral infection (Table II).

Discussion

Activation of telomerase seems to be one of the molecular events that underlie the multigenetic process of hepatocarcinogenesis (21-23). In the current study, the mean level of *hTERT* circulating DNA was significantly more elevated in HCC patients than in healthy controls. Our data is in support of previous studies which clearly demonstrated that *hTERT* DNA levels in lung cancer patients were significantly higher than those found in healthy donors and that circulating DNA decreased progressively in tumor-free patients during follow-up, while it increased in those in whom a recurrence occured (24). The presence of high amounts of tumor DNA in plasma or serum of patients with different tumors led to its possible utility as a valuable diagnostic and prognostic tool being suggested. Furthermore, the increasing tendency of plasma levels of *hTERT* DNA is in our series inversely associated with tumour differentiation, suggesting the association of higher *hTERT* DNA level with poorer tumor differentiation. This data is in agreement with a study of Iizuka *et al.* who showed that the level of cell-free DNA was significantly higher in poorly differentiated HCC than in well-differentiated or moderately differentiated HCC (25). No data are available on the role of circulating t-PA and PAI-1 in HCC carcinogenesis.

For the first time, we studied t-PA and PAI-1 plasma levels in HCC patients. In the present analysis, mean t-PA and PAI-1 levels were higher in patients compared to control values provided suggesting a direct role of these two factors in HCC carcinogenesis. Furthermore, 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 (26, 27). HBV or HCV infection can result in hepatocyte inflammation and high regenerative potential. For this reason, PAI-1, t-PA and hTERT circulating DNA have been analyzed in primary liver tumors with and without virus infection. Significantly, higher levels of PAI-1, t-PA and hTERT were observed when virus infection, both HCV and HBV were present. This confirms that the presence of virus increases risk for HCC. Moreover, this data suggested that these factors could be used better than or associated with the AFP test in the diagnosis of HCC. This test is, in fact, of limited use for screening of HCV-related HCC because of the unstable cut-off value and high rate of false-positive results. Moreover, in our analyses, no association was observed between PAI-1, t-PA and hTERT molecular markers and AFP, suggesting that the mechanisms underlying high levels of DNA in plasma are different from the mechanism by which the serum level of AFP is increased, raising the possibility that our molecular markers could be of better clinical use than AFP, which also failed to discriminate between virus infected and uninfected patients. Our hypothesis is that the increased levels of PAI-1 and t-PA, particularly in HCC patients with HCV+/HBV+ infection, could be due to: i) an increase of the release of PAI-1 from hepatocytes; ii) a reduction of PAI-1 clearance from the circulation; iii) the production of PAI-1 from the same tumor and a paracrine stimulation of normal liver parenchyma to produce more PAI-1; iv) a reduction of the hepatic receptor activity for t-PA and PAI-1 in HCC HCV+-related tumors. A study by Ren *et al.* correlated high plasma DNA levels with outcomes of patients with HBV-related HCC but failed to discriminate between HCC patients and at-risk patients with HBV infection (28). Furthermore, the study by Wu *et al.* did not show the efficacy of cell-free DNA analysis as a strategy to screen for HCC even if the definition of its applications in clinical and population studies need better knowledge of the mechanism of DNA release in blood and timing of its occurrence with respect to disease progression (29).

The present study is the first to show that detection of cell-free DNA, PAI-1 and t-PA in association with AFP in plasma can serve as tumor markers for the screening of HCC. Furthermore, the most valuable perception from genome-wide expression profiles of HCC was that HCC represents several distinct subtypes of liver cancer defined by distinct gene expression profiles (30-32). By means of molecular biology, Stahl *et al.* confirmed that HCC contains at least two subtypes, which may be distinguished by expression of β-catenin (33). Similarly, HCCs induced by chronic HBV or chronic HCV infection were demonstrated to display clearly distinct expression profiles and thus the conclusion was drawn that hepatocarcinogenesis due to HBV or HCV is driven by different pathophysiological mechanisms (34, 35). In this context, the difference observed in plasma levels of PAI-1, t-PA and *hTERT* DNA between HCC HCV–/HBV– and HCV+/HBV+ patients supports this conclusion.

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