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RESEARCH ARTICLE

Expression of insulin-like growth factor-II, matrix metalloproteinases, and their tissue inhibitors as predictive markers in the peripheral blood of HCC patients

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Abstract

Background/aim: Elevated relative expression of insulin-like growth factor-II (IGF-II) was observed in hepatocellular carcinoma (HCC) liver tissues with a role in neovascularization and associated with poor prognosis. IGF-II is influenced by the proteolytic cleavage of IGF-binding protein 3 and by matrix metalloproteinases (MMP), which are further regulated by their tissue inhibitors tissue inhibitor of metalloprotienase-1 (TIMP-1). Our aim is to study new molecular markers for HCC.

Patients/methods: RNA was extracted from the peripheral blood for evaluating the relative expression of IGF-II, MMP-9, and TIMP-1 in correlation with clinical staging of 39 HCC patients and 15 healthy controls using TaqMan real-time PCR.

Results: The relative expression of IGF-II and MMP-9 mRNA were significantly elevated in HCC patients compared with healthy controls; *P*-value <0.0001 for both. There was a significant correlation between MMP-9 and different HCC stages. On the other hand, TIMP-1 was significantly down-regulated in HCC patients; $P = 0.0003$ with the elevation of the IGF-II/TIMP-1 ratio. Significant correlation between TIMP-1 and HCC Stage III and Stage IV was found; *P*-value = 0.0138.

Conclusion: These results highlight the importance of profiling the expression of IGF-II, MMP-9, and TIMP-1 in the peripheral blood as prognostic molecular biomarkers in HCC.

Keywords: HCC, IGF-II, MMP-9, TIMP-1, PBMCs

Introduction

Recent researches are focusing on finding new molecular markers for diagnosis and prognosis of different cancers especially the hepatocellular carcinoma (HCC). Yao et al. studied the transforming growth factor (TGF)-β1 mRNA expression in peripheral blood of HCC patients, which was significantly higher than in other patients of liver diseases. Its sensitivity and specificity were as high as 90% and 94%, respectively, for HCC diagnosis but did not show any correlation with alpha fetoprotein (AFP) levels or tumor sizes (Yao et al., 2006). Combination of TGF-β1 and serum AFP was useful for detecting HCC with detection rate up to 97% (Dong, 2006). In addition to, hepatoma-specific gamma-glutamyltransferase (HS-GGT) derived from gamma-glutamyl transpeptidase where its elevated circulating mRNA and protein levels were found in HCC especially in the hypomethylated form helped in diagnosis or differentiation of HCC (Yao et al., 1998, 2000). Heat shock protein (HSP), another promising HCC biomarker where its overexpression was found

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to be highly correlated with HCC formation, and is considered as a vital marker indicating the progression and aggravation of HCC (Srivastava, 2005). Increased HSP gp96, member of the HSP90 family, in HCC tumor cells was closely related to the cell survival by preventing cell apoptosis. The gp96 expression in HCC tissues was correlated with degree of tumor differentiation and tumor size (Yao et al., 2006). Vascular Endothelial growth factor (VEGF) was investigated as an angiogenic biomarker in HCC (Jie et al., 2010) and found to be overexpressed in peripheral blood of many diseases where it enhanced angiogenesis (Bottomley et al., 1999; Hojo et al., 2000).

The insulin-like growth factor (IGF) system is composed of IGF-I, IGF-II, and insulin substrates in addition to their receptors. IGF-II is a mitogenic polypeptide hormone that is characterized by being closely related to insulin (Scharf et al., 2001), is highly expressed in the fetal liver and early after birth, and is strongly reduced in adulthood (Li et al., 1996).The IGF axis has important autocrine, paracrine, and endocrine roles in the promotion of growth and cell biology (Daughaday, 1990) and thus have been involved in the pathogenesis of several malignancies (Giovannucci, 1999) including Wilms tumors, colon (Baghdiguian et al., 1992), lung, breast (Toropainen et al., 1995), and prostate carcinomas (Chan et al., 1998), as well as HCC in addition to its important role as a mediator of proliferation and response to chemotherapy (Breuhahn et al., 2006). High levels of IGF-II peptide were detected in primary HCC and dysplastic foci of HCC bearing liver compared with normal liver tissue (Fiorentino et al., 1994). IGF-II plays an important role in neovascularization of HCC through increasing VEGF mRNA and protein levels as well (Bae et al., 1998).

IGF-I and IGF-II are presumably peptides that are required for normal fetal, postpubertal growth and are synthesized primarily by the liver (Norstedt et al., 1988; Stuver et al., 2000). Both exist in a complex form with their binding protein, insulin-like growth factor binding proteins 1–6 (IGFBPs 1-6), which are responsible for the regulation of IGFs bioavailability (McCusker et al., 1991). Matrix metalloproteinases (MMPs) are proven to act on the IGFBPs and decrease their bioavailability in the circulation (Cohen et al., 1992), and are regulated by their tissue inhibitor TIMP-1 (Milani et al., 1994; Benyon et al., 1996). The interactions within the IGF system, MMP, and TIMP were analyzed in a previous transgenic study. The overexpression of TIMP-1 led to an increase in IGFBP-3 through inhibition of their target proteins MMP-9 and MMP-2, therefore decreasing the IGF-II protein bioavailability (Martin et al., 1999). IGFBP-3, which is the most abundant IGFBP in the circulation, often serve to attenuate the effects of IGF at the receptor level thereby limiting IGF-induced cell growth and differentiation (Gong et al., 2000). In HCC, IGFBP-3 is lost or underexpressed due to the effect of MMPs and TIMPs resulting in the direct transcriptional induction and overexpression of IGF-II (Huynh et al., 2002).

The increase of proteolytic activity of MMP protein and the imbalance between MMP and TIMP plays a critical role in tumor invasion and in metastasis formation (Giannelli et al., 2002). Moreover, the significantly higher MMP-9/MMP-2 ratio observed in advanced, inoperable HCC patients, compared with those in early stage HCC patients, is useful in distinguishing between early and advanced stages of HCC (Kuyvenhoven et al., 2003; Yeh et al., 2010).

The regulation of expression of IGF-II was never examined in the peripheral blood of HCC patients except in a single qualitative study where the authors observed the relative mRNA expression of IGF-II in the peripheral blood of HCC patients but not in the peripheral blood of healthy controls (Dong et al., 2005).

It has been recommended using the appropriate single tumor marker or combination of other markers in order to improve the effectiveness in screening of HCC patients (Qin and Tang, 2004). In this study, we are concerned with exploring new molecular markers in the peripheral blood due to the lack of the reliable biomarkers with the exception of AFP, which is known to be a useful serological marker for diagnosis of HCC, but it gives false positive rates up to 40% when used in early diagnosis or the finding of small size HCC (<3 cm) (Yao et al., 2000). Recent researches have observed new potential HCC biomarkers.

Little is known about IGF-II, MMP-9, and TIMP-1 mRNA expression in the peripheral blood of HCC patients. Since IGF-II potentiates VEGF overexpression (Bae et al., 1998) that supports further angiogenesis upon their reactivation, evaluating their expression in peripheral blood could be considered a promising mean of a noninvasive diagnostic and prognostic biomarkers of HCC.

Patients and methods

Patients

The study group comprised 39 patients with HCC who were recruited from the Medical Oncology Department, National Cancer Institute, Cairo, Egypt in addition to 15 normal healthy control subjects who were sero-negative for both HBsAg and HCV Ab. All patients were recently diagnosed pretreated patients without exposure to any previous anticancer therapy at the time of collecting venous peripheral blood samples. All participants gave their written informed consent. All patients were subjected to clinical assessment, routine laboratory investigates (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]), serum albumin, hepatitis markers for HBV (HBsAg) and HCV (HCV Ab), and serum AFP. The diagnosis of HCC was based on histopathological examination of liver biopsies obtained by needle biopsy. Tissue sections were reviewed and analyzed for the tumor differentiation (well, moderate, poor) defined according to Edmondson and Steiner criteria. Clinical staging according to TNM staging system was assessed as well. Most of the recruited pretreated HCC patients (39 nos.) were predominantly males (77%) with an age ranging from 42 to 70 years. The demographic and laboratory features of HCC patients are described

in Table 1. HCC occurred in postviral (HCV and HBV) liver cirrhosis in 75% of cases. Most of the patients (74%) had more than one focal lesion as assessed by ultrasonographic examination with vascular invasion in few cases (Tables 3). Tumor staging revealed stages I, II, and III, that is, no spread to nearby lymph nodes or distant sites in 80%, and 20% had stage IV. Relative quantitative expression of IGF-II, MMP-2, MMP-9, and TIMP-1 mRNA were evaluated in peripheral blood of all HCC patients (39 nos.) and control (15 nos.) groups.

Isolation of total RNA and synthesis of cDNA

Total RNAs was isolated from the whole blood under sterile conditions using QIAamp RNA Mini Kit (Qiagen, Germany) following the manufacturer's instruction. All the RNA samples have an optical density 260/280 ratio ranging from 1.81 to 1.875 and 1% agarose gel electrophoresis showed two sharp bands of 28S and 18S rRNA, which confirms good quality of the RNA. RNA samples of OD 260/280 ratio <1.8 were excluded from the study. For synthesis of cDNA, 10 μL of total cellular RNA was reverse-transcribed into single-stranded complementary DNA (cDNA) using the high-capacity cDNA reverse transcription kit (Qiagen, Germany) according to the manufacturer's instruction. The synthesized cDNA samples were stored in –20°C until use.

Quantification of gene expression

Relative expression of IGF-II, MMP-2, MMP-9, and TIMP-1 was quantified using TaqMan real-time quantitative polymerase chain reaction and TaqMan gene expression assays, IGF-II (ABI Assay ID: Hs01005963_m1), MMP-9 (ABI Assay ID: Hs00234579_m1), MMP-2 (ABI Assay ID: Hs00234422_m1), TIMP-1 (ABI Assay ID:Hs00171558_m1*), and the housekeeping gene GAPDH (ABI Assay ID: Hs00266705_g1) (Applied Biosystems, Foster City, CA). The amount of mRNA expression for each parameter of each sample was normalized relative to the amount of GAPDH, which represents the housekeeping gene, in the same sample. The slope values were –3.38 for IGF-II, –3.4 for MMP-9, –3.36 for MMP-2, –3.35 for TIMP-1, and –3.36 for GAPDH. The PCR efficiency was calculated using $E = 10^{-1/\text{Slope}} - 1$. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All PCR reactions including controls were run in duplicate reactions.

Statistical analysis

Results of gene expression were represented as relative quantitation ($RQ = 2^{-\Delta\Delta Ct}$) value. RQ value reflects the relative mRNA expression of the studied parameter for each patient. Due to wide variation in the values, Mann–Whitney *U* test (nonparametric) was used to compare IGF-II, MMP-2, MMP-9, and TIMP-1 mRNA expression among different sample groups. One-way analysis of variance (one-way ANOVA) was used to correlate MMP-9 mRNA expression and tumor staging for each individual patient. Spearman method of correlation was used to correlate IGF-II, MMP-9, and TIMP-1 mRNA expression and serum AFP levels of each individual HCC patient. A *P*-value of <0.05 was considered statistically significant. Analysis was nonparametric and two-tailed and was performed using the GraphPad Prism 5.00 Software.

Table 1. Characteristic features of hepatocellular carcinoma (HCC) patients.

Age: mean	56
Sex: male/female	30/9
Aspartate aminotransferase (AST) (U/L)	76.9 ± 54.36
Alanine aminotransferase (ALT) (U/L)	84.8 ± 50.34
Serum albumin (g/dL)	3.1 ± 0.59
HCV Ab	51%
HBs Ag	24%
Serum AFP (ng/mL)	28.3 ± 14.69
Stages of HCC: I/II/III/IV	14/3/14/8

Results

The quantitative expression of IGF-II, MMP-2, MMP-9, and TIMP-1 did not show any statistical difference in reference to the age or gender variation of the recruited patients. Values are expressed as median; ***$P < 0.001$ and **$P < 0.01$.

Expression of IGF-II mRNA in peripheral blood of HCC patients and healthy controls

The relative expression level of IGF-II mRNA was found to be significantly up-regulated up to 170 folds; in HCC patients compared with healthy controls, *P*-value <0.0001 and >54% of HCC patients showed >4-fold increase in IGF-II expression as shown in Figure 1.

Table 2. Relationship between peripheral blood insulin-like growth factor-II (IGF-II), matrix metalloproteinase (MMP)-9, and TIMP-1 mRNA or IGF-II/TIMP-1 ratio and serum alpha fetoprotein (AFP) level or hepatocellular carcinoma (HCC) stage.

HCC Stages	Avg. AFP	*n*	Avg. IGF-II[a]	Avg. MMP-9[b]	Avg. TIMP-1	Avg. IGF-II/TIMP-1[c]
Healthy controls	0.184 ± 0.017	15	1.207 ± 0.28	1.58 ± 0.39	1.421 ± 0.336	1.46 ± 0.387
Stage I	14.69 ± 1.152	14	6.228[a] ± 2.757	12.4[b] ± 1.95	0.55 ± 0.233	110.137[c] ± 49.03
Stage II	32.3 ± 7.2	3	10.108[a] ± 3.3	48.83[b] ± 9.4	0.269 ± 0.18	2520.4[c] ± 2497.8
Stage III	29.5 ± 1.8	14	175.754[a] ± 141.5	447.87[b] ± 60	0.46[d] ± 0.14	1403.89[c] ± 855.7
Stage IV	51.825 ± 5.3	8	13.46[a] ± 6.39	2984.39[b] ± 462.9	0.265[d] ± 0.099	143.85[c] ± 82.49

[a]IGF-II mRNA average RQ value of the four HCC Stage groups was correlated to average AFP levels; P = 0.018.
[b]MMP-9 mRNA average RQ value was correlated to average AFP levels; P < 0.0001.
[c]IGF-II/TIMP-1 average ratio was correlated to average AFP levels; P = 0.048.
[d]TIMP-1 mRNA average RQ values for Stage III and stage IV groups were correlated with HCC tumor staging; P = 0.0138.
Results are represented as mean±SEM.

Expression of MMP-9 and MMP-2 mRNA in peripheral blood of HCC patients and healthy controls

Relative expression of MMP-9 and MMP-2 mRNA was compared in the peripheral blood of HCC patients and healthy controls. MMP-9 mRNA expression was significantly up-regulated up to 490 folds in peripheral blood of HCC patients compared with healthy controls; *P*-value <0.0001; >56% of HCC patients had up to 327-fold increase on MMP-9 expression as demonstrated in Figure 2. On the other hand, a nonsignificant up-regulation of the relative expression of MMP-2 mRNA was observed in HCC patients compared with the healthy control group; *P*-value = 0.29; only 33.3% of HCC patients had 3-fold increase of MMP-2 (Figure 3).

Table 3. Tumor stage and size for each individual hepatocellular carcinoma (HCC) patient.

Case No.	HCC tumor stage	Tumor size
1	Stage IIIC	Multifocal HCC
2	Stage IV	Multifocal HCC
3	Stage IIIB	Unifocal mass 8 × 9 cm
4	Stage IV	Unifocal mass 6 × 5 cm
5	Stage IIIA	Multifocal HCC
6	Stage IV	Multifocal HCC
7	Stage IIIA	Multifocal HCC
8	Stage IIIA	Multifocal HCC
9	Stage II	Unifocal mass 1.9 × 1.6 cm
10	Stage IV	Unifocal mass 6 × 6 cm
11	Stage IV	Multifocal HCC
12	Stage I	Multifocal HCC
13	Stage II	Unifocal mass 3 × 3 cm
14	Stage II	Multifocal HCC
15	Stage I	Multifocal HCC
16	Stage I	Unifocal 1.5 × 1.5 cm
17	Stage I	Unifocal mass 3 × 3 cm
18	Stage I	Unifocal mass 1.8 × 1.8 cm
19	Stage I	Multifocal HCC
20	Stage I	Multifocal
21	Stage I	Unifocal mass 1.4 × 1.6 cm
22	Stage I	Multifocal HCC
23	Stage I	Unifocal mass 1.8 × 1.8 cm
24	Stage I	Unifocal mass 2 × 2 cm
25	Stage I	Multifocal HCC
26	Stage I	Multifocal HCC
27	Stage IIIA	Multifocal HCC
28	Stage IIIC	Multifocal HCC
29	Stage IV	Unifocal mass 4.7 × 5 cm
30	Stage IIIC	Unifocal mass 5 × 5 cm
31	Stage IIIC	Multifocal HCC
32	Stage IV	Unifocal mass 5.5 × 5 cm
33	Stage III	Multifocal HCC
34	Stage III	Multifocal HCC
35	Stage II	Unifocal mass 1.7 × 1.4 cm
36	Stage IV	Multifocal HCC
37	Stage IIIC	Multifocal HCC
38	Stage IIIB	Multifocal HCC
39	Stage IIIB	Multifocal HCC

Expression of TIMP-1 in peripheral blood of HCC patients and healthy controls

In the studied 39 HCC patients, the expression of TIMP-1 mRNA was significantly down-regulated (3-fold) compared with healthy controls; *P*-value = 0.003 as shown in Figure 4.

Correlation between IGF-II, MMP-9, and TIMP-1 and stages of HCC in peripheral blood of HCC patients and healthy controls

In the present study, HCC patients were staged according to the extent of tumor invasion, presence of lymph node involvement, and metastasis into stages I–IV. IGF-II, MMP-9, and TIMP-1 expression was determined in the control group expressed as RQ values.

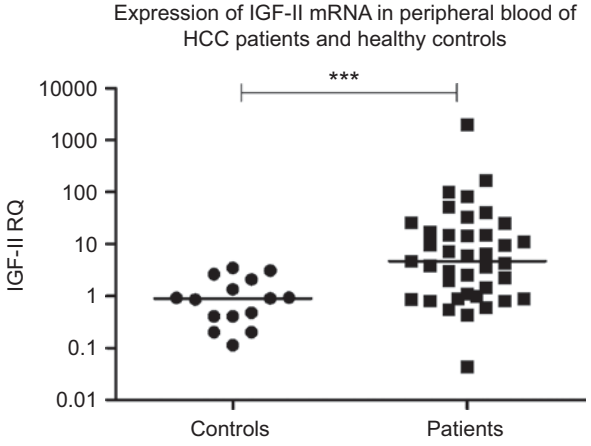

Figure 1. Relative expression of insulin-like growth factor-II (IGF-II) mRNA in peripheral blood mononuclear cells (PBMCs) of hepatocellular carcinoma (HCC) patients and healthy controls. IGF-II relative expression was significantly up-regulated in HCC patients as compared with controls (*P*-value <0.0005). Values are expressed as median. ***$P < 0.001$. The bar represents the median value.

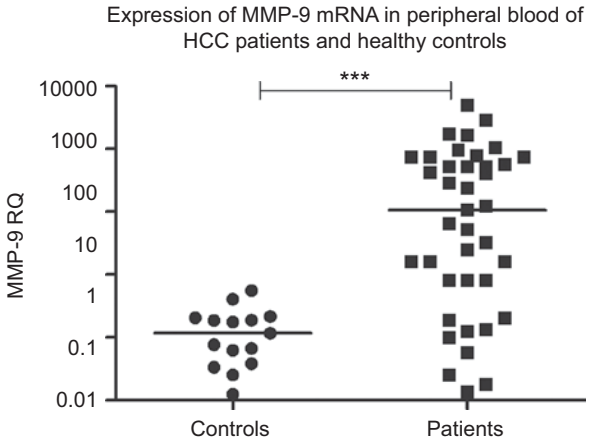

Figure 2. Relative expression of matrix metalloproteinase (MMP)-9 mRNA in peripheral blood mononuclear cells (PBMCs) of hepatocellular carcinoma (HCC) patients and healthy controls. MMP-9 relative expression showed a significant up-regulation in HCC patients as compared with controls (*P*-value <0.0001). Values are expressed as median. ***$P < 0.001$. The bar represents the median value.

Levels of IGF-II mRNA expression were not correlated with the stage of HCC (Supplementary data, Figure 2*) as well as with IGF-II/TIMP-1 ratio (Supplementary data, Figure 3*). On the other hand, a positive correlation was found between MMP-9 RQs (i.e. RQ value reflects the relative mRNA expression of MMP-9 for each patient) and the different stages of HCC.

The average RQ value for MMP-9 in the control group was 2.86 (range 0.13–5.59). The lowest average RQ value was 12.4 (range 0.14–24.67) demonstrated in patients with stage I HCC (36% of the 39 HCC patients), which showed overlap with healthy controls values. An average value of 48.83 (range 32.55–65.11) and an average value of 447.87 (range 106.34–789.39) were found in stage II (7.5% of the 39 HCC patients) and stage III (36% of the 39 HCC patients), respectively. The highest average RQ value was 2984.39 (range 969.17–4999.61), which was noted in patients with stage IV (20.5% of the 39 HCC patients) as shown in Figure 5.

Another positive correlation between TIMP-1 RQs (i.e. RQ value reflects the relative mRNA expression of TIMP-1 for each patient) and the different stages of HCC. The average RQ value for TIMP-1 in the control group was 1.42 (range 0.31–4.4). Stages I and II groups of patients showed overlap with the control group with average RQ values of 0.558 (range 0.012–2.83) and 0.26 (range 0.0014–0.6), respectively. Stage III patients had average RQ value of 0.46 (range 0.004–1.6) and stage IV patients had average RQ value of 0.26 (range 0.004–0.75) as shown in Figure 6.

IGF-II/TIMP-1 mRNA expression in HCC patients and healthy controls

The ratio of IGF-II/TIMP-1 mRNA expression was calculated to observe whether if there is a correlation that might explain the regulatory effect of TIMP-1 on IGF-II (Figure 7). A significant increase of IGF-II/TIMP-1 ratio was restrictedly demonstrated in HCC patients and was not exhibited in the control group; P-value <0.0001. This finding highlights the relation between the high expression of IGF-II and down-regulation of TIMP-1.

Correlating IGF-II, MMP-9, and TIMP-1 mRNA expression and serum AFP levels

Using Spearman method of correlation, IGF-II mRNA expression was found to be significantly correlated to serum AFP levels (i.e. they increase together in each individual HCC patient); P-value = 0.018 as shown in Figure 8. MMP-9 mRNA expression was even more significantly correlated to serum AFP levels; P-value <0.0001 as shown in Figure 9.

TIMP-1 mRNA expression did not show any sort of correlation with serum AFP levels; P-value = 0.69 (Supplementary data, Figure 4*). However, IGF-II/TIMP-1 ratio showed a significant correlation with AFP levels; P-value 0.048 as shown in Figure 10; ***$P < 0.001$ and **$P < 0.01$.

Discussion

AFP is well-known to be a useful serological marker for diagnosis of HCC, but one of its drawbacks is the false positive rates that reach up to 40% (Yao et al., 2000). Recent researchers have observed the circulating hepatoma-specific AFP subfraction, TGF-β1, HS-GGT, as more specific markers than total AFP level for early diagnosis for HCC but these are not well-established biomarkers (Yao et al., 2007). The main concern of our work is to investigate the IGF axis (IGF, IGFBPs, and IGF receptors) due to their role in the development of HCC (Scharf et al., 2001). IGF-II and its regulatory proteins the MMP-9, MMP-2, and TIMP-1 were not investigated in the peripheral blood of HCC except in single study by Dong et al., where they investigated qualitatively the IGF-II expression. IGF-II mRNA was

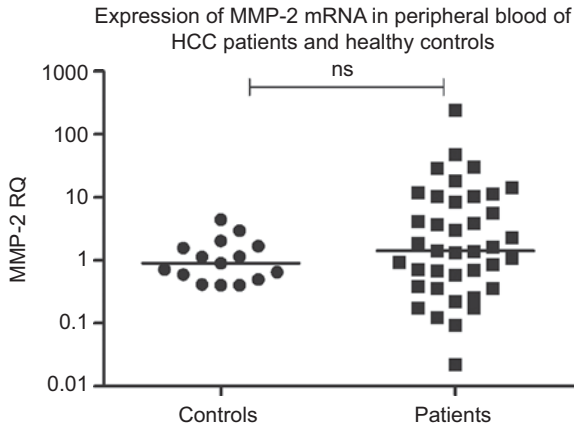

Figure 3. Relative expression of MMP-2 mRNA in peripheral blood mononuclear cells (PBMCs) of hepatocellular carcinoma (HCC) patients and healthy controls. MMP-2 relative expression showed a nonsignificant increased expression in HCC patients as compared with controls (P-value = 0.29). Values are expressed as median. ns = statistically nonsignificant. The bar represents the median value.

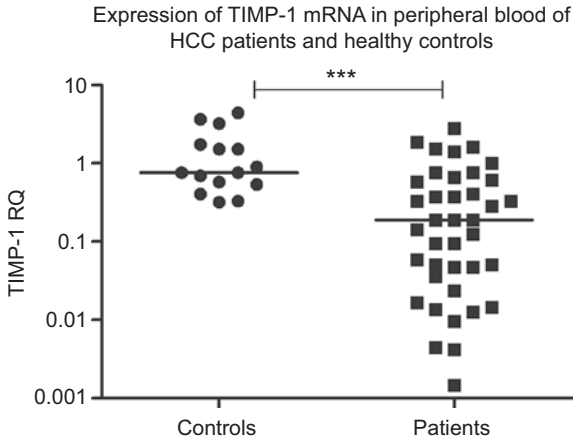

Figure 4. Relative expression of TIMP-1 mRNA in peripheral blood mononuclear cells (PBMCs) of hepatocellular carcinoma (HCC) patients and healthy controls. TIMP-1 relative expression was significantly down-regulated in HCC patients as compared with controls (P-value = 0.003). Values are expressed as median. ***$P < 0.001$. The bar represents the median value.

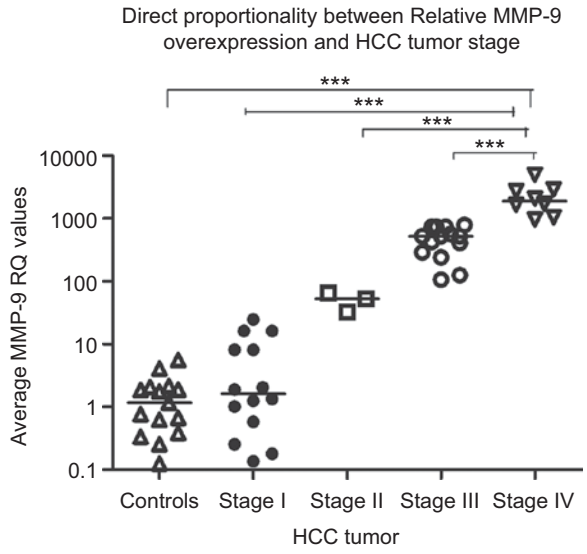

Figure 5. Correlation between MMP-9 RQ values and staging of hepatocellular carcinoma (HCC) patients. A direct proportionality has been observed between MMP-9 RQs expression and HCC stage. MMP-9 RQ values significantly increased in a linear manner with the staging as the tumor staging progress. Healthy controls showed a wide overlap with 14 HCC patients of stage I. MMP-9 expression was significantly higher in stage IV compared with healthy controls, stage I, stage II, and stage II, respectively (***$P < 0.0001$). Highest RQ values were found in eight HCC patients with stage IV HCC. Values are expressed as median. ***$P < 0.001$.

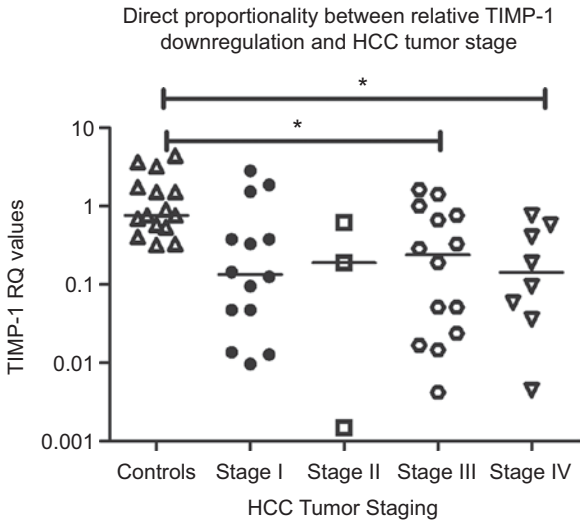

Figure 6. Direct proportionality between relative TIMP-1 downregulation and hepatocellular carcinoma (HCC) tumor stage. A direct relationship has been observed between TIMP-1 RQs expression and HCC stage. TIMP-1 RQ values significantly increased in a linear manner starting from stage III with the staging as the tumor staging progress. Healthy controls showed a wide overlap with 14 HCC patients of stage I, three HCC patients of stage II. TIMP-1 expression was significantly lower in stages III and IV compared with healthy controls (*P-value = 0.0138). Values are expressed as median. The bar represents the median value.

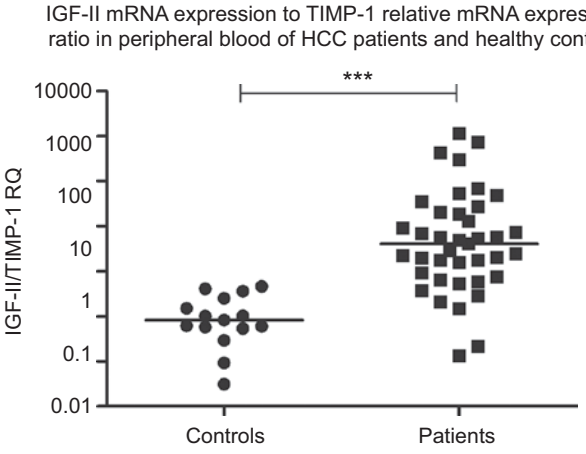

Figure 7. Insulin-like growth factor-II (IGF-II)/TIMP-1 mRNA expression in hepatocellular carcinoma (HCC) patients and healthy controls. Significant increase in the IGF-II/TIMP-1 ratio was obvious in HCC patients and was not exhibited in the control group (P-value <0.0001). Values are expressed as median. ***$P < 0.001$. The bar represents the median value.

only detected in HCC peripheral blood and showed no amplification in other patients with benign liver diseases, extrahepatic tumors and healthy controls. In addition, circulating IGF-II was found to be significantly higher in HCC peripheral blood compared with patients with liver cirrhosis and chronic hepatitis (Dong et al., 2005).

In this study, we analyzed several molecular targets in the peripheral blood that may serve as molecular biomarkers for HCC prognosis and diagnosis. First, we analyzed the expression profile of IGF-II, which is normally expressed in fetal life and highly re-expressed during hepatocarcinogenesis (Kiess et al., 1994), as well as MMP and TIMP relative expression in 39 patients with HCC.

IGF-II mRNA was significantly overexpressed in peripheral blood of HCC patients compared with the healthy control group. A frequency of 54% of the HCC patients demonstrated >4-fold expression of IGF-II mRNA (shown in Figure 1). In contrast to the previous study by Dong et al. (2005), they found the frequency of circulating IGF-II mRNA was demonstrated in 34.2% in peripheral blood mononuclear cells (PBMCs) of HCC patients (Dong et al., 2005). Moreover, our finding showed a basal level of IGF-II mRNA expression in healthy controls, which also contradicts the same previous study (Dong et al., 2005) where Dong et al. showed no IGF-II expression in normal PBMCs. In addition, our study shows no correlation between the expression pattern of IGF-II mRNA and the clinical staging. On the other hand, IGF-II mRNA expression showed a significant correlation with serum AFP levels, P-value = 0.018 (Figure 8).

Special interest has been devoted to the proteolytic MMP family, especially MMP-2 and MMP-9 and their tissue inhibitors, TIMP-1 with special concern during development of HCC. This is partly attributed to their ability to degrade type IV collagen, the major structural component of ECM, their role in cellular invasion, and thus the metastatic potential of tumor cells (Curran and Murray, 1999). Furthermore, TIMPs are capable of inhibiting invasion, metastasis, and angiogenesis, which are promoted by IGF

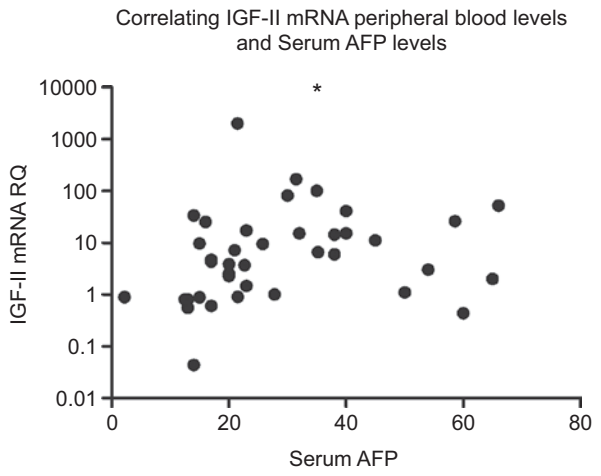

Figure 8. Correlating insulin-like growth factor-II (IGF-II) mRNA peripheral blood levels and serum AFP levels using Spearman nonparametric method of correlation, serum AFP levels are significantly correlated to IGF-II mRNA pattern of expression (*P-value = 0.018). The bar represents the median value.

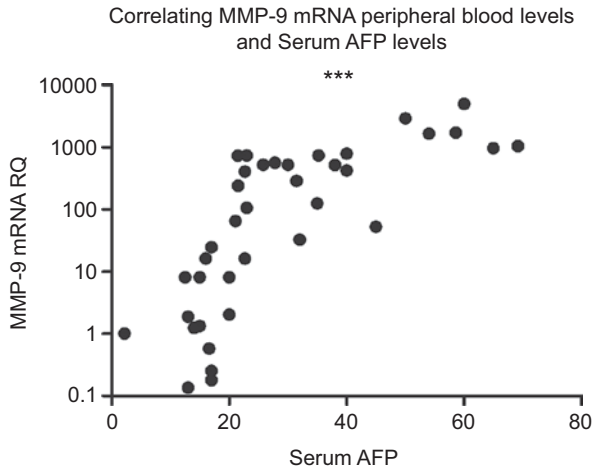

Figure 9. Correlating MMP-9 mRNA peripheral blood levels and serum AFP levels using Spearman nonparametric method of correlation, serum AFP levels are highly significantly correlated to MMP-9 mRNA pattern of expression; (***P-value <0.0001). ***$P < 0.001$. The bar represents the median value.

and thus the combined outcome of TIMP-1 elevation may be to suppress multiple stages of tumor development and progression (Martin et al., 1999). MMP-2 and MMP-9 and their tissue inhibitors, TIMPs mRNA expressions were not frequently investigated in the peripheral blood expect once in peripheral mononuclear leukocytes of chronic active hepatitis C patients (CAH) by Lichtinghagen et al. (2000) using RT-PCR where they reported an elevation in MMP-9 and lowering of TIMP-1 mRNA expression of CAH patients but they never studied in HCC peripheral blood.

Moreover, the significance of MMP-9 expression in HCC was previously verified and was found to be a predictor of HCC invasiveness, recurrence, and metastasis but in HCC liver tissues not in HCC peripheral blood (Sun et al., 2005; Zhang et al., 2006). Circulating MMP-9 was

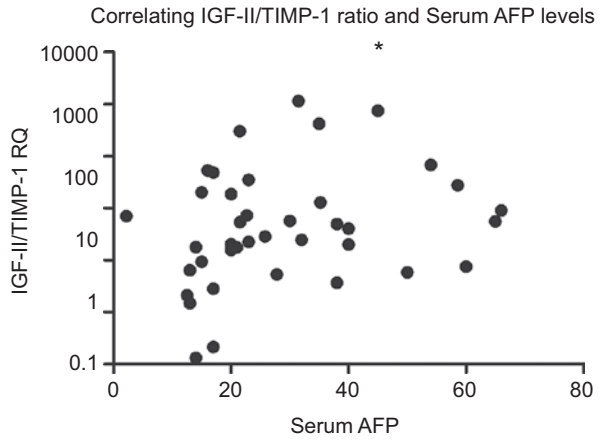

Figure 10. Correlating insulin-like growth factor-II (IGF-II)/TIMP-1 mRNA peripheral blood levels and serum AFP levels using Spearman nonparametric method of correlation, serum AFP levels are significantly correlated to IGF-II/TIMP-1 ratio (*P-value 0.048). The bar represents the median value.

found to be up-regulated in HCC patients compared with healthy controls. In contrast, serum MMP-2 showed no significant difference in serum levels of HCC patients and healthy controls (Yeh et al., 2010). HCC with advanced stages, that is, with capsular infiltration (Arii et al., 1996) and macroscopic portal vein invasion (Määttä et al., 2000) demonstrated stronger expression of MMP-9 RNA as well as more expression was relevant to the degree of dysplasia (Hayasaka et al., 1996). Furthermore, positive association between MMP-9 expression in the liver and histopathology parameters that indicate poor prognosis were demonstrated reflecting the predictive poor prognostic outcome with MMP-9 high staining percentage in HCC (Nart et al., 2010). In a previous study to assess the role of plasma MMP-9 in assessing and monitoring of HCC patients, the tumor size, grade of histological differentiation, serum AFP levels, or the application of any effective treatment for HCC were not related to MMP-9 (Murawaki et al., 2000). In contrast, our study shows significant MMP-9 mRNA up-regulation in most of the peripheral blood of the HCC patients compared with healthy controls; P-value <0.0001 (Figure 2). Moreover, these levels were significantly correlated with the stage of HCC. The degree/extent of the MMP-9 overexpression was represented as RQ value. The value of each individual patient was directly proportional to the stages of HCC. The highest RQ values were shown in stage IV, whereas the extremely low values were observed in stage I HCC revealing a wide overlap and comparable values between healthy controls and stage I HCC patients (shown in Figure 5). This direct relationship between the MMP-9 expression levels and the tumor staging might rely on the fact that MMP-9 is involved in collagen breakdown found in the cell membrane of the peripheral blood cells and thus tumor spread (Lichtinghagen et al., 2000). In addition to a highly significant correlation between MMP-9 mRNA expression and serum AFP levels; P-value <0.0001 (Figure 9).

In contrast to the significant up-regulation of MMP-9, our results demonstrated a nonsignificant up-regulation of MMP-2 mRNA expression (shown in Figure 3) in the HCC patients, which can be explained depending on the fact that MMP-2 mRNA is found almost exclusively in the liver, whereas MMP-9 mRNA is more expressed in leukocytes (Lichtinghagen et al., 2000).

The expression of TIMP-1 mRNA in liver tissues of HCC was significantly ($P < 0.00001$) up-regulated compared with the corresponding non-neoplastic liver (Ylisirniö et al., 2001) and TIMP-1 concentration was significantly correlated with the grades of differentiation, showing higher levels in poorly differentiated than in well and moderately differentiated HCCs; P-values <0.01 and <0.02, respectively (Matsumoto et al., 2004). Thus, lower grades of HCCs differentiation, stronger potential of growth, invasion, and metastasis were evident by TIMP-1 overexpression (Zhu et al., 1998), which was not previously shown in peripheral blood.

The potential involvement of TIMP-1 in the development of HCC was demonstrated in our study by the significant down-regulation of TIMP-1 with P-value = 0.003 in the peripheral blood of HCC patients (shown in Figure 4). In addition to significant elevation in IGF-II/TIMP-1 ratio in HCC patients compared with healthy controls highlighting the relation between the high expression of IGF-II and down-regulation of TIMP-1 needs further investigation. TIMP-1 mRNA pattern of expression did not show any correlation with serum AFP levels (Supplementary data, Figure 4*). However, IGF-II/TIMP-1 ratio was significantly correlated to serum AFP levels (Figure 10). In addition, TIMP-1 mRNA expression was significantly correlated with stages III and IV of HCC with P-value = 0.0138 as shown in Figure 6.

Elevated TIMP-1 mRNA expression was found in HCC tumor tissues (Matsumoto et al., 2004). This could be explained that expression of certain genes might differ from one organ to another, for example, MMP-9 was found to be exclusively expressed in peripheral blood cells and barely detected in liver tissues; however, MMP-2 showed the opposite where it was strongly detected in liver tissues and not in peripheral blood of cirrhotic patients (Lichtinghagen et al., 2000).

In conclusion, significant up-regulation of IGF-II as well as MMP-9 mRNA expression was found in peripheral blood of HCC patients compared with healthy controls. MMP-2 mRNA expression did not show a significant increase; however, there was a tendency of increase in the peripheral blood of HCC patients compared with healthy controls. MMP-9 relative expression and tumor staging showed a significantly positive correlation with direct proportionality between increased MMP-9 expression and the increase in staging of HCC. In addition, TIMP-1 relative expression showed a direct relation with HCC tumor stages III and IV. These results highlight the importance of IGF-II, MMP-9, and TIMP-1 as predictive prognostic molecular markers for HCC.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the article.

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