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Regulation of Cancer Stem Cell Marker (CD133) by Transforming Growth Factor Beta in Hepatocellular Carcinoma

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ABSTRACT
Hepatocellular Carcinoma (HCC) is the major form of primary liver cancer and the third leading cause of cancer mortality worldwide. HCC is frequently diagnosed at an advanced stage, resulting in rather poor survival rates. CD133, a trans-membrane glycoprotein, is an important cell surface marker for both stem cells and Cancer Stem Cells (CSCs) in various tissues including the liver. CD133 has been used as the Tumor Initiating Cells (TICs) marker in HCC. Further identification and characterization of CSCs or TICs in HCC are necessary to better understand hepatic carcinogenesis. So, the objective of this study was to estimate the level of the specific markers as CD133 and transforming growth factor β (TGF-β) in HCC. Fifty peripheral blood samples were collected from HCC patients. The CD133 protein expression was analyzed by flow cytometry and TGF-β levels were assayed by ELISA. This study revealed a higher expression of CD133+cells in all HCC patients. The expression of CD133+cells was positively correlated with clinical, pathological and laboratory parameters. A mild to moderate positive correlation was found with tumor size, male gender, pathological grade, *Hepatitis C virus* and Alpha-fetoprotein and showed a strong positive correlation between age, TGF-β, Lymph node metastasis, portal vein tumor thrombosis and CD133+cells in peripheral blood. CD133+cells might represent true CSCs in HCC which could allow a better understanding of HCC initiation and progression, as well as possibly bear great therapeutic implications. Moreover, the multifunctional cytokine TGF-β plays a crucial role in the regulation of CD133 expression in HCC.

Key words: Hepatocellular carcinoma, CD133, transforming growth factor β, alpha-fetoprotein

INTRODUCTION
Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer death (El-Serag, 2001). Hepatocellular carcinomas consist of a heterogeneous group of cells that have varying ability to proliferate and seed new tumors (Fausto, 2004). It has been proposed that a sub population of cells variously called tumor initiating cells, cancer progenitor cells, or Cancer Stem Cells (CSCs) serves as a proliferation reservoir, is able to seed new tumors with very low inoculum levels and is responsible for recurrence and metastases (Abou-Shady *et al.*, 1999). While these cells are not true pluripotent stem cells, they possess

characteristics of stem cells in that they can give rise to all the cell types in the tumor. They are relatively chemotherapy resistant and are a strong candidate for the source of intrahepatic HCC recurrence post liver transplantation (Lee *et al.*, 2009). There is no general consensus on the best markers to identify these cells and as a result there is no clear consensus of whether they play a major role in HCC. Recently, HCC progression has been thought to be driven by CSC through their capacity for self-renewal, production of heterogeneous progeny, resistance to chemotherapy and unlimited division. Several prior studies have examined CD133, CD44, CD90, CD13 and EpCAM as possible candidate CSC markers in HCC (Zhang *et al.*, 2010).

The most important causes leading to HCC are the *Hepatitis B virus* (HBV) and the *Hepatitis C virus* (HCV) infections, heavy alcohol consumption, aflatoxin B1, age and gender (males are more susceptible than females), race (Asian and African over 20 years), tobacco consumption, obesity associated with nonalcoholic fatty liver disease and the increase of the diabetes II mellitus (that rises the risk factor between 2 and 3), genetic hemochromatosis, primary biliary cirrhosis and alpha1-antitrypsin deficiency and autoimmune hepatitis (Tong *et al.*, 2011).

HCC is one of the most aggressive cancers. Patients who show progress over the terminal stage have 1 year survival of less than 10%. The choice of the therapy and the prognosis are dictated by the severity of the liver function, portal hypertension and medical comorbidities. National and international consensus was established to choose the best treatment adapted for each case and obtain the best prognosis (Yamashita *et al.*, 2010). Circulating Tumor Cells (CTCs) defines specifically the tumor cells detected in blood or lymphatic vessels. These circulating cells either in the blood stream or in the lymphatic system are considered to be micro-emboli (CTM) and represent a collective migration (Robrechts *et al.*, 1998). The terms Disseminated Tumor Cells (DTCs) and Isolated Tumor Cell (ITC) can be also found in the literature but are usually used to define the cells that can be detected in both the organs and the blood stream (Libbrecht *et al.*, 2001).

Among the inflammatory factors promoting proliferation are TGF-β, Fibroblast Growth Factor (FGF) and Epithelial Growth Factor (EGF). TGF-β is synthesized by mast cells, macrophages and lymphocytes as an inactive precursor that is activated by proteases in inflammatory microenvironments (Bissell, 2001). TGF-β promotes mesenchymal cell proliferation and facilitates tumor invasion and metastasis of cancer cells (Durnez *et al.*, 2006). Forty years after its discovery, serum AFP remains the most useful tumor marker in screening HCC patients. The serum concentration of 20 ng mL^{-1} is the most commonly used cut-off value to differentiate HCC patients from healthy adults in clinical researches. HCC patients with a high AFP concentration (≥400 ng mL^{-1}) tend to have greater tumor size, bi-lobar involvement, massive or diffuse types, portal vein thrombosis and a lower median survival rate. This is partially caused by the expression of ephrin-A1 (an angiogenic factor) and the ability of AFP to elicit the escape of carcinoma cells from the host's lymphocytes immune-surveillance (Matsuura *et al.*, 2010). So, the aim of this study was to focus on the specific markers of HCC used to detect CTC as CD133 and TGF-β in HCC patients.

MATERIALS AND METHODS

This study was conducted in Mansoura University Hospital (Internal Medicine and Oncology Departments), Egypt. The study included 50 HCC patients (18 females and 32 males) and 20 healthy subjects (11 females and 9 males) as a control selected to match the study group in age and gender. HCC was diagnosed by biochemical tests, sonograms and Computed Tomography (CT) scans, with histological confirmation. Tumor size was recorded as the greatest dimension of

each specimen. Tumors were staged by TNM classification of malignant tumors according to the International Union against Cancer (UICC) (Sobin and Wittekind, 2002) while tumor grading was performed as described by (Edmondson and Steiner, 1954). The study, in agreement of WMA of Helsinki declaration (WMA, 2013), was approved by the Ethical Commission and Institutional Review Board of Mansoura University Hospital in Egypt. A written informed conscious consent was obtained from all patients before their participation. All participants were subjected to the following:

- History and physical examinations
- Routine laboratory investigations including: CBC, PT, PTT, liver profile (Saab *et al.*, 2001), kidney profile and HCV antibody, HBs Ag (Daw *et al.*, 2002)
- Triphasic C-T abdomen with contrast
- US-Guided Biopsy from liver mass and pathological assessment
- **Measurement of serum TGF-β:** Five milliliter venous blood withdrawn by venous puncture from each subject. Plasma concentrations of TGF-β are quantifiable by an Enzyme Linked Immunosorbent Assay (ELISA) (cut-off value, 800 pg mL^{-1}) (Coulouarn *et al.*, 2008)
- **Measurement of serum AFP:** Serum AFP levels of subjects were measured by Nephelometry using high sensitivity AFP reagent (Behring Marburg, Germany Diagnostics). The detection limit of the assay (cut-off value of 200 ng mL^{-1}) (Abou-Shady *et al.*, 1999)
- **Flow cytometric assay for CD133:** CTC in the peripheral circulation occur at an estimated number of one CTC per 10^{5-7} Peripheral Blood Mononuclear Cell (PBMC) were separated by ficoll hypaque density gradient centrifugation (Sigma Chemicals). It is necessary to concentrate the sample. 100 µL of whole blood was pipette to each tube. The cells per 100 µL of blood were pre-incubated with 10 µL of Florescin Isothiocyanate (FITC) conjugated monoclonal mouse antihuman CD133 antibodies affinity purified human FcγR-binding antibody staining for 30 min on ice prior to staining (100 µL of a cell aliquot containing 5-8×105 nucleated cells). The recommended quantity of each primary antibody in an appropriate volume was combined of flow cytometry. Cells stained with isotypic controls for IgG2-PE, were used as negative controls. Flow cytometric analysis was performed by kit supplied by DAKO. Data were displayed on two histograms: Histogram I: Two-parameter histograms (FS vs. SS). Analyzed with Lysis II Coulter CellQuest software (Ailles and Weissman, 2007)

Statistical analysis: Data were analyzed using SPSS software Version 17.0. Quantitative data were expressed as (Mean±SD) while qualitative data were expressed as number and percentage. Continuous data are expressed as median (range) and were evaluated by appropriate statistical tests; Chi Square Test and Mann-Whitney's U Test (for paired data). Proportions were compared by means of Fisher's exact test. Correlations were evaluated using the Spearman rank correlation coefficient test. Kruskal-Wallis one way analysis of variance (ANOVA) compares more than two groups. Subgroups (percentages of patients) were compared by using the McNemar test. A value of $p<0.05$ was considered statistically significant. Sensitivity, specificity and predictive values were calculated to study the overall predictability of other techniques.

RESULTS

The comparison between CD133 expression, serum TGF-β and AFP levels in HCC patients and control group was shown in Table 1 and Fig. 1. There is a strong and statistically significant positive expression in peripheral sample of CD133 in HCC than control group (64.7±15.8 vs.

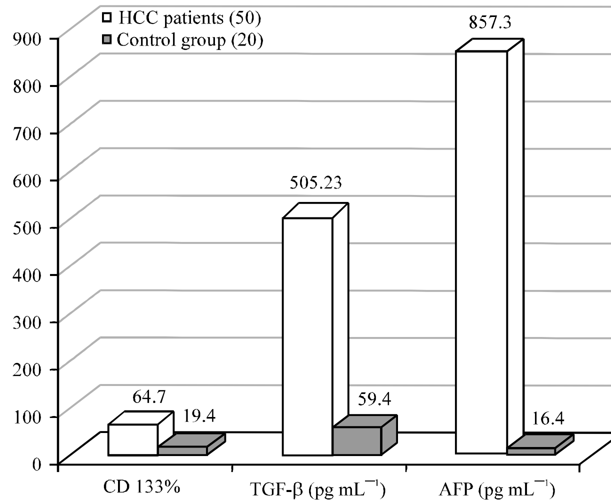

Fig. 1: Comparison between CD133+cells, serum Transforming Growth Factor-**β** (TGF-**β**) and Alpha Fetoprotein (AFP) levels in HCC patient and control group

Table 1: Comparison between level of CD133, serum Transforming Growth Factor-β (TGF-β) and Alpha Fetoprotein (AFP) in HCC patient group and control group

			Test of significance	
Parameters	Patients (50) M±SD	Control (20) M±SD	t	p
CD 133%	64.70±15.80	19.4±3.40	12.6	0.001
TGF-β (pg mL^{-1})	505.23±97.90	59.4±45.8	19.4	0.001
AFP (ng mL^{-1})	857.30±124.6	16.4±2.80	30.2	0.001

Table 2: Correlation between CD133 and other clinical and laboratory parameters (r: Spearman rank correlation coefficient test)

	CD133%	
Parameters	r	p
Age	0.66	0.44
Gender	0.22	0.66
Transforming growth factor-β (pg mL^{-1})	0.75	0.35
Alpha fetoprotein (AFP) (μg mL^{-1})	0.55	0.23
Pathological grade	0.45	0.25
Tumor size	0.38	0.25
Hepatits C virus	0.48	0.55
Lymph node metastasis	0.86	0.32
Portal vein tumor thrombosis	0.91	0.06

19.4±3.4; p = 0.001). Also, TGF-**β** and AFP levels was statistically significantly higher in patients with HCC than control subjects (p = 0.001). Figure 2 showed the flowcytometric results of CD133+ve cells in control group (Fig. 2a) and HCC patient group (Fig. 2b). There was a highly significant expression of CD133 in HCC patients compared with control group.

The expressions of CD133+cells were correlated with other clinical and laboratory parameters as shown in Table 2, a mild positive correlation was found with tumor size and male gender (r = 0.38 and 0.22, respectively) and moderate positive correlation with pathological grade, HCV

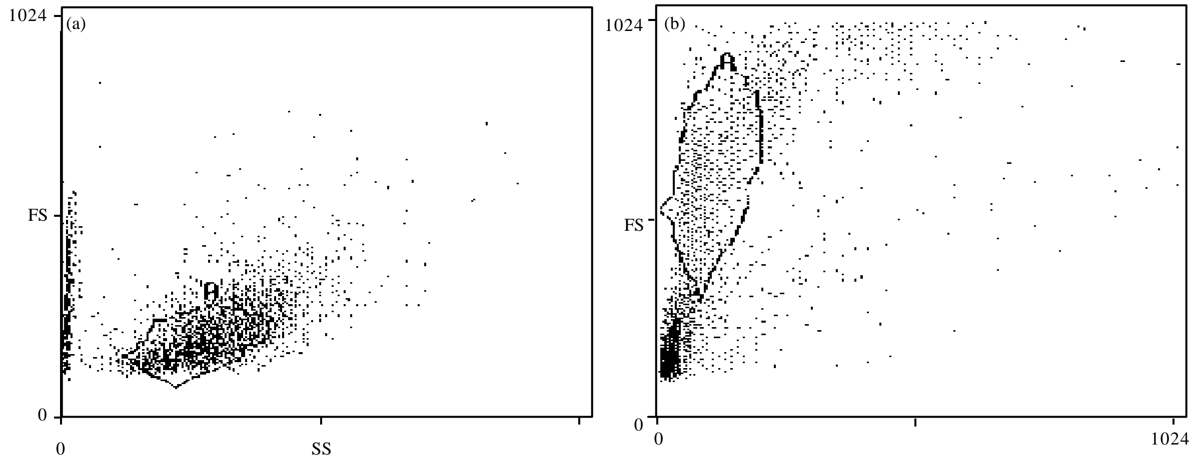

Fig. 2(a-b): (a) Flowcytometric results of CD133+ve cells expression in control group and (b) Flowcytometric results of CD133+ve cells expression in HCC group

and AFP but statistically non significant (r = 0.45, 0.48, 0.55, respectively). Also, CD133+cells in the peripheral blood of HCC patients were strongly positively correlated with age, TGF-β, lymph node metastasis and portal vein tumor thrombosis with (r = 0.66, 0.75, 0.86 and 0.91, respectively).

DISCUSSION

Hepatocellular carcinoma has a number of characteristics that are compatible with the existence of a circulating Cancer Stem Cell (CSC) population (Llovet *et al.*, 2003). Previous studies and reports have suggested that CD133, originally classified as a hematopoietic stem cell marker, could be used for enrichment of Cancer Stem Cells (CSCs) in human HCC (Zhu *et al.*, 2010). It was also noted that not all of CD133 (+) cells were representative of CSCs. Further identification and characterization of CSCs or tumor initiating cells in HCC are necessary to better understand hepatic carcinogenesis (Song *et al.*, 2008).

The results of our study showed that all of these markers CD133, TGF-β and AFP were elevated in HCC patients when compared to control. the increased expression of CD133 in all HCC patients at initial diagnosis. Yin *et al.* (2007) reported that CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. CD133 expression was detected in HCC-adjacent lesions as well as liver cirrhotic tissues but not in normal human liver. In cirrhotic tissues, CD133 was expressed only in some small hepatocytes at periphery of pseudo lobules, occasionally in trabeculae-like hepatocytes extending inside pseudo lobule. Of particular interest, CD133 was also expressed in some cells at portal areas in a form of ductule-like structures, extending into periportal hepatocytes of pseudolobules. These serum markers are useful to follow-up the development and the prognosis of the HCC but useless to follow-up circulating tumor cells in blood.

In the study by Haraguchi *et al.* (2010) reported that, although multiple publications demonstrated that CD133 is a marker of CSCs with tumorigenic properties from diverse tissues, a recent study indicated that both CD133+ and CD133-metastatic colon cancer cells were capable of initiating tumor formation. Wen *et al.* (2013) indicated that CD133 by itself might not be critical for tumor initiation. He proposes that further investigations are required before the role of CD133 in liver cancer initiation and progression is fully elucidated.

Yamashita *et al.* (2009) passes parallel with our results and reported that, the presence of CTC reflects the aggressiveness nature of a solid tumor. Many attempts have been made to develop assays that reliably detect and enumerate these cells. The clinical results obtained with such assays suggest that in some tumor types, CTC detection and identification can be used to estimate prognosis and may serve as an early marker to assess antitumor activity of treatment. In addition, CTC can be used to predict progression-free survival and overall survival. Also in our study, the levels of circulating TGF-β were significantly higher in the HCC patients. The combined detection of TGF-β and serum AFP could raise the detection rate of HCC up to 97%. Both of circulating TGF-β could be used as sensitive biomarkers for diagnosis and prognosis of HBV-induced HCC Unfortunately, TGF-β was poorly studied and further investigations have to be done to use circulating TGF-β as a marker of circulating tumor cells in HCC.

HCC tumor has shown to secret a lot of cytokines related to the development of the tumor, like Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor-beta 1 (TGF-β1), Interleukin 8 (IL-8), or Tumor-specific Growth Factor (TSGF). During chronic liver injury, Transforming Growth Factor β (TGF-β) plays an important role in fibrosis progression (Matsuzaki *et al.*, 2000).

Previous reports indicate that TGF-β expression is decreased in early-stage HCC and increased in late-stage HCC. A more recent report indicated that dysregulation of the TGF-β pathway leads to HCC through disruption of normal liver stem cell development. Lee *et al.* (2012) stated that TGF-β family plays a vital role in the control of proliferation and cellular differentiation in both stem cells and cancer cells. Yamashita *et al.* (2009) have shown that impaired TGF-β signaling by the activation of interleukin-6 (IL-6) in hepatic stem/progenitor cells can contribute to altered differentiation patterns and thus, HCC development. Similar results were found in EpCAM+Liver CSCs because the targeting of the pathway using the indirect modulation of IL-6/STAT3 was found to be effective for the eradication of EpCAM+liver CSCs. In addition (Mishra *et al.*, 2009), also provided evidence that TGF-β can regulate the expression of CD133+liver CSCs through an inhibition of the expression of DNA methyltransferases. In addition to resistance to chemo and radiation therapies, CSCs seem to be particularly adept in stimulating angiogenesis to promote tumor growth and increase the overall tumor aggressiveness before and after therapy. In fact, recent clinical studies have shown enhanced antitumor cell effects when anti-angiogenic therapy is combined with radiation or chemotherapy, suggesting that possibly radio resistance, chemotherapy resistance original articles that have documented a link between liver CSCs and angiogenesis. The first report, by Yang *et al.* (2008) found that high expression levels of hepatic stem/progenitor cell biomarkers, such as cytokeratin, CD133, nestin and CD44, are related to tumor angiogenesis and are indicative of high tumor recurrence and poor prognosis of surgically resected HCC. Through a systematic comparison of the gene expression profiles between sorted CD133 subpopulations in HCC cells by genome-wide microarray analysis, he has also found that CD133+liver CSCs produce much higher levels of IL-8 than the CD133non-CSC population. In our study, we found positive correlation between CD133 and other clinical and laboratory parameters as age, HCV infection, tumor size, portal vein thrombosis, pathological grade, TGF-β and AFP. Tang *et al.* (2008). The serum AFP level is correlated with the tumor size. 80% of small HCC (<2 cm) do not express AFP. In the other hand, AFP level can be elevated in patients with chronic liver disease with high degree of hepatocytes regeneration such as HCV-infection that show a high level of AFP in absence of malignancy. Gurtsevitch (2008) for these reasons, used some additional serological markers in combination with AFP seem to improve the performance of this

biomarker alone, especially in term of sensitivity. But there is no study that correlates their serum levels and the circulating tumor cell during the HCC development. Although the mechanisms leading to intra and extra-hepatic recurrences are still unknown, some observations suggest that Bone Marrow (BM) could also be a specific reservoir of CTC. Indeed, several reports have suggested that tumor cells are from BM origin (Gurtsevitch, 2008).

Hepatic tumor stem cells take advantage of the potential for stem cell support of the BM microenvironment. Galliher *et al.* (2006) more recently, advanced Pancreatic Ductal Adenocarcinoma (PDAC) and HCC share Transforming Growth Factor-β (TGF-β) as a common key-signaling mediator, involved in epithelial-to-mesenchymal transition, invasion and stroma-tumour dialogue. Recently, novel drugs blocking the TGF-β pathway have entered clinical evaluation demonstrating activity in patients with advanced PDAC and HCC (Neuzillet *et al.*, 2013). Though the measurement of AFP serves as an important tool in screening HCC patients, some reports have indicated that it has limited utility of differentiating HCC from benign hepatic disorders for its high false-positive and false negative rates and patients with acute exacerbation of viral hepatitis but no HCC may also have markedly increased AFP levels. Libbrecht *et al.* (2001) demonstrated that various solid tumors, such as colon, brain, ovarian, thyroid and prostate cancers are derived from CD133+CSCs. Specifically within colon cancer, CD133 expression is an independent prognostic marker for poor survival (Yuen *et al.*, 2002).

Park *et al.* (2012) reported that, TGF-β1 is a negative growth factor which correlates with cellular immune-suppression during the progression of HCC and its serum levels in HCC patients have been shown to be elevated compared with those in controls ($p<0.0001$). These researches have indicated that TGF-β1 may be a good supplementary to AFP in the diagnosis of HCC.

CONCLUSION

Given all of these findings, we postulate that reactivated CD133 positive cells are frequently present in HCC. Additionally, increased CD133 expression corresponds with higher stage tumors in HCC, thus indicating a poor prognosis for patients. These data, in this study, explored the role of the multifunctional cytokine TGF-β which could play a critical role in the regulation of CD133 expression in HCC patients.

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