Circulating endothelial cells and risk of progression in patients with hepatocellular cancer receiving sorafenib

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Aim: We investigated the behavior of circulating endothelial cells (CEC) in patients with hepatocellular carcinoma (HCC) receiving sorafenib, and whether CEC levels were associated with time to progression (TTP). **Materials & methods:** CECs in advanced HCC patients receiving sorafenib were counted at baseline and every 4 weeks. **Results:** Twenty four HCC patients were enrolled in the study. Median TTP was 3.2 months (1–6). Median baseline CEC levels were 67 cells/ml, with an increase of 169.8% after 4 weeks of treatment. Any time CEC levels in patients with a TTP lower than 4 months were higher, but not statistically significant, compared with those in patients with TTP more than 4 months. **Conclusion:** Treatment with sorafenib changed CEC levels in HCC patients.

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Angiogenesis is the process by which new microvessels are formed from pre-existing vessels [1,2]. Vasculogenesis is the process by which endothelial progenitor cells, contained predominantly in the postnatal bone marrow and derived from mesenchymal cells, are recruited and *in situ* differentiate to mature endothelial cells to form a primitive vessel network [1,2]. Both of these processes are necessary and involved in postnatal life in situations requiring an expanded vessel network. Neoangiogenesis is one of the key mechanisms involved in neoplastic transformation and tumor growth. The VEGF, which is overexpressed in many tumors, is the crucial regulator of this process. The anti-VEGF antibody bevacizumab and the receptor tyrosine kinase inhibitors of the VEGF receptor family, sunitinib and sorafenib have shown consistent results in clinical practice in metastatic colorectal cancer, renal carcinoma and hepatocellular carcinoma (HCC) [3,4]. Nevertheless no specific biomarker of their efficacy has been validated.

Almost 70% cases of HCC are diagnosed in an advanced stage, causing the death of majority of the patients [5]. No benefit for systemic cytotoxic chemotherapy with single-agent or combination regimens was observed in patients with advanced HCC [6]. Sorafenib (Nexavar[®]) is a small molecule that inhibits tumor cell proliferation and tumor angiogenesis [7]. Its action is based on inhibition of the serine-threonine kinases, Raf-1 and B-raf and the receptor tyrosine kinase activity of VEGFRs 1, 2, 3 and PDGFR- β [7]. In two large Phase III, randomized placebo-controlled trials performed in western countries (SHARP trial) and in Asia-Pacific (Asia-Pacific trial), sorafenib has been proved to be effective in patients affected by advanced HCC [7,8]. Anyway, it is unclear which patients will benefit most from the treatment.

KEYWORDS

• angiogenesis inhibitors
• CECs • hepatocellular carcinoma • sorafenib

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In an attempt to better understand which condition or factors facilitate to limit the efficacy of anti-angiogenic treatments, the analysis of circulating endothelial cells (CEC) and circulating endothelial progenitors (CEP, characterized by CD34, CD31 and VEGFR2) could represent a feasible approach for the selection of patients and the monitoring of activity of the treatments. The CEPs are released from the bone marrow following ischemia and may contribute to local angiogenesis supplied by existing endothelium. Their mobilization is influenced by growth factors, such as VEGF, enzymes, ligands and surface receptors [9]. Several studies have demonstrated that some pathological conditions, such as diabetes mellitus Type II and risk factors for ischemic cardiovascular disease are characterized by a decreased number of CEPs [1,9,10]. In contrast, limb ischemia and acute myocardial infarction may cause a rapid increase in the number of CEPs [1,11]. CECs are shed from vessel wall probably reflecting endothelial damage or dysfunction. Additional markers are used in several preliminary studies which are claimed to be specifically present on CECs (e.g., CD13, CD144, CD146), but until now there are no systematic or validation studies published, defining the phenotype and the numbers of EPCs or CEPs in healthy individuals or patients. Is a rare cell population in healthy individuals, and high levels of CECs seem to be associated to tumor progression [12]? Our attempt was to evaluate the possible role of CECs as a predictive factor during treatment with sorafenib in patients affected by advanced HCC.

Patients & methods

Patients affected by HCC Child–Pugh liver

function class A, unsuitable for any locoregional treatment and treated with sorafenib were eligible prospectively. A recovery of at least 8 weeks from locoregional treatments (radiofrequency, chemoembolization) and major surgery was necessary. The inclusion criteria were: age ≥18 years, an Eastern Cooperative Oncology Group performance status (ECOG PS) ≤2, Child–Pugh class A liver cirrhosis, if any. Patients with diabetes mellitus Type II, diabetic retinopathy and/or peripheral microangiopathy, myocardial infarction, severe/unstable angina, coronary/peripheral artery bypass, symptomatic congestive heart failure, cerebrovascular accident, transient ischemia, episode of severe hypertension, pulmonary embolism or deep vein thrombosis within the past 6 months, were excluded of the study. Chronic treatment with anti-aggregants and/or anticoagulant drugs, chronic treatment with statins and prior systemic treatment for advanced HCC were also exclusion criteria. All patients received sorafenib 400 mg, two-times a day (b.i.d.) daily until progression of disease or major toxicity. CECs and HPCs were evaluated at baseline and every 28 days. We evaluated HPCs for a control of the full-blood four-color flow cytometric method [12].

From December 2010 to November 2011, 224 patients, 23 males and one female, with the above characteristics were enrolled in the study. Median age was 70 years (range: 52–83 years). 20 patients presented alcoholic cirrhosis, and four chronic hepatitis C virus infection. BCLC stage C locally advanced HCC in ten patients, BCLC stage C HCC with extrahepatic disease in two patients, multinodular BCLC stage B HCC, not amendable to any further locoregional treatment in 12 (Table 1).

Table 1. Characteristics of the patients.		
Characteristics	Patients (n)	Percentage (%)
Males	23	958
Females	1	42
Etiology of disease:		
– Hepatitis C only	4	166
– Alcohol only	20	834
BCLC stage:		
– B (intermediate)	12	50
– C (locally advanced)	10	416
Extrahepatic spread	2	84
Child–Pugh class:		
– A	24	100
– B	0	0
– C	0	0

Table 2. Circulating endothelial cells and HPC.

		CEC (median cells/ml)	HPC (median cells/ml)
All patients (24 patients)	Baseline	6755	130,048
	4 weeks	18,227	37,311
TTP >4 and <12 months (5 patients)	Baseline	297	1164
	4 weeks	17,756	32,682
	16 weeks	9051	
TTP <4 months (19 patients)	Baseline	839	135,091
	4 weeks	18,227	44,294
	16 weeks	14,157	
TTP >12 months (3 patients)	Baseline	3558	
	4 weeks	133	
	20 weeks	4276	

Before study entry all patients provided written informed consent in accordance with the national and institutional recommendations, according to the principles of the declaration of Helsinki and ICH/good clinical practice.

Blood from patients with HCC and treated with sorafenib was collected in Ethylenediaminetetraacetic acid tubes and CECs and HPCs were counted using a full-blood flow cytometric method [12]. Based on the available literature and our preliminary experience with available antibodies, the CECs population was characterized by the presence of the following markers: CD34⁺, CD31^{bright}, VEGFR2⁺ and CD45^{-/low}, while 7-amino actinomycin D (7-AAD) was used to discriminate between live and late apoptotic (or dead) cells [13]. The hematopoietic stem cell population was measured in the same analysis and was characterized by CD34^{bright} and CD45^{dim} staining.

Results

A total of 17 patients presented progressive disease with a time to progression (TTP) of 3.2 months (range: 1–6 months), four interrupted sorafenib for toxicity after 22 days (range: 15–35 days): severe asthenia, anorexia and weight loss. Three patients presented a clear and significant clinical benefit with a time to progression of 16, 18 and 48 months. Median baseline CECs and HPCs respectively were 67 cells/ml (range: 10–141 cells/ml) and 1300 cells/ml (range: 342–2546 cells/ml). After 4 weeks of sorafenib median CECs and HPCs respectively were 18,227 cells/ml and 37,311 cells/ml, observing an important increase in median CEC levels and a decrease in HPCs levels, while as expected, no leucopenia was observed during sorafenib. Regarding posttreatment changes and

considering patients which received sorafenib for more than 4 months (five patients, group A), we observed a median CECs at baseline, after 4 and 16 weeks respectively of 297 cells/ml, 17,756 cells/ml and 9051 cells/ml, while 19 patients who received sorafenib for less than 4 months (group B) presented respectively counts of 839 cells/ml, 18,227 cells/ml and 14,157 cells/ml. In the three patients (group C) with significant clinical benefit and time to progression more than 12 months, median CECs at baseline was 3558 cells/ml, after 4 weeks 133 cells/ml, and after 20 weeks 4276 cells/ml (Table 2).

Nevertheless no statistically significant difference was observed comparing in a log rank test the CEC levels in baseline, after 4 and after 16 weeks of treatment, in patients with a TTP >4 months, versus patients with TTP <4 months. The p-value respectively was 0.16, 0.79 and 0.17.

Discussion

Both CECs and CEPs are a rare cell population involved in neoangiogenesis and are useful biomarkers of vascular damage. CECs are defined as mature endothelial cells shed from the vasculature. The mobilization, recruitment and homing of CEPs into tumors are independent multistep events in the process of the tumor vasculogenesis, involving multiple growing factors, tumor and stromal cells [14]. The role of CECs and CEPs to the vasculature of solid tumors and prognosis of cancer patients is still controversial. Nevertheless, a recent meta-analysis evaluated these cell populations as prognostic factor in patients with lung cancer, evidenced that high counts of CECs are correlated with worse 1-year overall survival, while high levels of pretreatment CEPs are associated with both worse progression-free and overall survival [15].

Proangiogenic factors (VEGFR, PDGF) have a strong expression in HCC and cirrhotic tissue and may regulate and promote both endothelial cell proliferation and CEP mobilization and homing into tumoral and cirrhotic tissue [16]. Furthermore an increased vessel density and a major number of CEP is presented in cirrhotic and adjacent tissue than in HCC tissue [14,16]. A different kinetic of CEC population as an effect of the anti-angiogenic agent has been observed in a Phase II study combining a receptor tyrosine kinase inhibitor erlotinib and sorafenib for non-small-cell lung cancer [17]. Indeed, after 7 days of sorafenib/erlotinib a threefold increase of CECs was observed, while in patients receiving only erlotinib the CEC count did not change. Moreover, CD133⁺/HPCs (CD34^{bright}/CD45^{dim}) markedly decreased during sorafenib/erlotinib, while erlotinib alone did not modify this cell population. In this study, lower pretreatment levels of CD133⁺/HPCs were observed in responding patients and correlated with TTP [17]. Similarly, in a study evaluating the activity of sunitinib in patients affected by HCC, high levels of CECs were also associated with rapid progression or mortality [18]. A more recent Phase I trial evaluates sorafenib after liver transplantation in 14 patients with high-risk HCC, higher levels of CECs were observed in patients who did not recur and with a better prognosis [19]. Moreover, in two papers it was evaluated that the role of CEPs in patients treated with sorafenib and metronomic chemotherapy, or with sorafenib alone, reported high levels of CEPs which were correlated with a poor prognosis [20,21].

In our study we evaluated the modification of CECs and HPCs at baseline and every 28 days in 24 real-life patients affected by HCC during treatment with sorafenib. We choose to evaluate only two potential biomarkers to decrease the risk of false-positive relations. Our patients' population was quite homogeneous regarding clinical conditions, stage of the neoplastic disease, previous treatments and demographic characteristics. We evaluated the CECs' modulation according to the response to sorafenib and time to progression (TTP). We performed a prospective analysis dividing the patients in three groups: those with a TTP less than 4 months, TTP more than 4 months and less than 12 months, and those with a TTP more than 12 months. Nineteen patients were in the first group, five in the second and three patients in the third group. An increased number of CECs was observed in all 24 patients after 4 weeks of treatment, and a subsequent reduction was observed after 16 weeks. Median number of CEC at baseline and after 4 weeks, respectively was 67,55/ml and 18,227/ml, with an opposite effect on HPCs (130,048 cells/ml vs 37,311 cells/ml). CECs was higher at baseline in the second group of patients, almost threefold higher than patients of the first and third group. Nevertheless no statistically significant difference was observed comparing in a log rank test the CEC levels at baseline, after 4 and after 16 weeks of treatment in patients with a TTP >4 months, versus patients with TTP <4 months. As for all similar previous reports, the small number of patients probably is the major limit of this study.

Figure 1. Circulating endothelial cells and HPCs modulation. Black and white will be fine

Conclusion

CECs increased in all patients affected by HCC after 4 weeks of treatment with sorafenib, with a subsequent different/opposite kinetic among two group of patients: those who presented or not a clinical benefit (Figure 1). However, we did not demonstrate any statistically significant impact of CECs modification in TTP. We believe that it is not feasible that a new study with major number of patients enrolled to evaluate only CEP/CECs and that the research of potential non-invasive predictive and prognostic biomarkers probably is still not near the conclusion.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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