Digestive and Liver Disease 48 (2016) 76–80

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Liver, Pancreas and Biliary Tract

Different expression of VEGF and EGFL7 in human hepatocellular carcinoma

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ARTICLE INFO

Article history:
Received 24 June 2015
Accepted 30 September 2015
Available online 9 October 2015

Keywords:
Epidermal growth factor like domain 7 (EGFL7)
Hepatocellular carcinoma (HCC)
Liver cirrhosis
Vascular endothelial growth factor (VEGF)

ABSTRACT

Background: Vascular endothelial growth factor (VEGF) is one of several angiogenic factors expressed in cirrhosis and during progression to malignancy, that seem to play a major role in hepatocellular carcinoma development. Lately, another angiogenic factor, epidermal growth factor-like domain multiple 7 (EGFL7), has attracted interest due to its possible relationship with hepatocellular carcinoma metastasis.
Aims: To evaluate expression of VEGF and EGFL7 in human hepatocellular carcinoma, compared to corresponding cirrhotic surrounding tissue.
Methods: Tumoural and cirrhotic tissue was harvested from 12 consecutive patients undergoing surgical resection. VEGF and EGFL7 were assessed by immunofluorescence and quantitative reverse transcriptase-polymerase chain reaction, compared with normal controls.
Results: Both angiogenic factors were over-expressed in cirrhotic livers compared to normal controls. VEGF and EGFL7 expressions did not differ according to disease aetiology, nodule size or other clinical variables. While VEGF expression was constant, regardless of tumour differentiation stage and unchanged compared to surrounding cirrhotic tissue, EGFL7 expression increased in less differentiated hepatocellular carcinoma.
Conclusions: The preferential expression of EGFL7 in less differentiated hepatocellular carcinoma compared to VEGF, suggests a possible important role of this angiogenic factor in a later oncogenic and infiltrative/metastatic phase.

1. Introduction

After lung and stomach cancer, hepatocellular carcinoma (HCC) is the third cause of cancer deaths worldwide. Moreover, its incidence in many countries is rising as hepatitis C virus (HCV)-related liver disease increases, especially in its most severe form [1]. HCC occurs sporadically in normal livers and liver cirrhosis represents the major known risk factor. Management of the disease is based on a multidisciplinary approach providing surveillance, loco-regional treatment, surgery including liver transplant, and more recently chemotherapy [2]. However, in nearly 60% of cases, HCC is diagnosed at an advanced stage when the majority of current therapeutic options are of reduced utility or not applicable. In 2008, the use of sorafenib, a kinase inhibitor of Raf, vascular endothelial growth factor receptor and platelet-derived growth factor receptor, was shown to have a three-month survival benefit in patients with advanced HCC [3]. These results have led to increased interest in molecular targeted therapy and the cellular pathogenesis of HCC.

In this context, recent studies have focused on the tumoural microenvironment of HCC. Several biological processes are triggered in the small space surrounding the tumour, comprising angiogenesis, immunity, and repair processes characterized by fibrosis deposition [4].

Angiogenesis, in particular, seems to play a major role in HCC growth, as it is also altered in chronic liver disease and present from the early phases of tumour development [5–7]. Several angiogenic factors are involved in the process of tissue injury and repair associated with chronic liver disease. Among these the vascular

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http://dx.doi.org/10.1016/j.dld.2015.09.019
1590-8658/

endothelial growth factor (VEGF) seems to play a major role [8,9]. Whether other known or as yet unknown angiogenic factors may provide an important contribution to the onset of HCC in humans is, at present, unclear.

Recently, epidermal growth factor-like domain 7 (EGFL7) has emerged as a major factor regulating vascular development. Interestingly, at variance with other angiogenic molecules, EGFL7 expression seems to be restricted to endothelial cells and exerts its activities in an autocrine fashion [10]. Its levels rise during embryogenesis, as well as in physiologic or pathologic angiogenesis. On the basis of this background we sought to evaluate the comparative expression of VEGF and EGFL7 in liver tissue of patients affected by HCC. The assessment of the two angiogenic factors was conducted both in neoplastic and surrounding cirrhotic tissue.

2. Materials and methods

2.1. Patients

Twelve consecutive patients admitted to our liver unit for surgical resection of HCC were included in the study. All subjects gave their written informed consent to participation in the research, which was approved by our local ethical committee. HCC liver specimens and surrounding tissue were harvested during surgery. Normal liver specimens were harvested from patients (n. 3) undergoing liver resection for hepatic metastasis from colon cancer. All samples were immediately snap frozen in liquid nitrogen and then transferred to our laboratory facilities for further analysis. Histological staging of liver tumours was performed in the Hospital Pathology Department according to the WHO grading system [11] as follows: G1 = well differentiated; G2 = moderately differentiated; G3 = poorly differentiated HCC. Individual and clinical features of patients were also collected for analysis.

2.2. Quantitative reverse-transcriptase (RT) PCR

Total RNA was extracted from each sample using TRIZOL Reagent Kit (Invitrogen, Milan, Italy). Possible DNA contaminants were removed employing RNeasy Mini Kit (Qiagen, Milan, Italy). RNA quality and quantity was evaluated by using agarose gel (1%) electrophoresis and spectrophotometric absorbance reading, employing a Nanodrop ND-100 model, respectively. Synthesis of cDNA was achieved using random primers and the Superscript Reverse Transcriptase III kit (Invitrogen, Milan, Italy) following the manufacturer's specifications. Gene expression was measured using the Real Master Mix SYBR ROX (Eppendorf, Hamburg, Germany) and the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems). Gene expression was quantified using the $\Delta\Delta$Ct method and normalization to GAPDH expression. Specific primers for EGFL7, VEGF and GAPDH were designed using the Primer Express software (Applied Biosystems in Life Technologies, Monza, Italy) Primer sequences were as follows:

EGFL7: (forward) 5′-TCGTGCAGCGTGTGTACCAG3′;
EGFL7: (reverse) 5′-GCGGTAGGCGGTCCTATAGATG-3′;
VEGF: (forward) 5′-ATGACGAGGGCCTGGAGTGTG-3′;
VEGF: (reverse) 5′-CCTATGTGCTGGCCTTGGTGAG3′;
GAPDH: (forward) 5′-TCGGAGTCAACGGATTTGGT-3′;
GAPDH: (reverse) 5′-GAATTTGCCATGGGTGGAAT-3′.

2.3. Immunofluorescence

For immunofluorescence studies, fresh liver biopsies were directly embedded in OCT and kept in liquid nitrogen vapours until solidified. Cryostat sections (5 μm) were fixed in MeOH (5 min at −20 °C) and air-dried. Non-specific antibody binding was blocked by incubation with 10% donkey serum in PBS (1 h at room temperature). Specimens were then incubated with primary antibodies or non-specific IgG from the same animal in which the primary antibody was developed (overnight +4 °C). After repeated washes in PBS, sections were exposed to fluorescently labelled secondary antibodies (1 h, at room temperature).

All antibodies used for immunofluorescence analysis were diluted in PBS containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich). Cell nuclei were counterstained with Hoechst33342 (Sigma-Aldrich), and mounted with Möwiol mounting medium (Sigma-Aldrich). All images were acquired using an Axioplan 2 imaging microscope (Zeiss, Thornwood, NY, USA). Primary antibodies employed were the following: polyclonal goat anti-EGFL7 from R&D Systems (Wiesbaden, Germany) and from Santa Cruz Biotechnology (Santa Cruz, CA, USA) used at 0.2 μg/ml; monoclonal mouse anti-VEGF (Abcam 1:50). Secondary antibodies were Cy3 donkey-anti-goat (Jackson ImmunoResearch, West Grove, PA, USA) 4 μg/ml concentration and Alexa Fluor568 goat-anti-mouse; (Invitrogen in Life Technologies, Monza, Italy) 4 μg/ml concentration. Hoechst staining was employed to visualize cell nuclei in section.

Table 1
Overall patient population features.

		Patients $n = 12$
Males		9 (75%)
Mean age (years)		65.4 ± 7.7
Liver disease aetiology	HCV	6 (50%)
	HBV	3 (25%)
	Ethanol	2 (16.7%)
	Cryptogenic	1 (8.3%)
HCC staging (WHO system[a])	G1	4 (33.3%)
	G2	4 (33.3%)
	G3	4 (33.3%)
Nodule size		
Large > 5 cm		9 (75%)
Small < 5 cm		3 (25%)

SD, standard deviation; HCV, hepatitis C virus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.
[a] G1 = well differentiated; G2 = moderately differentiated; G3 = poorly differentiated hepatocellular carcinoma.

2.4. Statistical analysis

Data were stored and analyzed using the IBM-SPSS statistic software package. Statistical analysis was carried out using unpaired Student *t*-test for continuous variables and Fisher test for discrete variables. One-way ANOVA was used for multiple group comparison, followed by Bonferroni *t*-test to assess differences between individual groups. For each test a $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Patient characteristics

Patient and tumour characteristics are summarized in Table 1. All patients had compensated Child–Pugh class A cirrhosis, minimal or absent portal hypertension and presented a single nodular HCC lesion without metastasis. Tumour size was larger than 5 cm in 9 patients and less than 5 cm in 3 patients (Table 1). Pathological assessment demonstrated a trabecular or trabecular-globular pattern in the majority of tumours with loss of these features and of sinusoidal space in less differentiated nodules.