

## The multifaceted role of podoplanin expression in hepatocellular carcinoma

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### Abstract

The role of podoplanin in hepatocellular carcinoma (HCC) is not clear yet. The aim of our study was to evaluate the expression of podoplanin in HCC and to determine its role in hepatocarcinogenesis. We performed immunohistochemistry with monoclonal D2-40 antibody, on paraffin-embedded tissue sections of 72 patients diagnosed with HCC. Lymphatic vessels density (LVD) was increased in patients who had vascular invasion at the time of diagnosis ( $P=0.018$ ) and in those with associated cirrhosis ( $P=0.006$ ). Tumor cells showing podoplanin expression were correlated with histological grade ( $P=0.040$ ). Podoplanin-expressing cancer associated fibroblasts (CAFs) were correlated with both LVD ( $P=0.019$ ) and tumor cells ( $P=0.015$ ). Our results sustain the dual role of podoplanin in HCC by its involvement in both HCC tumorigenesis, lymphatic neovascularization and tumor invasion invasiveness. A possible crosstalk between epithelial and stromal tumor cells in HCC tumor microenvironment may be mediated by podoplanin, but this hypothesis needs further studies to elucidate this interrelation.

### Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent cancers worldwide. Despite the intense efforts in understanding the mechanisms and finding a cure, HCC remains one of the leading causes of worldwide cancer-related deaths.<sup>1</sup> The treatment options in HCC are limited and finding new therapeutic targets is imperative.

Podoplanin is a 162-amino acid transmembrane sialoglycoprotein that belongs to the family of type-1 transmembrane sialomucin-like glycoproteins.<sup>2</sup> It is expressed in various normal human tissues such as lymphatic endothelium, heart, lung, placenta, skeletal muscle, glomerular podocytes, mesothelial cells, Schwann cells, osteoblast, follicular dendritic cells, reticular fibroblasts, myofibroblasts and myoepithelial cells.<sup>3-7</sup> The monoclonal antibody D2-40 can detect podoplanin and currently is the most used antibody to demonstrate lymphatic endothelium. In addition to staining lymphatics, podoplanin was found to be positive in a relatively large spectrum of cancers including angiosarcomas, mesotheliomas, follicular dendritic cell sarcomas, testicular tumors, squamous cell carcinoma, thymomas and some subtypes of the central nervous system tumors.<sup>8-13</sup> Also, podoplanin positivity was identified in CAFs of various malignancies and it seems that its expression in these cells plays an important role in tumor progression.<sup>14</sup>

The purpose of our study was to evaluate LVD in a series of human hepatocellular carcinoma cases by using monoclonal antibody against podoplanin (clone D2-40). In addition, we analyzed CAFs and tumor cells D2-40 immunoreactivity, in order to determine their role in hepatocarcinogenesis.

### Materials and Methods

#### Patients and tissue samples

Our study included a total number of 72 patients, diagnosed with HCC, between 2002-2014, who underwent partial hepatectomy in the Department of Surgery of the "Prof. Dr. Octavian Fodor" Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, Romania. Tumor samples, as well as clinical data, were obtained from the Institute database according to protocols approved by the Ethical Committee and protocols followed the guidelines by the Helsinki Declaration. Surgically resected samples were fixed in 10% buffered formalin for 48 h and then they were embedded in paraffin followed the routine automated flow of this procedure. One section from each case was stained with haematoxylin and eosin for histopathology. Based on preliminary microscopic evaluation given by two independent pathologists for diagnosis, slides for immunohistochemistry were selected for each case.

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Key words: Hepatocellular carcinoma; podoplanin; lymphatic vessel density; cancer associated fibroblasts.

Received for publication: 29 July 2016.

Accepted for publication: 3 February 2017.

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European Journal of Histochemistry 2017; 61:2707  
doi:10.4081/ejh.2017.2707

### Clinicopathologic features

Our study included samples from 72 patients, composed of 50 males (70%) and 22 females (30%), aged between 30-91 years (mean 64.13 years). In relation to tumor size, HCCs were divided into 2 categories: tumors smaller than 5 cm and tumors greater than 5 cm. Of the 72 HCCs included in this study, 28 tumors (39%) were larger than 5 cm and 44 (61%) were smaller than 5 cm. According to Edmondson and Steiner system, tumor grade was divided into three groups: well differentiated (grade I-8 cases), moderately differentiated (grade II-30 cases) and poorly differentiated (grades III and IV-34 cases). Tumor stages were classified according to TMN classification of tumors of the liver as follows: I (23 cases), II (26 cases), III (16 cases), and IV (7 cases). Among the 72 HCCs, cirrhosis was present in 32 cases (44%), including hepatitis B in 12 cases and hepatitis C in 7 cases. Vascular invasion was noted in 25 cases (35%).

### Immunohistochemistry

Three- $\mu$ m thick sections were cut from each case. Quality control of the specimens selected for immunohistochemistry was performed by applying immunostaining for vimentin clone V9 (Dako Agilent Technology, Carpinteria, CA, USA). Absence of vimentin immunoreaction was considered as an exclusion criterion. All immunohistochemical steps were performed by using BOND MAX Autostainer system with rigorously controlled steps from dewax to nuclear counterstain by settings of proper pre-designed program from

the menu. For our purpose, we selected a program including dewax, hydrogen peroxide inhibition, 30 min incubation time for primary antibodies and we used a working system based on Bond Polymer Refine Detection System Brown Kit (Leica, Microsystems, Newcastle, UK), a standardized HRP free kit for automated immunohistochemistry including nuclear counter-stain step. There were selected as primary antibodies anti human podoplanin antibodies (clone D2-40, Dako Agilent Technology) Slides removed from the Autostainer were automatically cover-slipped by using Leica CV5030 Fully Automated Glass Coverslipper (Leica).

Image acquisition and data analysis were performed using Nikon Eclipse E 600 microscope (observed by using 200x and 400x magnification) and Lucia G software for microscopic image analysis.

### Evaluation of D2-40 staining

The lymphatic microvascular density (LVD) was quantified intratumoral and peritumoral, by counting the podoplanin-positive vessels in three 'vascular hot spots', at high magnification (x200). 'Hot spot' areas were selected and defined as microscopic fields of 200x magnification containing the highest number of D2-40 positive lymphatic vessels. In these areas (three for each case) we counted D2-40 positive structures having a lymphatic vessels morphology with large lumen lined by D2-40 positive endothelial cells. In addition, we evaluated the intensity of podoplanin in tumor cells and in stroma cancer-associated fibroblasts (CAFs). The score was quantified on a scale of 1-3+ as follows: low (+), moderate (++) or intense (+++). The final score was assessed based on two criteria: intensity of the immunostaining and percent of positive cells. For this purpose, we used the morphometry software (NIS-Elements D 2.30, Laboratory Imaging s.r.o., Prague, Czech Republic) and a method previously described by Suci *et al.*,<sup>15</sup> modified for cytoplasmic expression. Briefly, the software allowed us to apply a semi-automated method able to differentially assess positive and negative podoplanin expressing cells by detecting different intensities and counting positive podoplanin expressing cells. This method was used for tumor cells. The reaction to podoplanin was scored as follows: 0 (negative), absent or weak present in <10% of tumor cells; 1+ (low), weak in >10% of tumor cells; 2+ (moderate), weak or high expression in >10% of tumor cells but less than ≤30% of tumor cells; and 3+ (intense), high expression in >30% of tumor cells.

### Statistical analysis

Statistical analysis was performed to determine the relationship between podoplanin expression and the clinicopathologic parameters such as gender, age, presence of cirrhosis, chronic hepatitis, tumor stage, histological grading, tumor size and vascular invasion using Spearman's test. P-values of less than 0.05 values were considered statistically significant. All statistical analysis was performed using the SPSS 22.0 software.

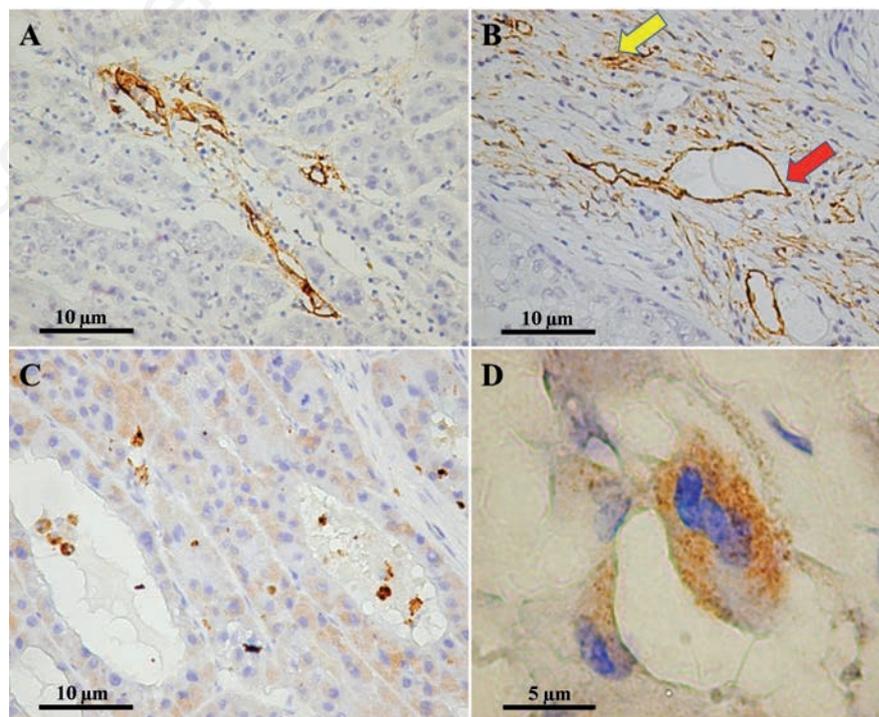
## Results

### Immunohistochemical findings

Of 72 HCCs analyzed for lymphatic vessels, 32 (44.4%) showed podoplanin-positive vessels within the intratumoral tissue, while 57 (79%) revealed podoplanin-positive vessels within the peritumoral tissue (Figure 1A). Podoplanin positivity revealed that LVD was greater in peritumoral tissue (mean 14.6) compared with

intratumoral tissue (mean 5.7). LVD was significantly related to background cirrhosis ( $P=0.006$ ). We found that LVD was increased in patients who had vascular invasion at the time of diagnosis ( $P=0.018$ ). Relationship between LVD and clinicopathological data are shown in Table 1.

We observed podoplanin expression in stromal spindle cells that were morphologically and immunohistochemically (vimentin positive) identified as CAFs (Figure 1B). CAFs were positive for podoplanin in 41 cases (57%) and were correlated with both LVD ( $P=0.019$ ) and podoplanin-expressing tumor cells ( $P=0.015$ ). Moreover, examination of tumor cells showed cytoplasmic positivity for podoplanin (Figure 1 C,D) in 23 HCCs (32%) including low expression in 14 cases (60%), moderate expression in 7 cases (31%), and intense expression in two cases (9%). Podoplanin-positive cells were significantly correlated with the histological grade ( $P=0.040$ ). Relationship between podoplanin expression by tumor cells and clinicopathological parameters are shown in Table 2.



**Figure 1. Podoplanin expression in human hepatocellular carcinoma showing: A) Positivity for lymphatic vessels and its absence in tumor cells; B) Positivity for cancer associated fibroblast (CAFs, yellow arrow) and stromal lymphatics (red arrow); C) Podoplanin cytoplasmic staining of tumor cells at low magnification; D) Podoplanin cytoplasmic staining at high magnification.**

## Discussion

Tumor associated-lymphangiogenesis was identified as an important factor in the development and progression of various human cancers.<sup>16-19</sup> Moreover, some pre-clinical experimental studies showed that inhibition of lymphangiogenesis decreases the rate of metastasis by 50-70%.<sup>20</sup> While angiogenesis in HCC was extensively analyzed and nowadays it is increasingly well

understood, little is known about the role of lymphangiogenesis in this malignancy. In an HCC experimental model, Thelen *et al.* proved that lymphangiogenesis is involved in tumor growth and metastatic spread.<sup>21</sup> In addition, they demonstrated for the first time that LVD has a key role in tumor progression, tumor recurrence and survival in human HCC.<sup>22</sup> By using D2-40 monoclonal antibody, we demonstrated intratumoral lymphatic vessels in 44.4% (32/72) of HCCs and peritumoral lymphatic vessels in

79% (57/72) of our cases. Similarly to the Thelen *et al.*<sup>23</sup> study, we showed that LVD is significantly associated with cirrhosis (P=0.006). In addition, our study demonstrated that high lymphatic density is correlated with vascular invasion (P=0.018), a parameter that typically predicts a more advanced tumor stage. Thus, we believe that tumor-associated lymphangiogenesis is involved in tumor growth, contributing to neovascularization and tumor invasion in human HCC.

**Table 1. Relationship between lymphatic vessel density and clinicopathological parameters.**

Clinicopathological parameters	No. of cases	LVD peritumoral (Range)	LVD intratumoral (Range)	P-value
Gender				
Males	50	14.92 (0-45)	5.93 (0-28)	>0.05
Females	22	12.27 (0-60)	5.61 (0-24)	
Age				
<60 years	21	14.19 (0-35)	2.76 (0-12)	>0.05
>60 years	51	14.47 (0-60)	6.94 (0-28)	
Grade				
Well differentiated (grade I)	8	8.62 (0-28)	5.37 (0-28)	>0.05
Moderately differentiated (grade II)	30	14.10 (0-35)	5.60 (0-25)	
Poorly differentiated (grades III, IV)	34	15.73 (0-60)	6.14 (0-25)	
Stage				
I	22	13.72 (0-29)	8.04 (0-28)	>0.05
II	26	13.69 (0-35)	4.15 (0-25)	
III, IV	26	13.56 (0-60)	5.11 (0-20)	
Tumor size				
<5 cm	46	16.30 (0-45)	7.06 (0-28)	>0.05
>5 cm	26	11.34 (0-60)	4.57 (0-20)	
Vascular invasion	25	15.32 (0-60)	3.60 (0-20)	0.018
Cirrhosis	32	15.84 (0-45)	7.90 (0-25)	0.006
Chronic hepatitis	19	11.21 (0-29)	5.00 (0-28)	>0.05

LVD, lymphatic vessel density.

**Table 2. Relationship between podoplanin expression by tumor cells and clinicopathological parameters.**

Clinicopathological parameters	No. of cases	Podoplanin expression		P-value
		Positive	Negative	
Gender				
Males	50	15 (30%)	35 (70%)	>0.05
Females	22	7 (32%)	15 (68%)	
Age <60 years	21	10 (48%)	11 (52%)	>0.05
>60 years	51	13 (25%)	38 (75%)	
Grade				
Well differentiated (grade I)	8	0 (0%)	8 (100%)	0.040
Moderately differentiated (grade II)	30	9 (30%)	21 (70%)	
Poor differentiated (grades III, IV)	34	14 (41%)	20 (59%)	
Stage				
I	22	7 (32%)	15 (68%)	>0.05
II	26	11 (42%)	15 (58%)	
III, IV	26	5 (19%)	21 (81%)	
Tumor size				
<5cm	46	14 (30%)	32 (70%)	>0.05
>5 cm	26	9 (35%)	17 (65%)	
Vascular invasion	25	6 (24%)	19 (76%)	>0.05
Cirrhosis	32	13 (41%)	19 (59%)	>0.05
Chronic hepatitis	19	9 (47%)	10 (53%)	>0.05

Expression of podoplanin in tumor cells was found to play an important role in carcinogenesis, cell motility and tumor invasion. In addition, high podoplanin expression was strongly associated with an aggressive tumor behavior, with higher metastatic potential and with a poor survival in a wide variety of tumors.<sup>24-29</sup> In the present study, we showed for the first time that podoplanin is expressed in the tumor cells of HCC and we found that high podoplanin expression is significantly correlated with a high histological grade ( $P=0.040$ ), suggesting a role of podoplanin in hepatocarcinogenesis. Our findings are in good agreement with other studies that link podoplanin expression of tumor cells to histological grade in human cancers.<sup>23,30-32</sup> Atsumi *et al.* demonstrated that podoplanin is a potential marker of tumor-initiating cells (TICs) with stem-cell-like properties in squamous cell carcinoma.<sup>33</sup> In human HCC, cancer stem cells (CSCs) have been successfully identified and they are thought to be responsible for tumor recurrence and treatment failure.<sup>34-36</sup> Kato *et al.* developed a cancer-specific mAb against podoplanin and demonstrated its effectiveness in an experimental model of glioblastoma.<sup>37</sup> Thus, further studies targeting TICs in podoplanin-expressing HCCs and inhibiting podoplanin functions may lead to new anti-tumor strategies.

CAFs represent a major component of the tumor stroma and they have a pivotal role in cancer development. Since CAFs are assigned with various pro-tumoral roles, they are attractive and promising targets for cancer therapy. However, little is known about the role of CAFs in the human hepatocellular carcinoma microenvironment. Jia *et al.* demonstrated that CAFs are able to promote HCC proliferation and to support the growth of tumor cells.<sup>38</sup> Moreover, their results show that CAFs support tumor cell survival in severe conditions, such as massive necrosis. Recent studies identified podoplanin as a marker of CAFs in various cancers and there is increasing evidence that its overexpression has a direct impact on both tumor growth and progression.<sup>14</sup> In our study, podoplanin expression by CAFs was found in 41 cases (57%) of HCC and it was significantly correlated with both LVD ( $P=0.019$ ) and podoplanin-expressing tumor cells ( $P=0.015$ ). It was already proved that podoplanin may mediate cancer cell migration.<sup>29,39</sup> The most postulated theory indicate that cancer cells lose their epithelial phenotype and acquire a mesenchymal one, resulting in an increased migratory and invasive potential.<sup>31</sup> To the best of our knowledge, the present study is

the first to identify an interaction between epithelial and stromal tumor cells in HCC tumor microenvironment mediated by podoplanin. Further studies are necessary to clarify the biological functions and mechanism of podoplanin in HCC microenvironment.

In conclusion, our study shows the diversity of podoplanin biology in HCC. On the one hand, podoplanin proved to be a valuable marker in highlighting lymphatic vessel density. Our results suggest that tumor-associated lymphangiogenesis is involved in tumor growth, contributing to neovascularization and tumor invasion in HCC. Based on our results, we hypothesize that podoplanin might play a role in orchestrating the cross-talk between tumor cells and CAFs in human HCC microenvironment, although further studies are needed to elucidate this interrelation.

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