IS MDV-CD34 A POOR PROGNOSIS PREDICTOR OF HEPATOCELLULAR CARCINOMA?

POMPILIA RADU[1], IOANA RUSU[2], TEODOR ZAHARIE[2], VASILEANDREICA[1,2], ZENO SPÂRCHEZ[1,2]

[1]University of Medicine and Pharmacy Iuliu Hațieganu Cluj-Napoca
[2]Regional Institute Of Gastroenterology and Hepatology, Cluj-Napoca

Abstract

Aim. Angiogenesis is an important process for growth, recurrence and metastasis of hepatocellular carcinoma (HCC). Thus, indentifying the molecular markers associated with angiogenesis may help create a scoring system that could predict the risk for recurrence and metastasis of HCC. This study was designed to investigate whether MDV-CD34 (microvessel density as determinated by CD34) could serve as a valid prognostic marker.

Methods. Tissue samples from 18 patients with cirrhosis and HCC tumors were immunohistochemically stained with antibodies of anti-CD34. For statistical analysis we used Kaplan–Meier method, with the log-rank test for comparison.

Results. Of the 18 tissue samples of HCCs that were analyzed, 9 were graded as grade 2 and 9 samples as grade 3, according to Edmondson Steiner. No significant difference was identified between expression of MDV-CD34 in tumor and Edmonson Steiner grade. MVD-CD 34 was not correlated with tumor size (Pearson, p=0.65). In tumors without vascular invasion tended to have a lower MVD-CD34 compared with tumors with vascular invasion (p=0.02)

Conclusion. A significant difference between the expression of MDV-CD34 in tumor tissue versus nontumor tissue was observed. Also, a strong relationship was found between high MDV-CD34 and vascular invasion.

Keywords: Hepatocellular carcinoma, MDV-CD34, tumor tissue, nontumor tissue Edmondson Steiner.

Hepatocellular carcinoma (HCC) is a highly vascular solid tumor with an increased ability for vascular invasion and metastasis. It develops from dysplastic nodules in a cirrhotic liver. The development of dysplastic nodules to HCC is associated with a change in the blood supply from the portal vein to a predominantly arterial blood supply. These changes in vascularization are necessary to provide a route for the nutrients to reach the tumor and, in the same time, a way for the tumor to spread into circulation. An increase in tumor dimension beyond 0.5 mm induces the proliferation of vascular endothelial cells. For their new function, the new capillaries have a different morphology, which will help accomplish their function. They have leaky basement membranes, making them more accessible to tumor cells than mature vessels [1]. It has been demonstrated that increasing density of newly formed micro vessels in growing tumors correlated closely with increasing number of tumor cells shed into the bloodstream [2]. A prognostic influence of micro-vessel density (MVD) independent of conventional pathologic predictors has been demonstrated in a variety of cancers [3,4]. Several endothelial cell markers, including CD31, CD34, UEA-1, CD105, vWF have been investigated from this point of view [5,6,7,8,9,10].

The main problem after curative treatment for HCC is recurrence. Conventional predictors of recurrence are clinico-pathological parameters (pTNM stages, tumor size, Edmondson Steiner (ES) grade, vascular invasion), but they are not trustful indicators of prognosis. Therefore, it is necessary to identify new markers that can predict prognosis.

It has been reported that microvessels increase gradually from cirrhotic nodules through low grade and high grade dysplastic nodules, with the greatest numbers recorded in HCC [8]. Conflicting data regarding the relation between CD34 and patient prognosis have been published so far.

The aim of the present study was: to compare the MDV status in cirrhotic tissue and tumoral tissue; to

Adresses for correspondence: radupompilia@yahoo.com

correlate MVD with tumor characteristics (tumor size, Edmonson Steiner grade) and to analyze the prognostic value of MDV-CD34.

Methods

18 tissue samples, collected from patients diagnosed with HCC and cirrhosis at the Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca were analyzed. The follow-up of this patient was calculated from the date of diagnostic to October 2012. All patients included in study were new diagnosed and none of them had undergone any previous treatment for HCC.

Grading of HCC was done according to Edmondson and Steiner criteria (ES) [11]. MVD-CD34 of tumorous and non-tumorous tissue sections was evaluated according to Gasparini's criteria [12] by two independent. At low power field (x 40), the tissue sections were screened and five areas with the most intense neovascularization (hot spots) were selected. Microvessel counts of these areas were performed at high power field (x 200).

Tumor specimens were taken from areas next to the margin of the tumors as well as from more central areas. Necrotic tissues were avoided. Adjacent non- tumorous liver tissues were also collected. All the specimens were formalin-fixed, paraffin-embedded and pathologically diagnosed to be HCC and evaluated by hematoxylin and eosin (H&E) for conventional histological assessment. Histological characteristics such as tumor type, tumor size, vascular invasion and necrosis were reviewed by two pathologists (ZT and IR), in order to minimize the inter-observer variation. Maximum tumor diameter was measured macroscopically in fresh specimens. Vascular invasion included both portal and hepatic venous invasions. Histological sections of 4-μm thickness were prepared for immunohistochemical study.

Immunohistochemical staining

Immunohistochemical staining (IHC) was performed using the streptavidin-biotin-peroxidase complex method. Formalin-fixed, paraffin-embedded sections were deparafinized in xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 20 minutes. Antigen retrieval was performed by microwave pretreatment in 0.01 M citrate buffer for 15 minutes. The sections were first incubated with normal goat serum at room temperature for 10-15 minutes and then incubated with the primary monoclonal antibodies: mouse antihuman CD34 monoclonal antibody (BioGenex, San Ramon, CA) diluted 1:10. Secondary biotinylated antimouse immunoglobulin was applied and reacted with peroxidase-labeled polymer. The sections were developed in 3,3-diaminobenzidine and counterstained with Mayer's hematoxylin. The sections were washed with phosphate-buffered saline at the end of each step. The negative control was obtained by substituting the primary antibodies with mouse immunoglobulin G.

The results of MDV-CD34 in cirrhotic versus tumoral tissue were presented as medians with interquartile ranges (IQR) and were compared using the Mann-Whitney test. Survival analysis was performed using the Kaplan-Meier method, with the log-rank test for comparison. Pearson correlation coefficients were presented for data with a normal distribution.

Results

The baseline characteristics of these patients are presented in Table I. The mean age of diagnosis was 62.7 ± 8.9 years. In our study, 16 males and 2 women were included. The etiology of cirrhosis was: infection with HCV in 14 cases, infection with HBV in 2 cases and alcohol intake in 2 cases.

Table I. Patients characteristics.

Variable	Value
Gender M/F	16/2
Mean (SD) age in years	62.7±8.9
Etiology HBV/HCV/Alcohol	2/16/2

M- male; F- female; HBV- hepatitis B virus; HCV- hepatitis C virus.

Of the 18 HCCs tissue samples that were analyzed, 9 were graded as grade 2 and 9 as grade 3, according to ES. No significant statistical relationship was identified between expression of MDV-CD34 in tumor tissue and ES grade (Table II).

Table II. Correlation between MVD and pathological characteristics in the tumoral tissue and nontumoral tissue.

Variable	Median MDV	p value

Edmonson grade
    2
    3
	
	42 (60-104)
	50 (79.0-106.8)
		
		NS

Vessels involvement
    Yes
    No
	
	102.1 (92.059-112.1)
	69.6 (43.8-95.3)
		
		p=0.02

Tissue
    Tumoral
    Non tumoral
	
	71.5 (46.8-103.5)
	0.9 (2.9-5)
		
		p<0.001

NS-Not significant; MDV- microvessels density

MDV-CD34 was found to be heterogeneously distributed within the tumor, and maximal density was seen at the periphery of the lesion, near the borders. Tumorous tissue demonstrated intense staining (mean MVD-CD34 was 78.9 ± 39, range 20-164). In non-tumorous cirrhotic tissue a low intensity staining was observed (mean MVD-MDV-CD34 was 3.18 ± 2.6 range 0-9). The expression of MVD-CD34 in non-tumorous liver tissue was significantly lower than the expression of tumor MVD-CD34 (p<0.001) (Table II).

MVD-CD34 was not correlated with tumor size (Pearson, p=0.65).

Considering that vascular invasion is a predictor for poor prognosis, MVD-CD34 was analyzed in tumors with

and without vascular invasion. Tumors without vascular invasion showed a lower MVD-CD34 expression compared with tumors with vascular invasion (p=0.02) (Figure 1).
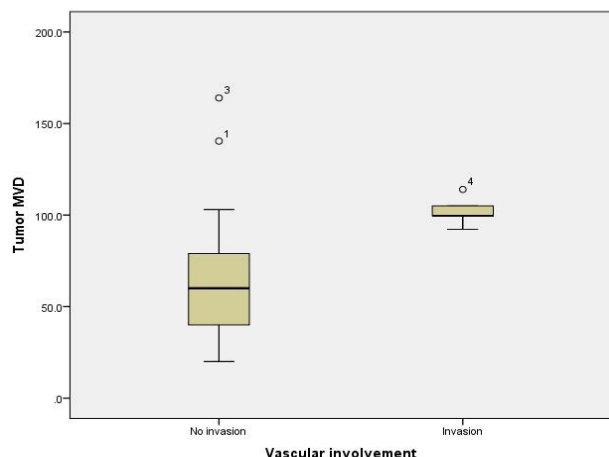

Figure 1. MDV CD 34 in tumoral tissue with vascular involvement versus MDV-CD 34 tumoral tissue without vascular involvement.

Discussions

Angiogenesis is a crucial process for tumor growth and metastasis. It is based on activation, proliferation and migration of endothelial cells: secreted angiogenic factors activate resting endothelial cells in adjacent blood vessels. Activated endothelial cells lost inter-endothelial cell contact and break down the surrounding basement membrane and extracellular matrix by secreting proteases. MDV-CD34 staining is known to be the most specific antibody for the detection of microvessels in HCC. Microvessels stained with anti-CD34 are capillary-like, rather than having the appearance of sinusoids in normal liver.

We found a statistically significant difference between the expression of MDV-CD34 in tumor and the expression of MDV-CD34 in the surrounding cirrhotic tissue (p<0.001, Wilcoxon test). This is in accordance with the findings of previous studies. Also, we found a correlation between MDV-CD34 expression in cirrhotic and tumor tissue.

No correlation was encountered between MDV-CD34 and ES grade. Also ES was not a prognostic marker for survival. This result differs from the findings of previous studies that managed to correlate ES grading with the prognosis. Moreover, Zhou et al. proposed the inclusion of ES grading in a scoring criteria in order to predict the prognosis of curatively resected HCC [13]. Possible explanations could be the reduced patient sample size included in the study and the lack of patients in stages 1 and 4.

To analyze the impact of MVD-CD34 on survival, we split the data in two groups: low MVD-CD34, below the median value, and high MDV-CD34, above the median value. Comparing the two above-mentioned groups, there

was no statistical significant difference in patient survival. In the literature there are several studies that analyzed the relationship between MVD and survival but results are still conflicting. Most reports have found a negative relation between high MVD and prognosis [8,10,14]. However, a positive relationship between high MDV and prognosis, or no correlation at all has also been published [15,16].

In our study we tried to assess the relation between MDV-CD34 and HCC size but we did not find any statistically significant data. This result differs from the findings of previous studies [17,18]. Correlation between MDV-CD34 and HCC size has previously been reported [13]. These discrepancies can probably be explained by differences in the studied groups: etiology of cirrhosis, tumor stage and method of MDV-CD34 count. Our study proves that HCC tumors with vascular invasion presented high MDV-CD34 expression while HCC tumors with no vascular involvement showed low MDV-CD34 expression (Figure 1). We found a difference between these variables and survival: patients with vascular invasion had a lower survival in comparison to patients with no vascular involvement (Table III and Figure 2). These results are in agreement with the findings of previous studies [13].

Table III. Survival time according tumor features.

Variables	Survival mean (months)	p Value
ES		NS (p=0.45, Log Rank test)
grade 2	38.1 (16.3-59.9)	
grade 3	25.4 (12.8-37.9)	
MDV in **tumoral tissue**		NS (p=0.64, Log Rank test)
< median values	13.2 (10.6-15.8)	
> median values	36.5 (17.9-55.1)	
MDV in non tumoral tissue		NS (p=0.35, Log Rank test)
< median values	11.2 (6.8-15.6)	
> median values	44.4 (29.4-59.5)	
MDV in tumoral tissue		NS (p=0.26, Log Rank test)
Invasion	19.3 (0-44.1)	
Non invasion	39.1 (17.7-60.6)	

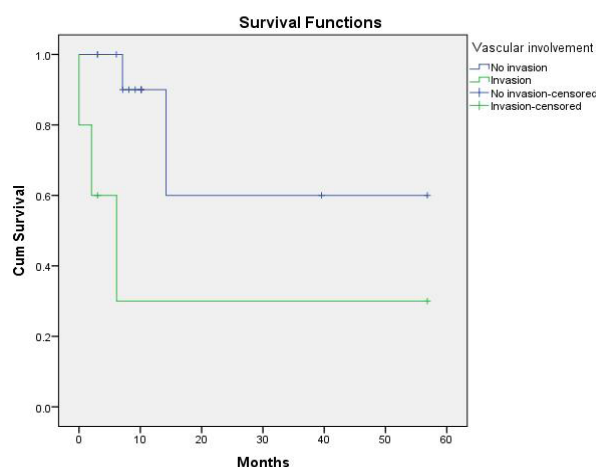NS - Not significant; MDV - microvessels density; ES - Edmonson Steiner.

Figure 2. Kaplan Meier survival-curve for patient with/without vascular involvement.

In our study, we demonstrated the involvement of MDV-CD34 in angiogenesis, but we must take in consideration that a low number of patients were included. Most of the tissue samples were advanced HCC thus we did not have the opportunity to observe the modifications of angiogenesis from early to advanced stage.

These associations are being evaluated in an ongoing study in order to establish the factors that can predict the prognosis of hepatocellular carcinoma.

In conclusion, we report a significant difference between the expressions of MDV-CD34 in tumoral tissue versus non-tumoral tissue. Also, a strong relationship was found between high MDV-CD34 levels and vascular invasion.

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