

Mast cells positive to tryptase, endothelial cells positive to protease-activated receptor-2, and microvascular density correlate among themselves in hepatocellular carcinoma patients who have undergone surgery

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Background: Mast cells (MCs) can stimulate angiogenesis, releasing several proangiogenic cytokines stored in their cytoplasm. In particular MCs can release tryptase, a potent in vivo and in vitro proangiogenic factor via proteinase-activated receptor-2 (PAR-2) activation and mitogen-activated protein kinase phosphorylation. Nevertheless, no data are available concerning the relationship between MC density positive to tryptase (MCDPT), endothelial cells positive to PAR-2 forming microvascular density (PAR-2-MVD), and classical MVD (C-MVD) in hepatocellular carcinoma (HCC) angiogenesis. This study analyzed the correlation between MCDPT, PAR-2-MVD, and C-MVD, each correlated to the others and to the main clinicopathological features, in early HCC patients who underwent surgery.

Methods: A series of 53 HCC patients with early stage (stage 0 according to the Barcelona Clinic Liver Cancer Staging Classification) were selected and then underwent surgery. Tumor tissue samples were evaluated by means of immunohistochemistry and image analysis methods in terms of number of MCDPT, PAR-2-MVD, and C-MVD.

Results: A significant correlation between MCDPT, PAR-2-MVD, and C-MVD groups, each correlated to the others, was found by Pearson *t*-test analysis (*r* ranged from 0.67 to 0.81; *P*-value ranged from 0.01 to 0.03). No other significant correlation was found.

Conclusion: Our in vivo pilot data suggest that MCDPT and PAR-2-MVD may play a role in HCC angiogenesis and could be further evaluated as a target of antiangiogenic therapy.

Keywords: tumour angiogenesis, stromal cells, translational research

Introduction

Mast cells (MCs) can play a role in tumor angiogenesis, and their involvement has been demonstrated in several animal and human malignancies.^{1,2} MCs can secrete several classical proangiogenic factors, including vascular endothelial growth factor, fibroblast growth factor-2, thymidine phosphorylase, and interestingly a nonclassical proangiogenic factor named tryptase, stored in their secretory granules.³⁻⁷ With special reference to tryptase, it induces in vitro microvascular endothelial cells (EC) proliferation in the matrigel assay and displays in vivo capillary growth on the chick embryo chorioallantoic membrane, which is conversely suppressed by tryptase inhibitors.⁸ This proangiogenic stimulus induced by tryptase is mainly mediated via protease-activated receptor-2 (PAR-2), which belongs to the G-protein-coupled receptor

family.^{9–12} Four forms of PARs have been reported (PAR-1 through PAR-4). In particular, PAR-2 can be activated by proteases such as trypsin and tryptase. These proteases cleave the N terminus to generate a tethered ligand, which interacts and activates the receptor.^{13–20} Signaling via PAR-2 expressed on ECs elicits activation of the major members of the mitogen-activated protein kinase phosphorylation family and induces EC proliferation. PAR-2 activation also leads to the production of other proangiogenic factors, such as vascular endothelial growth factor, interleukin-8 (IL-8), IL-6, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor.^{21–27}

In literature, no data have been published on the relationship between MC density positive to tryptase (MCDPT), ECs positive to PAR-2 forming microvascular density (PAR-2-MVD), and classical MVD (C-MVD) in hepatocellular carcinoma (HCC) angiogenesis.^{28–30}

In this pilot study, we analyzed the number of MCDPT, PAR-2-MVD, and C-MVD to correlate to each other in primary tumor tissue from HCC patients who underwent surgery.

Materials and methods

Study populations

A series of 53 HCC patients with early stage (stage 0 according to the Barcelona Clinic Liver Cancer staging classification) were selected and underwent curative liver resection: segmentectomies and left and right hepatectomies.^{31–34} The pretreatment evaluation included biochemical liver function, indocyanine green clearance test, complete blood count, coagulation profile, dose serum alpha-fetoprotein, chest X-ray, liver ultrasound with contrast medium (contrast-enhanced ultrasound), and a computed tomography scan of the abdomen. The diagnosis of HCC was histologically confirmed by echo-guided needle aspiration or, alternatively, by classic imaging findings for HCC associated with a pathological increase of alpha-fetoprotein levels higher than the cutoff of 200 ng/mL. In the global series, there were 53 HCCs.^{35,36} The clinicopathological features of the patients are summarized in Table 1. Full ethical approval and signed consent were obtained from individual patients. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the “Mater Domini” Hospital, “Magna Graecia” University, Catanzaro (2011.61; December 13, 2011).

Immunohistochemistry

For the evaluation of MCDPT, PAR-2-MVD, and C-MVD, a three-layer biotin–avidin–peroxidase system was utilized.³⁷

Table 1 Clinicopathological features of 53 patients with hepatocellular carcinoma

| | |
|---------------------------------------|---|
| Age (years), median value | 68 |
| Sex (M/F) | 33/20 |
| Etiology (HCV/HBV/alcoholic/NASH) | 31/14/4/4 |
| Child-Pugh A | 53 |
| Serum AFP (ng/mL), median value | 69 |
| Serum bilirubin (mg/dL), median value | 1.7 |
| Serum AST (IU/L), median value | 53 |
| Serum ALT (IU/L), median value | 36.5 |
| Histologic grade | G ₁₋₂ : 46; G ₃ : 7 |
| Liver segments | |
| IV | 5 |
| II | 12 |
| VI | 14 |
| V | 9 |
| VII | 13 |
| Performance status 0 | 53 |
| BCLC stage 0 | 53 |

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; F, female; HBV, hepatitis B virus; HCV, hepatitis C virus; IU, international unit; M, male; NASH, nonalcoholic steatohepatitis.

Briefly, 4 µm thick serial sections of formalin-fixed and paraffin-embedded tumor samples and adjacent normal liver tissue were cut. Sections were then microwaved at 500 W for 10 minutes, after which endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Tumor sections were incubated with the following primary antibodies: antitryptase (clone AAI; Dako, Glostrup, Denmark) diluted 1:100 for 1 hour at room temperature, anti-PAR-2 (C-17, sc-8205; Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:50 for 1 hour at room temperature, and anti-CD34 antibody (QB-END 10; Bio-Optica, Milan, Italy) diluted 1:50 for 1 hour at room temperature as a pan-endothelial marker, respectively. The bound antibody was visualized using a biotinylated secondary antibody, an avidin–biotin peroxidase complex and liquid permanent red (LPS, K0640; Dako). Nuclear counterstaining was performed with Gill’s hematoxylin no 2 (Polysciences, Warrington, PA, USA). The primary antibody was omitted in negative controls.

Morphometrical assay

Light microscopy integrated with an image analysis system (Quantimet-500 Leica, Wetzlar, Germany) was utilized.³⁷ In both tumor sections and adjacent normal liver sections, immunostained areas (hot spots) were selected at low magnification (×100), then MCDPT (Figures 1A and 2A, respectively), PAR-2-MVD (Figures 1B and 2B, respectively), and C-MVD (Figures 1C and 2C, respectively) were assessed at ×400 magnification (0.19 mm² area).

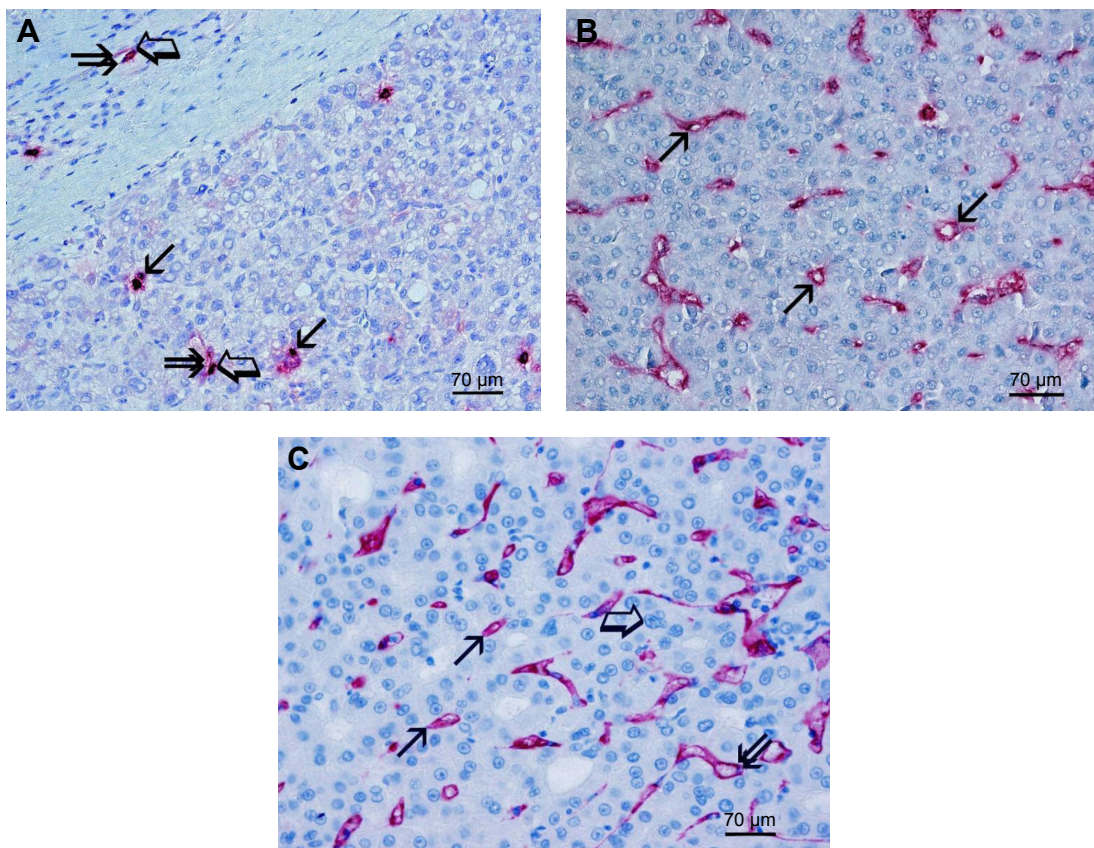


Figure 1 Primary hepatocellular cancer tissue sections.

Notes: Magnification: $\times 400$ at light microscopy. **(A)** Several scattered red immunostained mast cells positive to the antitryptase antibody. Single small arrows indicate single red immunostained mast cells. Big arrows indicate single mast cells adjacent to a microvessel and double small arrows indicate red immunostained tryptase forming a microvessel with its internal lumen. **(B)** Many red immunostained microvessels positive to the anti-PAR-2 antibody. Small arrows indicate single microvessels. **(C)** Many red immunostained microvessels positive to the anti-CD34 antibody. Small arrows indicate single microvessels, big arrow indicates a blue stained nucleus of a liver cancerous cell, and double arrow indicates a red immunostained vessel with many red blood cells in its lumen.

Abbreviation: PAR-2, proteinase-activated receptor-2.

Statistical analysis

Mean values ± 1 standard deviation (SD) of all the evaluated tissue parameters are reported in Table 2. Correlations between MCDPT, PAR-2-MVD, and C-MVD were calculated using Pearson's (*r*) analysis. Correlations among all the analyzed parameters and the main clinicopathological features listed in Table 1 were performed by the chi-square test. $P < 0.05$ was considered significant. All statistical analyses were performed with the SPSS statistical software package (SPSS, Inc., Chicago, IL, USA).

Results

The clinicopathological features of the patients with data expressed as median values are summarized in Table 1. In particular, a series of 53 HCC patients with early stage disease (stage 0 according to the Barcelona Clinic Liver Cancer staging classification) were studied.

In tumor tissue, mean values ± 1 SD of MCDPT, PAR-2-MVD, and C-MVD were 11.02 ± 4.73 , 22.64 ± 7.12 , and

25.34 ± 8.76 , respectively. In adjacent normal liver tissue, mean values ± 1 SD of MCDPT, PAR-2-MVD, and C-MVD were 3.71 ± 1.22 , 6.30 ± 2.28 , and 11.44 ± 4.67 , respectively. With special regard to tumor tissue, a significant correlation between MCDPT and C-MVD ($r = 0.77$, $P = 0.02$), between PAR-2-MVD and C-MVD ($r = 0.81$, $P = 0.01$), and between MCDPT and PAR-2-MVD ($r = 0.67$, $P = 0.03$) (Figure 3) was found. The results described above are summarized in Table 2. Furthermore, no correlation concerning MCDPT, PAR-2-MVD, and C-MVD and the main clinicopathological features was found.

Discussion

HCC is the fifth leading cause of cancer mortality in the world. HCC is a well-established hypervascular tumor with a high rate of angiogenesis.³⁸ In recent years, MCs have been revealed to be involved as important players in tumor angiogenesis by means of the release of proangiogenic factors stored in their secretory granules. However, the role of

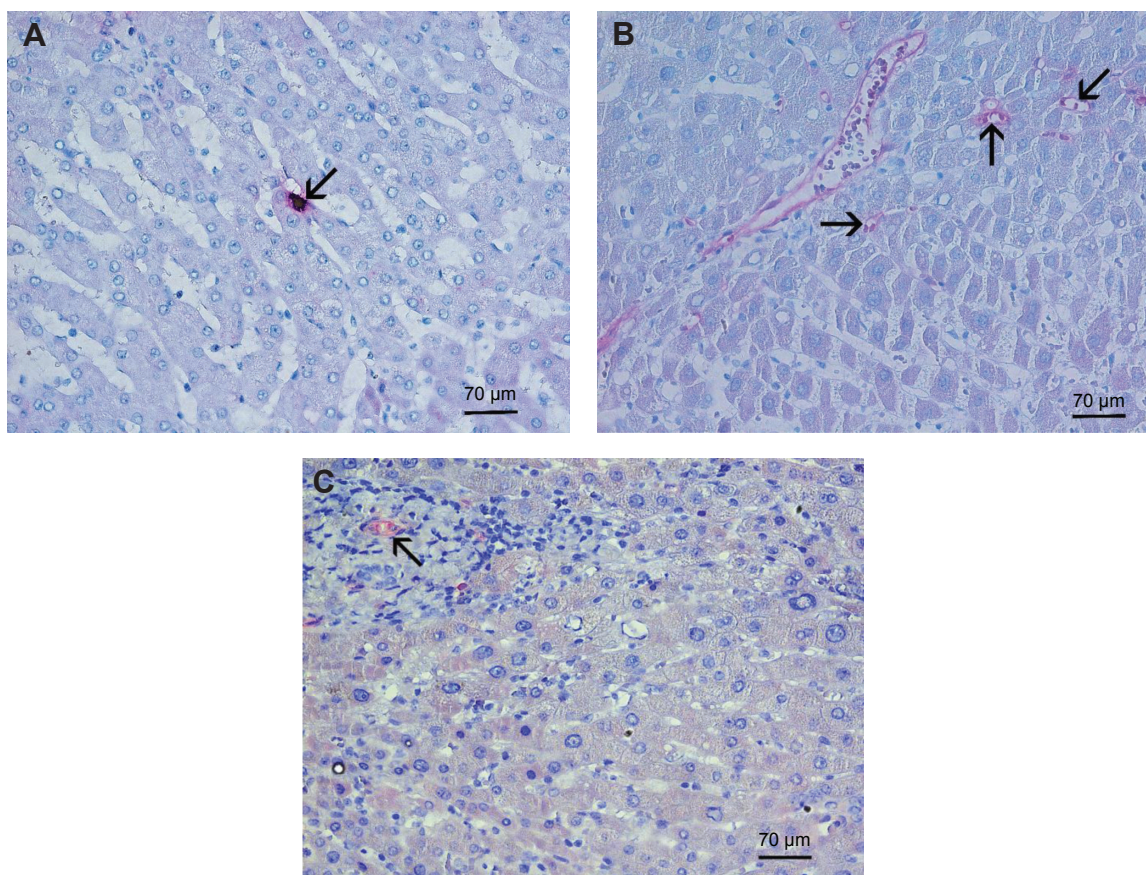


Figure 2 Normal liver tissue sections.

Notes: Magnification: $\times 400$ at light microscopy. **(A)** Arrow indicates the red immunostained mast cell positive to the antitryptase antibody. No other immunostained mast cells are evident. **(B)** Few immunostained microvessels positive to the anti-PAR-2 antibody. Arrows indicate single microvessels. **(C)** Arrow indicates the red immunostained microvessel positive to the anti-CD34 antibody. No other immunostained microvessels are evident.

Abbreviation: PAR-2, proteinase-activated receptor-2.

MCDPT in HCC angiogenesis has not been well investigated, and no data have been published regarding MVD in terms of PAR-2 endothelial expressing cells.³⁹

In the tumor microenvironment, MCs can be activated in different ways such as: c-kit receptor activation and phosphorylation by stem cell factor, immunoglobulin E mechanism

mediated by T lymphocyte–MC interaction, and other microenvironmental stimuli.^{40,41} After activation, intensive or piecemeal degranulation of secretory granules occurs depending on the MC activation mechanism, and MC-derived proangiogenic factors are released into the tumor microenvironment stimulating angiogenesis.⁴² Among them, tryptase has been characterized as a powerful nonclassical angiogenic factor in recent years.^{7,43–45}

Table 2 MCDPT, PAR-2-MVD, and C-MVD (mean \pm standard deviation) as a function of HCC primary tumor tissue adjacent to normal tissue

| Tissue | MCDPT 400 \times (0.19 mm ²) | PAR-2-MVD 400 \times (0.19 mm ²) | C-MVD 400 \times (0.19 mm ²) |
|----------------|---|---|---|
| Primary tumor | 11.02 \pm 4.73 | 22.64 \pm 7.12 | 25.34 \pm 8.76 |
| Normal control | 3.71 \pm 1.22 | 6.30 \pm 2.28 | 11.44 \pm 4.67 |

Abbreviations: C-MVD, classical microvascular density; MCDPT, mast cell density positive to tryptase; MVD, microvascular density; PAR-2, proteinase-activated receptor-2.

Tryptase is an agonist of PAR-2 in vascular ECs, which stimulates their proliferation. Signaling via PAR-2 on ECs elicits activation of the major members of the mitogen-activated protein kinase phosphorylation family and contributes to proliferation of ECs and then angiogenesis. Experimental data also suggest that PAR-2 activation leads to the production of other proangiogenic factors, such as vascular endothelial growth factor, interleukin-8, IL-6, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor.^{23–27,46–48}

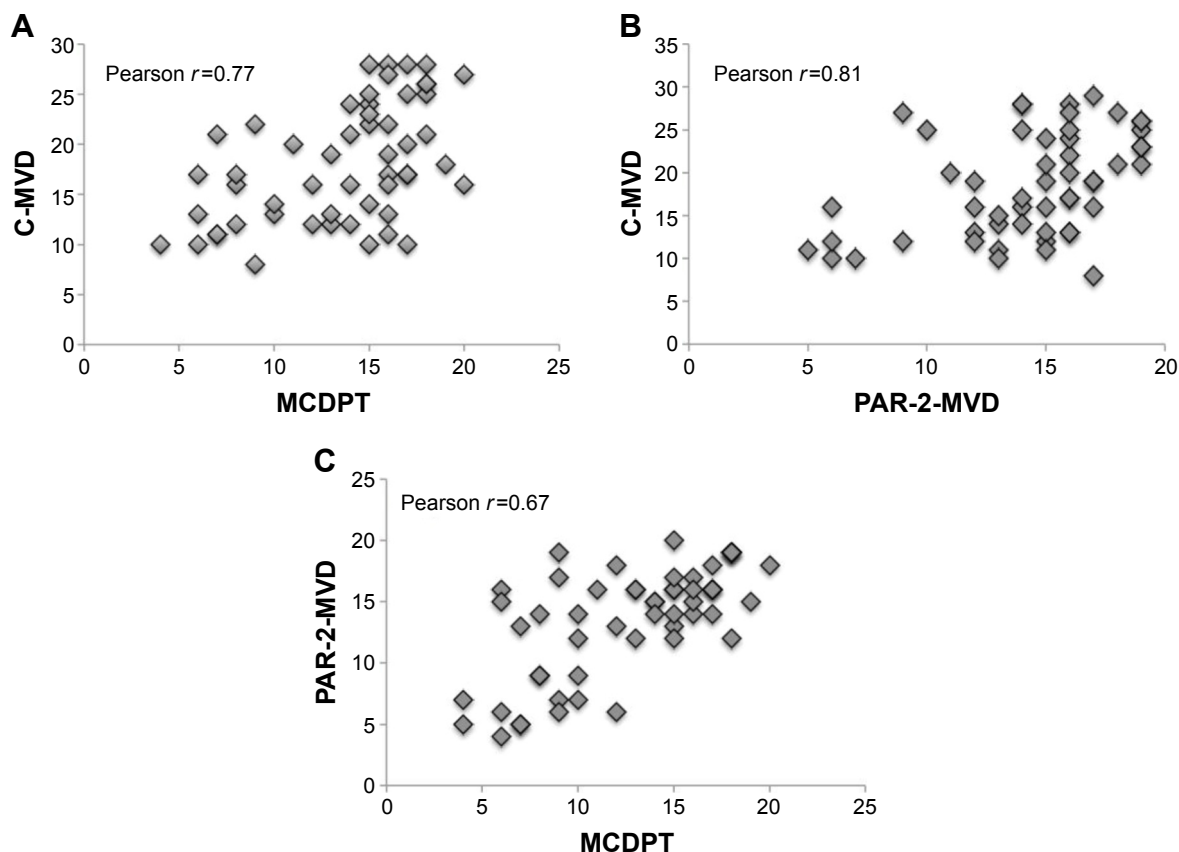


Figure 3 Correlation (A) between MCDPT and C-MVD ($r=0.77$, $P=0.02$), (B) between PAR-2-MVD and C-MVD ($r=0.81$, $P=0.01$), and (C) between MCDPT and PAR-2-MVD ($r=0.67$, $P=0.03$).

Abbreviations: C-MVD, classical microvascular density; MCDPT, mast cell density positive to tryptase; MVD, microvascular density; PAR-2, proteinase-activated receptor-2.

Among the few available data regarding the role of MC tryptase and angiogenesis in HCC patients, Grizzi et al⁴⁹ have demonstrated that MC accumulation at the tumor site may lead to increased rates of tumor vascularity and, consequently, increased rates of tumor growth and metastasis. In this study, no immunohistochemical method was employed in that MC density was evaluated by a histochemical method using toluidine blue stain, and tumor vascularization was also evaluated by Direct-red 80.⁴⁹

In order to analyze the role of MC tryptase, Goffredo et al suggested the potential biomarker role of tryptase in 30 HCC patients, and the study showed decreased serum tryptase levels after hepatic transarterial chemoembolization treatment, indicating that tryptase is concentrated in primary tumor tissue.²⁸

Here, our pilot results demonstrate an association between MCDPT, PAR-2-MVD, and C-MVD, supporting the central role of tryptase as a main proangiogenic factor in primary HCC. Based on these data, it is possible to speculate that the inhibition of tryptase by means of gabexate mesilate or

nafamostat mesilate could be a novel antiangiogenic strategy worthy of clinical investigation.⁵⁰⁻⁵³

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Author contribution

Michele Ammendola and Girolamo Ranieri conceived and designed the study; Rosario Sacco, Bruno Nardo, Giuseppe Sammarco, Michele Ammendola, and Alessandra Zullo performed the surgery; Cosmo Damiano Gadaleta, Ilaria Marech, Tullio Piardi, and Patrick Pessaux analyzed the data; Valeria Zuccalà, Rosa Patruno, Alberto Crovace, Nicola Zizzo, and Girolamo Ranieri contributed reagents/materials/analysis tools and immunohistochemistry. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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