

# The tyrosine kinase inhibitors effects on metastatic tumor graft in the chick chorioallantoic membrane assay

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## Abstract

**Background:** Due to its heterogeneous nature, pancreatic cancer has a higher incidence and a clinical treatment failure. In this study, we present the effects of Avastin, Rapamycin and their combination on the pancreatic liver metastatic human tumor graft in the chick chorioallantoic membrane (CAM) assay. **Materials and Methods:** We conducted this study with 33 fertilized chicken eggs, incubated at 37°C, divided into three working groups: control (three eggs), first (10 eggs), second (10 eggs), and third group (10 eggs). A cell suspensions derived from human liver metastasis of pancreatic tumor were implanted on the CAM, in the ring. First group was treated with 2 µL Avastin (Bevacizumab 25 mg/mL), the second with Rapamycin and the third with Avastin and Rapamycin combination on days 10, 12, 14 of incubation. The immunohistochemical techniques using vascular endothelial growth factor A (VEGFA), CD34, podoplanin, platelet-derived growth factor subunit A (PDGFA) and epidermal growth factor receptor (EGFR) as primaries antibodies were performed on metastatic tumor and metastatic tumor graft. **Results:** Our results showed that the unique treatment with Avastin gave rise to metastases on CAM xenograft, due likely to inflammatory infiltrate and vascular remodeling. The lowest immunoexpression of CD34, podoplanin, PDGFA, EGFR has been noticed in the Rapamycin-treated group without important differences correlated to dosage and time. In the third group, decreased value was found for PDGFA only. The periphery of the tumor graft malignant cells intensely expressed VEGFA, podoplanin and EGFR. **Conclusions:** The inhibitory therapy with mechanistic target of Rapamycin (mTOR) and Avastin may favor the epithelial to mesenchymal transition by podoplanin and phosphatase and tensin homolog (PTEN) pathways in liver metastasis pancreatic graft to CAM.

**Keywords:** tyrosine kinase, metastatic tumor graft, pancreatic adenocarcinoma, mesenchymal transition.

## Introduction

The chick chorioallantoic membrane (CAM) as an experimental model used for tumor growth presents many advantages: vascularization of the CAM, reduced cost, simplicity, incomplete development of the chick's immuno-competent system. In the digestive area, the *in ovo* chick CAM assay was described as an efficient xenograft model of hepatocellular carcinoma and was used to test the pancreatic duct tumor cells lines [1].

The heterogeneous nature of the pancreatic cancer is sustained by the interrelations between the signaling pathways: angiogenesis [vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), related proteins family Ras, phosphatidylinositol 3'-kinase/Akt/mammalian target of Rapamycin (PI3K/Akt/mTOR), Src, Janus kinase/signal transducer and activator of transcription (JAK/STAT)], cancer stem cells (Notch, Hedgehog, Wnt signaling), and stromal environment. All of the above-mentioned may explain the clinical treatment failure and why the incidence of pancreatic cancer remains higher and increased gradually, compared with those of other common cancer. Developments in detection and treatment did not increase the 5-year survival rate, which remained about 4% only [2].

It was noticed that more than 50% of patients with pancreatic cancer have liver metastases at the time of diagnosis and are associated with a poor prognosis [3, 4].

On the other hand, it was showed that the recurrence rate of pancreatic ductal adenocarcinoma was very high and the 5-year survival rate reduced to 10–20% [5]. Some data suggested that several pathways, mechanisms and molecules, such as: EGFR, E-cadherin, laminin gamma-chain, VEGF were associated with postoperative hepatic metastasis and poor prognosis in ductal pancreatic adenocarcinoma [6–9].

The murine experimental methods, morphological, immunohistochemical (IHC) and array data showed that VEGF expression rate was lower compared with primary tumor in liver metastasis, with a slight increase at the tumor invasion front [10]. The evaluation of the liver metastasis obtained by orthotopically injected of human pancreatic cancer AsPC-1 cells into immunodeficient/beige mice showed the highest suppression of metastatic tumor growth in mice receiving the combination therapy: Rapamycin with anti-VEGF antibody 2C3 [11].

From the four histological growth pattern described in liver metastasis with digestive origin (pushing, replacement, desmoplastic and mixed), the replacement type was described as the most frequent in the case of pancreatic primary tumor [12, 13].

The current standard therapy for patient with metastatic disease includes Gemcitabine, Nab-Paclitaxel, 5-Fluorouracil, Leucovorin, Irinotecan, Oxaliplatin. No standard chemotherapy for the patient after pancreatectomy has been established [5, 14]. Starting from these premises, in the present work, we studied the effect of Avastin,

Rapamycin and their combination on the pancreatic liver metastatic human tumor graft in the CAM assay.

## ☞ Materials and Methods

The first step of the CAM experimental model preparation included the incubation of 33 fertilized chicken eggs at 37°C. On day 3 of incubation, a small part of the eggs' shell was removed. The small window resulted in the eggs' shell was covered with Parafilm. The eggs were then incubated for another seven days. A ring of silicone was applied onto the CAM surface of each egg, on day 10 of incubation (day 1 of the experiment). The eggs were divided into three groups: control (three eggs), group 1 (10 eggs), group 2 (10 eggs) and group 3 (10 eggs).

The metastatic liver fragment was taken from the patient by performing excision of the tumor of the liver metastasis. All procedures were done according with the principles of the Declaration of Helsinki and were approved by the Institutional Review Board. A cell suspension derived from human liver metastasis of pancreatic tumor has been implanted on the CAM, in the ring. The remaining of the metastatic fragment was fixed in buffer formalin, in order to perform the morphological evaluation. After two days, when the tumor xenografts were visible, first group was treated with 2 µL of Avastin (Bevacizumab 25 mg/mL) on days 1 and 3 of the experiment (day 10, 12, 14 of incubation). The same dose of Rapamycin was applied to the CAM of the second group, in the ring. The combined doses of Avastin and Rapamycin were applied on the CAM from the group 3 with similar frequency.

On day 7 (day 16 of incubation), the experiment was stopped and the CAM of each egg was detached, fixed in 10% buffered formalin and paraffin embedded. Slides from each case were stained with Hematoxylin–Eosin (HE) for histopathological evaluation and also for case selection for IHC procedures.

For the IHC technique, cytokeratin (CK) 7 (monoclonal, clone RN7, ready to use – RTU, Leica Biosystems, Newcastle upon Tyne, UK), CK8/18 (monoclonal, clone 5D3, RTU, Leica Biosystems, Newcastle upon Tyne, UK), CK19 (monoclonal, clone b170, RTU, Leica Biosystems, Newcastle upon Tyne, UK), VEGFA (mouse, human VEGF-165 recombinant protein, 1:50 dilution, RELIAtech GmbH, Wolfenbuttel, Germany), CD34 (monoclonal, clone QBEnd/10, RTU, Leica Biosystems, Newcastle upon Tyne, UK), podoplanin (mouse monoclonal, clone 4D5aE5E6, 1:100 dilution, RELIAtech GmbH, Wolfenbuttel, Germany), platelet-derived growth factor subunit A (PDGFA, N-30, sc-128, purified rabbit polyclonal, 1:50 dilution, Santa Cruz Biotechnology, Inc., Europe), and EGFR (monoclonal, clone EGFR.25, RTU, Novocastra, Leica Biosystems, Newcastle upon Tyne, UK) were used as primary antibodies. Before the incubation with primary antibodies, the enzyme pretreatment (for CK19, for 10 minutes) and heat-induced epitope retrieval with Bond Epitope Retrieval Solution 2, RTU, solution pH 9 (Leica Biosystems, Newcastle upon Tyne, UK) were performed for 20 minutes. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 minutes. Bond Polymer Refine Detection System was used for visualization. As chromogen, 3,3'-Diaminobenzidine (DAB) dihydrochloride was applied for 10 minutes and

Hematoxylin for 5 minutes, as a counterstain. The entire IHC procedure was performed with Leica Bond-Max autostainer (Leica Biosystems, Newcastle upon Tyne, UK). The cytoplasmic expression was evaluated as positive reaction in both tumor and endothelial cells.

## ☞ Results

The morphological evaluation of liver metastases with human pancreatic origin revealed a poorly differentiated type of pancreatic ductal adenocarcinoma. The histological growth pattern noticed was mixed, with both, a pushing and replacement type components (Figure 1a).

The pancreatic origin of cell suspensions derived from liver metastasis and implanted on the CAM was proved by the IHC expression of CK7, CK19, and CK8/18 (Figure 1, b–d).

The IHC evaluation of VEGF in the liver metastasis with pancreatic origin showed a granular cytoplasmic pattern, with heterogeneous distribution. The intensity value of immunoexpression in the remnant liver was 3, while in the metastatic area the intensity values of expression decreased from central area, where isolated positive cells with value 3 of intensity were found, to the periphery, in the vicinity of the liver, where the value of immunoexpression intensity was 2. CD34 was expressed in the vessels of portal spaces, not in the hepatocytes. The vessels were characterized by the following aspects: the absence of the lumen in the blood vessels from the central area of metastasis, and the presence of intussusception in the vessels from the border: remnant liver-metastatic area. The number of podoplanin-positive lymphatic vessels was higher to the periphery, in comparison with central part of metastatic area. The podoplanin expression was noticed in the portal spaces and subcapsular. In the portal spaces, PDGFA was positive in the endothelial cells. For EGFR, a heterogeneous pattern was found with variable values of intensity between 1 and 3 in the tumor cells from metastatic area.

The macroscopic evaluation of the pancreatic liver metastatic human tumor graft in the chick CAM assay revealed a reduced bleeding in the ring, in the 10<sup>th</sup> day.

In the day 14, we noticed the increased dimensions for the tumor graft in all of the cases. The cases treated with Rapamycin were vascularized. The cases from the group 3, treated with combined therapy showed a heterogeneous distribution, with persistent blood vessels.

In the 16<sup>th</sup> day, the cases were characterized by the presence of the secondary metastasis on the CAM surface for the group previously treated with Avastin. The blood vessels were small, but still present. The Rapamycin application did not influence the tumor growth and vascularization. The Avastin and Rapamycin combined therapy applied on tumor graft had as consequences the decreased of tumor graft volume and vascularization. The blood vessels presented a heterogeneous distribution. All of these macroscopic aspects may be noticed in the Figure 2.

Morphological staining revealed a squamous differentiation of chorionic epithelium and the presence of inflammatory infiltrate in the tumor area, in the first



group, treated with Avastin. In the second group, treated with Rapamycin, tumor necrosis and bleeding in the adjacent tumor graft tissue were noticed.

In the Avastin treated group – day 14, the immunostaining for VEGF has a granular cytoplasmic pattern, with intensity value of 3 in the tumor cells disposed to the periphery of the pancreatic liver metastatic tumor graft, in the chick CAM. In the center of the tumor area, a reduced intensity of VEGFA expression (value 1) was found. In the same region, the VEGF expression in endothelial cell was heterogeneous, present in some cells and absent in others.

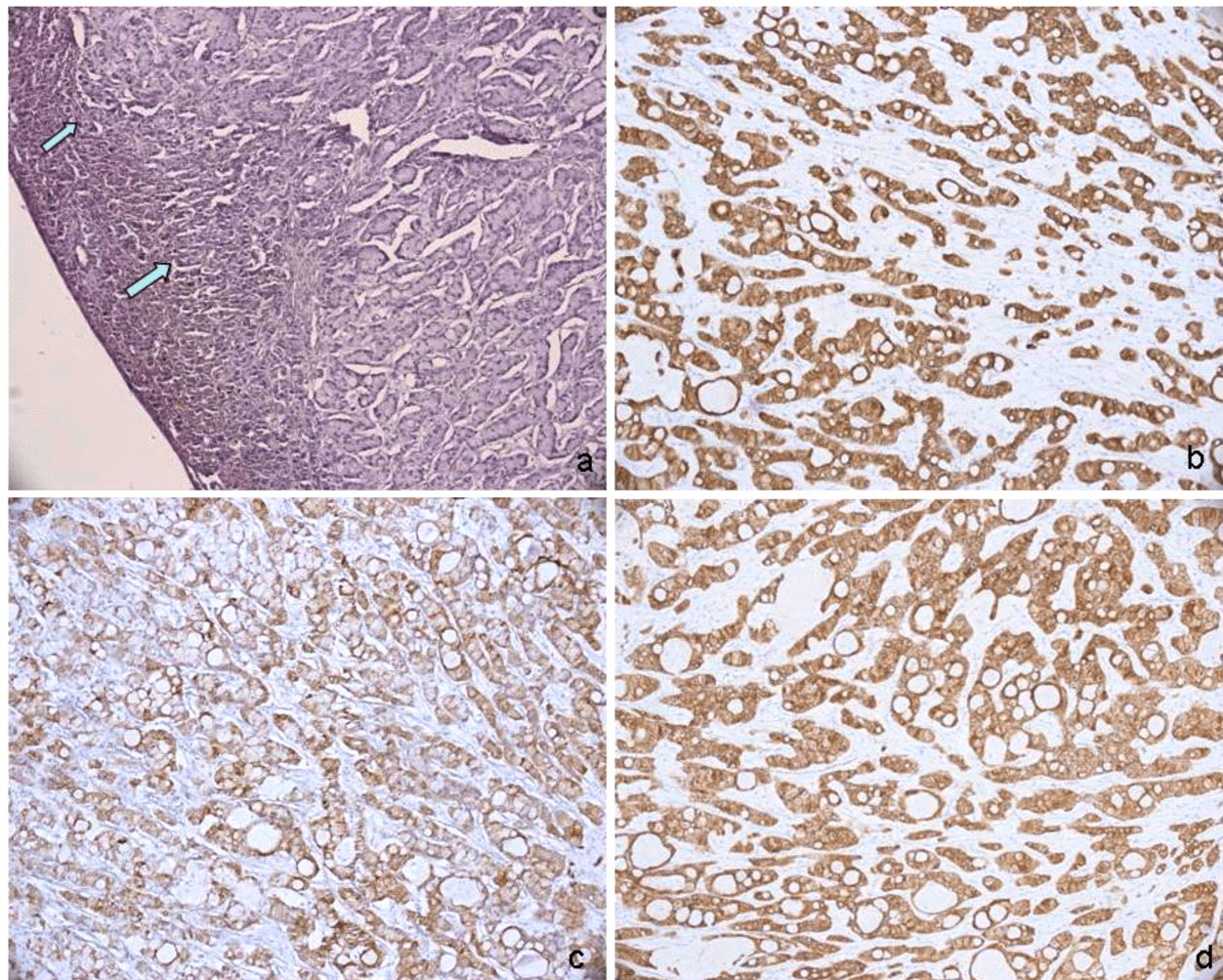
The effect of the Rapamycin to the same group was the complete VEGF absence in the endothelial cells of the tumor graft. In the tumor cells, the intensity value of VEGF IHC expression was 2, with homogeneous distribution in the entire tumor area and granular cytoplasmic pattern.

In the group 3, treated with Avastin and Rapamycin, VEGF expression was similar with those from Rapamycin-treated group in the graft tumor cells. The difference was noticed in the endothelial cells, where the VEGF expression was present.

The Avastin-treated group (16<sup>th</sup> day) was characterized by the decreased intensity and distribution of VEGF

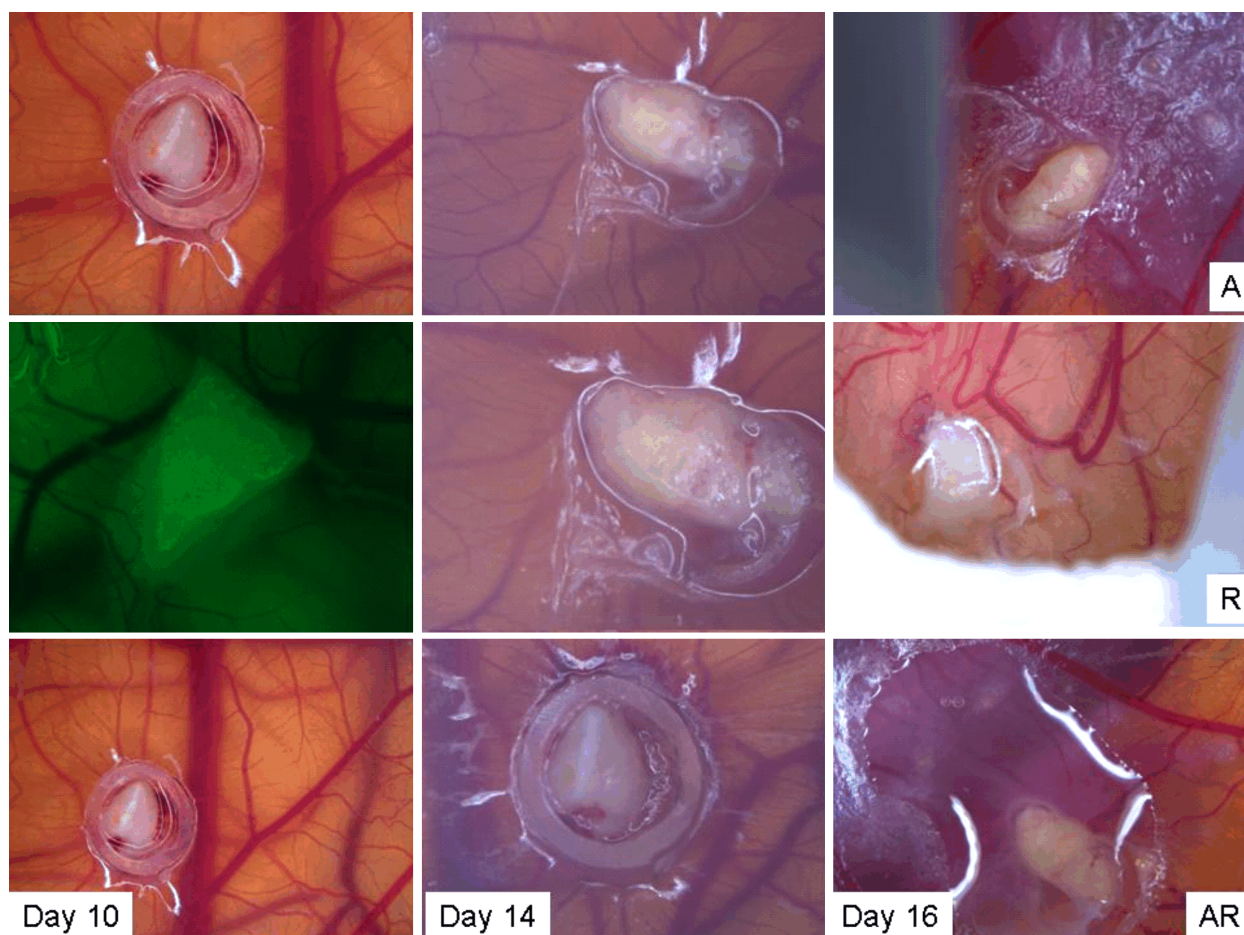
expression in the tumor cells of the graft. Small groups of VEGF-positive tumor cells with intensity value 1 and granular cytoplasmic pattern were noticed. Between them were found isolated tumor cell with intensity value 2. The blood vessels with alternating VEGF-positive and -negative endothelial cell were present. A tendency to keratinization was present also. Comparatively with the first group, the effect of the Rapamycin treatment in the second group was VEGF expression in endothelial cells. The combination of Avastin and Rapamycin was associated with VEGF expression in tumor cells graft and endothelial cells more intensely to the periphery (value 3) compared to the central area of the graft (value 2).

The immunoexpression of CD34 in the second group (14<sup>th</sup> day) treated with Avastin revealed a higher microvascular density to the periphery compared to inner area of tumor graft (Figure 3a). Blood vessels with intussusception phenomenon were present in the center and to the periphery of tumor graft. The same aspect was found after treatment with Rapamycin regarding the microvascular density, but the morphology of blood vessels was different: narrow or absent lumen and small dimensions (Figure 3b). The Avastin–Rapamycin combination was followed by the presence of vessels without lumen mainly, with distribution throughout the tumor area (Figure 3c).



**Figure 1 – Histological growth pattern of liver metastasis with pancreatic origin: (a) HE staining, ×100. Expression of CK7 (b), CK19 (c), and CK8/18 (d) in liver metastasis with pancreatic origin (IHC staining, ×100). HE: Hematoxylin–Eosin; CK: Cytokeratin; IHC: Immunohistochemical.**





**Figure 2 – The macroscopic aspect of the cases from the groups treated with Avastin (A), Rapamycin (R) and Avastin plus Rapamycin (AR), in the days 10, 14, and 16.**



**Figure 3 – Immunoexpression of CD34 in tumor xenograft, day 14, after treatment with Avastin (a), Rapamycin (b), and Avastin–Rapamycin combination (c). Anti-CD34 antibody immunomarking: (a)  $\times 100$ ; (b and c)  $\times 400$ .**

The continue applying therapy to the tumor graft, indicated in the day 16, in the first group, treated with Avastin, the absence of CD34 expression in the endothelial cell of tumor graft. The second group, treated with Rapamycin showed only a few vessels in the central area of tumor graft, without lumen. To the periphery of tumor graft, vessels with and without lumen, more numerous toward the centre, were found. The third group, treated with Avastin and Rapamycin had numerous blood vessels with and without lumen in the entire tumor area.

For all groups included in the study, in the 14<sup>th</sup> and 16<sup>th</sup> days, the immunoexpression of podoplanin in tumor cells had the value 3 of intensity to the periphery of the tumor graft. The most reduced intensity, value 1, was noticed in tumor cells from the central part of tumor

graft, in the second group, treated with Rapamycin, in the both, 14<sup>th</sup> and 16<sup>th</sup> days. For the first group and the third group, treated with Avastin and Rapamycin, a decreased of IHC expression from value 3, in 14<sup>th</sup> day, to value 2 in the 16<sup>th</sup> day was noticed.

PDGFA expression was influenced by the Rapamycin treatment, in days 14 and 16, the immunoexpression intensity value in tumor cells was 2. The first group, treated with Avastin, expressed PDGFA in the periphery of tumor graft, with intensity value 3. A decreased intensity (value 2) was noticed in the centre of metastatic area. The expression of PDGF was found in the endothelial cells also. The decreased value intensity from 3, in day 14 to value 2 in day 16, in the entire area of tumor graft was noticed in the third group, treated with Avastin and Rapamycin combination.

After the first dose of Avastin, the decrease of EGFR immunoexpression intensity (value 2) was found in the cells from the central part of tumor graft, compared with the periphery (value 3). The EGFR reaction in the tumor cells and endothelial cells was negative in the first group, day 16, in the second group, days 14 and 16, and in the third group. In this last group, treated with Avastin and Rapamycin combination, in the 14<sup>th</sup> day, a reduced number of positive tumor cells with intensity value 2 of reaction were noticed to the periphery of tumor area. In day 16, groups of positive tumor cells, intensity value 3 were found diffusely in the tumor graft.

## Discussion

VEGFA, the “vascular permeability factor”, has five major isoforms: 121, 145, 165, 189 and 206 [15, 16]. The first two isoforms are usually secreted while the last two are found in the extracellular matrix [16]. VEGF-165 is half secreted and half bound to the cell surface and the extracellular matrix [17]. VEGF plays a crucial role in tumor angiogenesis. Influenced by some extrinsic factors, mainly by hypoxia, pancreatic cells produce VEGF. Expression of VEGF is regulated mainly by hypoxia inducible factor-1 (HIF-1) signaling. In pancreatic ductal adenocarcinoma, the high levels of hypoxia and HIF-1 expression were correlated with prognosis and with VEGF expression [18, 19]. The last was associated with the presence of lymph nodes and liver metastases [20, 21], grade and mean microvascular density (MVD) [9]. Opposite results were found by others authors [22].

Niedergethmann *et al.* [10] showed immunoreactivity with a score of 3 in primary tumors – pancreatic ductal adenocarcinoma, whereas in the liver metastases, VEGF expression rate was lower with score of 2. The tumor invasion front showed a staining with a score of 2.8. In our study, we noticed VEGF-positive cells with intensity value of 2 in the metastatic area, with isolate tumor cells with higher intensity (value 3) localized in the central part of liver metastasis. The VEGF immunoexpression intensity value decreased near remnant liver. After the first dose of Avastin, in day 14, the tumor cells with higher intensity, value 3, were noticed to the periphery of tumor graft on the CAM, while in the central part positive tumor cells with intensity value 1 were found. In the tumor cells, VEGF production may be stimulated by insulin-like growth factor, activation of PI3K, activation of Akt, due to phosphatase and tensin homolog (PTEN) inactivation. The last one induced podoplanin expression. It was demonstrated that podoplanin was positive in a variety of tumors and its expression was associated with poor prognosis. As the malignant cells expressing podoplanin often show a mesenchymal phenotype reminiscent of an epithelial–mesenchymal transition (EMT) [23], podoplanin could be considered as an EMT inducer. In our study, to the periphery of tumor graft was noticed the highest intensity of podoplanin immunoexpression (value 3) in tumor cells from all treated group.

The tumor progression, immune suppression, angiogenesis and tumor metastasis are usually associated with inflammation process. In response to tumor-derived growth factors and chemokines, inflammatory cells of the immune system are recruited to the tumor micro-

environment [24, 25]. Mast cells, granulocytes and monocytes, provide the tumor with angiogenic factors, enzymes for extracellular matrix (ECM) remodeling and growth factors to create a pro-expansion and metastasis medium [26]. In our study, this influence may be an explanation for the second metastasis, which was present on the CAM surface, in the first group, treated with Avastin only, in the 16<sup>th</sup> day mainly. On the other hand, the alternative expression of VEGF, with positive and negative endothelial cells may be an explanation. The intussusceptive phenomenon, an adaptation response to stress and hypoxia, was the mainly mechanism of vessel generation and vascular remodeling noticed in our study. It was showed that in human colorectal cancer metastasized to the liver, following antiangiogenic therapy with Bevacizumab, the remaining resistant vessels are covered by pericytes. They are much larger in diameter in comparison to capillary vessels. In a genetically engineered mouse model of pancreatic neuroendocrine tumors, long-term treatment with a VEGF receptor-2 blocking antibody generated refractory tumors. Inside the refractory tumors, the abundance of pericyte-covered co-opted vessels was increased [27]. In the 16<sup>th</sup> day of Avastin treatment, blood vessels with lumen, with absence of CD34 expression in endothelial cells were found.

The treatment with Everolimus (RAD001) administered as a single agent to the patient with Gemcitabine-refractory metastatic pancreatic cancer showed minimal clinical activity. Dai *et al.* [28] showed that Rapamycin inhibited the proliferation of pancreatic carcinoma PC-2 cells *in vitro*, in a dose- and time-dependent manner. No differences correlates with the dose and time were found in our study, in the second group treated with Rapamycin. The absence of reaction was found in both, endothelial and tumor cells.

## Conclusions

The lowest immunoexpression values for CD34, podoplanin, PDGFA and EGFR noticed in the Rapamycin-treated group without important differences correlated to dose and time, support the presence of mTOR inhibitor in the therapeutic scheme liver metastasis of pancreatic ductal adenocarcinoma. Our findings may launch the hypothesis that inhibitory therapy with mTOR and Avastin combination favor the EMT by podoplanin and PTEN pathways in liver metastasis pancreatic graft to CAM.

## Conflict of interests

The authors declare that they do not have any conflict of interests and they do not have a financial relationship with the organization that sponsored the research.

## Availability of data and materials

The authors declare that the data supporting the findings of this study are available within the article.

## Ethics approval and consent to participate

All procedures were done according with the principles of the Declaration of Helsinki and were approved by the Institutional Review Board (Consent by the Municipal Emergency Hospital, Timișoara, Romania). All institutional and national guidelines for the care and use of laboratory animals were followed.

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