Pathology – Research and Practice 210 (2014) 901–908

Contents lists available at ScienceDirect

Pathology – Research and Practice

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

Original Article

# Serous neoplasms of the pancreas share many, but not all aspects of their microvascular and angiogenic profile with low-grade clear cell renal cell carcinomas

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ARTICLE INFO

*Article history:*
Received 30 July 2013
Received in revised form 26 March 2014
Accepted 19 June 2014

*Keywords:*
Pancreatic neoplasms
Serous neoplasms
Kidney neoplasms
Clear cell renal cell carcinoma
Angiogenesis

ABSTRACT

Similarly to clear cell renal cell carcinomas (CCRCC), serous neoplasms (SN) of the pancreas frequently show inactivation of *VHL* gene, clear cell histology and abundant microvasculature. Data on the microvascular and angiogenic profile of SN are scarce. Aiming to examine further the striking resemblance of clear cell epithelial neoplasia in pancreas and kidney, we compared the microvascular profile and expression of pro-angiogenic factors in SN and in CCRCC using immunohistochemical stains. SN and CCRCC shared a predominance of differentiated blood vessels, scarcity of lymphatic vessels, presence of CD105 and claudin-5 in tumoral vessels, expression of vascular endothelial growth factor (VEGF)-A, cyclooxygenase-2 (COX-2), carbonic anhydrase IX in tumoral cells, and lack of VEGF-C in tumoral cells. In contrast to CCRCC, SN showed lower pericyte coverage of vessels, lower blood vessel endothelial cell proliferaction fraction, more pronounced VEGF receptor (VEGFR)-2 and glucose transporter-1 expression, higher inducible (iNOS) but lower endothelial nitric oxide synthase (eNOS) expression, as well as presence of VEGFR-3 and D2-40 expression in epithelial cells. In conclusion, we found a significant similarity but not equality of microvascular biology of SN and CCRCC. We recognized VEGFR-2, VEGFR-3, COX-2, iNOS, eNOS and D2-40 as new markers of epithelial cells of SN of the pancreas.

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## Introduction

Serous neoplasms (SN) are relatively rare and almost always benign tumors of the pancreas. They are usually composed of hundreds of small cysts (microcystic serous cystadenomas) and only rarely form oligolocular or solid lesions. The cysts in SN are lined by glycogen-rich, clear cells and supported by subepithelial microvessels. SN may develop in patients with von Hippel–Lindau (VHL) disease as well as sporadically. Inactivation of *VHL* gene is the most prevalent genomic aberration in SN [14,23,31,44,69,75,78].

Clear cell carcinomas are the most common cancers of the kidney. Similarly to SN, clear cell renal cell carcinomas (CCRCC) may appear either in patients with VHL disease or sporadically. Inactivation of *VHL* gene (allelic deletion, mutation, promoter hypermethylation) is a common finding in CCRCC [6,7,21,47,78,81]. Abundant microvasculature is a classical histopathological feature of CCRCC [21], but the molecular mechanisms which lead to angiogenesis in CCRCC remained unknown just until recently.

*VHL* gene alterations are responsible for so-called 'pseudohypoxia' [10] in tumoral tissue of CCRCC, which results in an increase of pro-angiogenic factors. This oxygen-independent process is constitutive in the majority of both sporadic and VHL-disease-related CCRCC, and it is mediated by hypoxia-inducible factor-1α (HIF-1α). Stabilization of HIF-1α and subsequent induction of expression of specific target genes may result from 'true' tumoral hypoxia as well [28,37,60].

HIF-1α activates transcription of a number of genes involved not only in angiogenesis, but also in the regulation of cell growth, proliferation, apoptosis, glucose transport, and pH [7,28,37,60]. Among the most extensively studied HIF-1α target genes are: vascular endothelial growth factors (VEGF), nitric oxide synthases (NOS), glucose transporter-1 (GLUT-1), cyclooxygenease-2 (COX-2), and carbonic anhydrase IX (CA IX) [7,28,37,60,63].

The angiogenic profile of CCRCC is of paramount clinical significance. Anti-angiogenic agents are important therapeutic option for patients with CCRCC, particularly those who are not suitable for surgical resection of primary lesion and/or metastatic deposits, or suffer from tumor relapse [7,37,48,60].

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http://dx.doi.org/10.1016/j.prp.2014.06.033
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There is plentiful literature data on histopathological characteristics of microvasculature of CCRCC. There are many papers on the immunohistochemical profile of microvessels in tumoral tissue and also many reports devoted to the presence and distribution of pro-angiogenic factors in cancer cells [2,4–6,9,12,16,18,24,26,40,50,52,57–59]. In comparison, data on the microvascular and angiogenic profile of SN are scarce. Although the abundance of microvessels in SN was noticed early [3,14], the functional status of these vessels is still not well understood. In 2006, Yamazaki and Eyden described VEGF expression in SN [80]. In 2009, Thirabanjasak et al. [70] showed that SN express HIF-1α and some of its effectors (VEGF, GLUT1, and CA IX).

In the present paper, we describe the microvasculature and angiogenic profile of SN based on immunohistochemical stains. We examined the distribution and characteristics of microvessels in SN and verified the presence of selected pro-angiogenic factors in tumoral cells. The stains performed included: classical vascular markers (CD31, CD34 [45]), new vascular markers (endoglin/CD105 [15], claudin-5 [42]), lymphatic marker (podoplanin/D2-40 [27,43]), pericyte marker (smooth muscle actin, SMA [50]), and pro-angiogenic factors: VEGF-A, VEGF-C, VEGF receptor-2 (VEGFR-2), VEGF receptor-3 (VEGFR-3), COX-2, inducible NOS (iNOS), endothelial NOS (eNOS), GLUT-1, and CA IX [7,28,37,60]. Some vascular markers were examined simultaneously in pairs on single slides – these included CD31/CD34 double stain to highlight microvessel differentiation [79], CD34/SMA double stain to highlight pericyte coverage of vessels [12,17], CD31/D2-40 double stain to highlight the distribution of blood and lymphatic vessels [43], and CD31/claudin-5 stain to compare immunoreactivity for both vascular markers in the same vessels. Blood and lymphatic endothelial cell proliferation fraction (BECP% and LECP%, respectively) were assessed using CD34/Ki-67 and D2-40/Ki-67 double stains, respectively [4–6,72,73]. For comparative purposes, we also examined samples of low-grade, low-stage CCRCC. This allowed us to demonstrate similarities but also significant differences between microvascular and angiogenic status of SN and CCRCC, i.e. two clinically distinct but histologically similar lesions related to *VHL* gene alterations.

## Materials and methods

### *Study cases*

Twenty-three random cases of solitary SN diagnosed at our institution between 2000 and 2011 were included in the study. These cases were included in a cohort of 40 cases of SN, the clinicopathological details of which we have reported previously [46]. All cases included in the analysis represented the (micro)cystic serous adenomas [23,69], some of these tumors also had a peripheral macrocystic component [29], i.e. at least a single cyst larger than 1 cm. We also included 17 samples of solitary CCRCC among cases diagnosed in total or partial nephrectomy specimens during the same time period at our Department. All included CCRCC cases were pT1a tumors (diameter 4 cm or less, tumor limited to the kidney) according to the 2010 AJCC staging criteria [19]. All selected CCRCC showed low nuclear grade [2,5], i.e. Fuhrman [20] nuclear grade 1 or 2.

All the specimens were fixed in 10% buffered formalin. Specimen processing, paraffin embedding and hematoxylin and eosin staining were performed in a standard manner. For each study case, a single paraffin block with abundant tumoral tissue was selected. Double antigen stains were performed using conventional sections, whereas single antigen stains were performed in tissue microarrays (TMA).

Among 23 SN cases included in the present study, 8 cases were examined for microvascular status only (whole conventional sections, double stains), 10 cases were examined for pro-angiogenic factors only (TMA sections, single stains), and 5 cases were examined for both.

Among 17 CCRCC cases in the study, 5 cases were examined for microvascular status (whole conventional sections, double stains), and another 12 were examined for pro-angiogenic factors (TMA sections, single stains).

### *TMA construction*

TMA were prepared using a dedicated instrument (Manual Tissue Arrayer MTA I, Beecher Instruments, Sun Prairie, USA). Five tissue cores (diameter 1.5 mm) were extracted from each donor paraffin block and put into recipient blocks. Multiplication of cores taken from donor block allowed us to ensure representativeness of the cores for the whole block and minimized the risk of obtaining an inadequate tissue core (due to paucicellularity or potential loss during sectioning). That was particularly important for SN cases, since all represented cystic lesions.

### *Immunohistochemical stains*

Four-micrometer-thick sections were cut from conventional paraffin blocks and TMA blocks and put onto Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany). Following deparaffinization and rehydration performed in a routine manner, sections were heated for antigen retrieval, pre-treated for endogenous peroxidase blocking, and incubated with primary antibodies. The details of antigen retrieval procedures, as well as primary antibodies and detection systems used, are described in Supplementary Table 1. For double stains, Envision G2 DoubleStain System (Dako) was used according to the manufacturer's instructions, similarly to previous studies [4,5]. Diaminobenzidine and permanent red served as chromogens, and hematoxylin was a counterstain. Staining procedures were performed using automated machine (Dako). Appropriate external positive controls were established. For negative control, primary antibodies were omitted.

### *Interpretation of immunostains – microvasculature*

The immunochemistry slides were assessed simultaneously by two of the authors (L.L. and J.P.) using a double-headed microscope. In both SN and CCRCC, only intratumoral vessels without muscle layer detectable in routine hematoxylin and eosin staining were taken into consideration. For CD31/CD34 double stains, proportions of differentiated vessels (CD34-positive) among all CD31-positive vessels were assessed (in 5% increments) [79]. For CD34/SMA double stains, proportions of vessels with pericyte coverage (SMA-positive) among differentiated vessels (CD34-positive) were assessed (in 5% increments) [17]. In CD31/D2-40 double stain, distributions of blood and lymphatic vessels were recorded in a descriptive format. In CD31/claudin-5 stain, percentage of claudin-5-postive vessels among CD31-positive vessels was assessed (also in 5% increments). For BECP% and LECP% assessments, the percentages of Ki-67 positive cells among CD34-positive or D2-40-positive endothelial cells in the tumoral tissues, respectively, were calculated by counting 1000 cells. For CD105 stain, distribution of immunopositive vessels was documented descriptively. Due to reasons described in the discussion section, we did not examine blood microvessel density (MVD) and lymphatic (micro)vessel density (LVD) in the study samples.

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