UNIVERSITY OF MEDICINE AND PHARMACY

"VICTOR BABES" TIMIȘOARA
FACULTY OF MEDICINE
DEPARTMENT II HISTOLOGY

CĂLĂRAȘ ADELA MARIA

PhD THESIS

IDENTIFICATION OF PREDICTIVE MARKERS FOR RESISTANCE TO ANTIANGIOGENIC AND ANTIVASCULAR THERAPY IN KIDNEY CANCER

Scientific adviser

PROF. UNIV. DR. ANCA MARIA CÎMPEAN

Scientific adviser co-tutorship

CONF. UNIV. DR. EUGEN MELNIC

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INTRODUCTION

Kidney cancer, or more specifically, renal cell carcinoma (RCC), has some peculiarities. Clinically, it evolves for long periods indolently, without noisy symptoms and in many cases, patients go to the doctor in advanced stages of evolution. On the other hand, microscopic examination of these tumors reveals in most cases, relatively well-differentiated, uniform, tumor cells, having rare cases in which atypical mitosis and cell-nuclear anaplasia are observed. However, the growth rate of RCC is relatively high, with local progression, followed relatively quickly by distant, venous, and lymphatic extension. Searching for new diagnostic methods, potential therapeutic targets are a necessity in the current period for several reasons, partly due to the reduced survival of advanced cases, insufficient response to adjuvant therapy, and in addition, the increase of number of cases in recent decades.

One of the general characteristics of kidney tumors is represented by a very rich vascularization, both in the tumor area and also peritumorally. For a long time, it was thought that the vessels around the tumor were pre-existing, dilated blood vessels, and those inside the tumor were included in proliferation. Today we know that tumor cells are capable of secreting angiogenic factors and forming their own vessels that connect to the systemic circulation. This process of forming new vessels from preexisting ones is called angiogenesis and it is present in all solid human and animal tumors, as a necessity for local tumor progression.

Another important aspect is that the tumor cells are able to secrete angiogenic growth factors, which stimulate the formation of new blood vessels. Interestingly, these vessels are structurally and molecularly different from normal ones. Finally, the angiogenesis process can be inhibited by blocking growth factors and specific receptors, and by normalizing the network of blood vessels associated with the tumor.

Antiangiogenic therapy began more than 25 years ago, but the results obtained in experimental models have not been confirmed by clinical experience. The first humanized monoclonal antibody created against growth factor was bevacizumab (Avastin), which has not been shown to be effective in RCCs, although they are highly vascularized and secrete significant amounts of VEGF.

Given the unknown facts related to antiangiogenic medication in cases with RCC, in this paper we have studied with the help of molecular biology methods at protein level, elements with potential involvement in resistance to therapy, but also potential targets and elements with a role in predicting therapeutic response. We believe that this approach is at the forefront of knowledge of the molecular profile of RCC and can contribute to changing the therapeutic strategy.

There is now ample evidence, certifying on one hand, the role of angiogenesis in the progression of RCC, and on the other hand, the fact that inhibition of new vessel formation can limit tumor expansion. Unlike other localizations of neoplastic disease, in RCC angiogenesis, although intensively studied, is less understood as molecular evolution, and as the formation of types of vessels.

For these reasons, the objectives we have set ourselves in the present research are the following:

1. Definition of the process of angiogenesis and vasculogenesis in the tumor and peritumoral area of RCC.
2. Identification of types of blood vessels in the tumor area.
3. Clarifying the prognostic value of microvascular density and identifying vessels that may be a target for antivascular medication.
4. The correlation between newly formed vessel types and the expression of growth factors with angiogenic potential, considering in particular VEGF, PDGF, FGF.
5. Identification of the main molecular elements having protein expression that may be involved in resistance to antiangiogenic therapy.
6. Expression of predictors for favorable response to adjuvant medication.

1. MORPHOLOGICAL CHARACTERIZATION OF STUDIED RENAL TUMORS

In order to morphologically evaluate the cases and to establish the histopathological diagnosis, we used the technique of classical hematoxylin-eosin staining.

Based on the material we have studied, which contains 90 cases of renal malignancies, we have identified tumor lesion in 90 cases, normal renal parenchyma in 11 cases and modified renal parenchyma, bordering tumors, in 79 cases.

Renal carcinomas having clear cells identified (n=71), accounted for most cases and were characterized by the presence of round or polygonal cells, with abundant and clear cytoplasm due to increased glycogen content, with cores in varying evolutionary degrees, from small cores without nucleolus to large, globular cores with multiple nucleolus. Some cases presented areas of extensive necrosis, with hemorrhagic suffusion, cells with granular cytoplasm and hemosiderin contents with or without renal capsule invasion, and inflammatory infiltrate in varying proportions, located on the border between tumor tissue and normal kidney tissue. A particular aspect presented the cases of ccRCC with microcystic appearance, which were characterized by multiple microcysts, hemorrhagic suffusion, tumor cells arranged in islets and nests. In rare cases, we have identified forms, which presented metaplasia of pseudo cartilaginous type, and numerous vessels with intraluminal calcifications.
However, we also identified cases of ccRCC, which presented cells with eosinophilic cytoplasm, sometimes granulated, with an increased content in mitochondria.

Papillary RCC forms (n=11) were characterized by tumor cells arranged along well-vascularized connective axes, these cells having on the one hand a clearer basophilic cytoplasm, with low-grade cores, found in papillary RCC type 1 (n=4), and on the other hand, having an eosinophilic, granular, high-grade cytoplasm characteristic in papillary RCC type 2 (n=7).

In sarcomatous carcinoma (n=5) we observed a completely modified structure, represented by cells with rhabdoid appearance, sarcomatous, pleomorphic cells with acidophilic cytoplasm and longitudinal striations, but also stellate cells and thick cytoplasmic extensions, having abundant myofibroblasts, all cellular elements presenting frequent interconnections.

In one of the studied cases, we identified the form of renal cell carcinoma with collecting ducts, which was characterized by the presence of tubular or tubulo-papillary structures, lined by tumor cells.

The degree of differentiation, assessed according to the protocol mentioned in the general part, was predominantly represented by a degree ofG2 differentiation, characterized by eosinophilic cores visible under x400 microscopy, 19 cases had a degree of G3 differentiation with acidophilic nucleolus, enlarged in volume, prominent, visible under x100 microscopy, in 5 cases a degree of G1 differentiation was highlighted, with basophilia nucleolus, without highlighted nucleolus, the degree of G4 differentiation in which cells were multinucleated, giants with rhabdoid sarcomatous differentiation, was encountered in 7 cases.

1.1 IDENTIFICATION OF MICROVESSELS ASSOCIATED TO TUMOR AND PROGNOSTIC VALUE OF MICROVASCULAR DENSITY (MVD)

Following the immunoreaction for CD34, in the kidney, which was not invaded by the tumor, the presence of a very large number of vessels was observed, arranged orderly, with similar dimensions, over 90% having permeable lumen in the medullary, located among the tubules, disposition that is preserved both on cross and longitudinal sections, and in certain portions of the medullary the branches of the vasa recta are observed.

Regarding the results obtained on tumor and peritumoral tissue, we found the presence of peritumoral vessels that have a wide lumen, unequal in size and have intussusception phenomena. In the peritumoral area, already appear blood vessels without lumen, of distinct size and irregular, but minority shape. The vessels in the tumor area form a high-density network that numerically mimics the density of the normal wound. The vessels form a network with extensive branches and extensions, in most cases representing a contiguous.

In case of renal carcinoma having clear cells, on the same microscopic field, different types of vascular networks coexist. In rare cases, we identified vessels which showed intraluminal migrated tumor cells.

We could distinguish, in some cases, a form of network that we name segmented, in which, although there are many blood vessels on the histological section, most of them are not connected to each other, and most do not have well-defined lumen. In our casework, this aspect corresponds relatively constantly, with the form of papillary woundal carcinoma.

In the sarcomatous form, the vessels have a slower proliferation rate than tumor cells, and therefore the extensive network that we have observed in renal carcinoma having clear cells with good differentiation, is not formed. Most vessels are small, irregular, and some of them contain tumor cell embolism. This aspect of tumor vascular invasion is only rarely seen in renal carcinoma having well-differentiated clear cells. Occasionally, I observed large areas of section, which showed only positive CD34 elements, intratumorally, without lumen which, with the highest probability, signal processes of rapid endothelial budding. As a particular form, we mention the disposition and focal concentration of vessels, between extensive areas of tumor cells, and sometimes in these vessels, we have observed tumor cells reported as vascular invasion. We also found that the density of the vascular network decreases with increasing differentiation.

Microvascular density is the most frequently evaluated parameter for the study of intratumorally angiogenesis. After several years of enthusiasm over this method, several articles have been published in recent years, questioning the prognostic accuracy of MVD in kidney tumors. To elucidate this aspect, we have studied and calculated the MVD in the cases included in our study and correlated the results with clinical-pathological parameters of conventional prognosis. We have identified significant difference between the highest clear

cell carcinomas and other types of kidney tumors, without this being a diagnostic indicator. We evaluated the correlations between MVD and other clinical-pathological prognostic parameters and obtained the following data: with stage, p=0.023, with survival at 3 years, p less than 0.21; with degree of differentiation p=0.07; with histopathological form p less than 0.002, but only for sarcomatous form. MVD did not correlate with lymph nodal metastases (p=0.4), but strongly correlated with lung metastases (p less than 0.0001). It was also noted that all cases with lung metastases showed very high MVD values in the primary tumor, usually above 1000 per^{mm2}. This observation led us to the study of lymph vascular invasion. Among the cases included in the study, we identified lymph vascular invasion using the staining method, for CD34 in 19 cases.

Conclusions

The staining method for CD34 is useful for studying the morphology of intra- and peritumoral vessels in kidney carcinomas. It is the main method used to calculate MVD, but our results refute its prognostic value, except for the prediction of lung metastases. On the other hand, it is useful to improve the results related to vascular invasion with tumor emboli, an element of great prognostic importance.

1.2 TYPES OF VESSELS ASSOCIATED WITH KIDNEY TUMOR, PROGNOSTIC SIGNIFICANCE AND THERAPEUTIC IMPACT

To identify the types of vessels in the tumor area, we have applied double immunostaining with CD34, as endothelial marker, and smooth muscle actin, as endothelial cell marker. In the renal parenchyma in the vicinity of the tumor, most blood vessels are of mature type, both cortical and medullary. The signal for CD34 is more intense in the renal corpuscles because the cytoplasm of mesangial cells is smooth. The mature vessels have different shapes, the largest dimensions being observed at the level of the internal medulla and hilum.

The vessels in the medullary are almost all the mature type, the most intense signal for actin being present in the vasa recta and the weakest in the insulated tubular capillaries.

At the interface between the renal parenchyma and tumor in most cases of clear cell RCC, we have observed a massive accumulation of myofibroblasts that structurally resemble capsular ones, the thing being that here, they form a dense and thick syncytium. From the thick bundles of myofibroblasts are being detached small groups of actin-positive polygonal cells, located at the interface between tumor cells and stroma.

In all cases, several types of vessels were identified, endothelial cells in the form of buds without lumen, expressing only CD34 approved as immature vessels and/or leader cells, vessels with lumen of variable size colored for CD34, but without perivascular cells, these were associated with intermediate-type vessels. The mature vessels showed double endothelial and perivascular signals, always having permeable lumen.

Immature and mature vessels have always had the same appearance, unlike intermediate vessels which have numerous transitional elements. Aspects in which intermediate vessels have perivascular cells on one wall and not on the other, are common. From this point of view, we subclassified the intermediate vessels observed, into three subtypes: subtype 1, with no signal for perivascular cells, subtype 2, discontinuous signals on one of the walls, and subtype 3 with continuous signal for perivascular cells on at least 50% of the contour. We have not encountered such a subclassification in the literature, but we consider it useful for assessing the effectiveness of ant vascular therapy.

On large areas, intermediate and transitional vessels predominate, vessels which we have described in most cases of clear cell RCC, solid form. Mature vessels are present almost exclusively in thick connective septa in the tumor stroma and peritumoral space, they occur, only exceptionally, in the tumor area itself. In the same cases, in many situations, the moment of addition of perivascular cells to the external wall of the vessel is observed. In this situation, the perivascular, actin-positive cells are polygonal and ovoid and are grouped at one of the poles of the vessel.

These aspects, which we have described so far regarding clear cell RCCs (most of the cases in the study), show that the maximum density network consists predominantly of intermediate vessels. When the network is more spaced, lax, over 90% of the vessels are pure intermediates (subtype 1), thus completely devoid of perivascular cells. Regarding this, we identified perivascular cells apparently migratory, or which have not realized the position.

In clear RCC cells, the alternative with microcysts and lax network, most vessels are mature, although small, almost all perivascular cells are arranged in layer.

All these aspects lead to the idea that the vessels in the tumor area undergo a rapid morphological maturation process, which explains the reduced number of tumor cells related to each branch, this process being more evident at the periphery of the tumor, at the proliferation front. The higher the density of the vascular network in the tumor area, the more numerous are the mature vessels, even if from the dimensional point of view, they do not correspond to the initial definition.

In chromophobe carcinoma, the network is lax, made up of rare blood vessels, and most are mature type, even though many of them have very narrow or unidentifiable lumens.

In papillary carcinoma, the vessels in the tumor area are rare, elongated, with few branches, half at least mature type, with narrow lumen and do not form networks.

In sarcomatous carcinoma, over 90% of the identified vessels are of mature type and most likely represent pre-existing vessels of the stroma, invaded by the tumor.

As indicated above, kidney tumors are characterized by marked heterogeneity in terms of vascular network, which is much more obvious in the tumor area. The numerical evaluation encounters numerous technical problems on the colored sections using double immunoreaction, and currently, from the data available to us, there is no specialized software to differentiate the types of intratumorally vessels between them. For this reason, we propose to apply a qualitative observational test, based on the examination of 10 consecutive fields chosen at x200 magnification and the percentage assessment of vessels with a single *CD34 signal for (endothelium) and with double signal (CD34 for smooth muscle endothelium/actin for perivascular cells). From the data presented, the highest vascular density is confirmed in the group of clear cell carcinomas, but also in this group we observed marked vascular heterogeneity. Some cases showed maximum score, others had a significant number of mature vessels. This leads to the hypothesis of the existence of a substance that induces rapid maturation of newly formed vessels, even if they are very small in size. Given the relatively small number of chromophobia and papillary carcinomas, we believe larger series are needed to confirm these results. We did not observe any statistically significant correlation between the calculated score and the other clinical-pathological prognostic elements. This leads to utilizing this score as an individual prognostic element.

Conclusions

We describe three subtypes of intermediate blood vessels in the tumor area with potential implications for the effectiveness of ant vascular therapy.

The distribution of the network of mature and immature intermediate vessels is dependent on the histopathological form, but with relatively large variations, especially in clear cell RCC. Regardless of the type of tumor, vessel development occurs the same.

We propose for the first time a quantification score of vessel types in the tumor area, as a predictor for potential response to ant vascular therapy. The rate between immature, intermediate, and mature vessels may be an indicator of response to therapy, especially in clear cell RCC. Our results support that ant vascular therapy is not effective in cases of sarcomatous carcinoma. We premise the existence of an unknown substance that induces rapid maturation of vessels in the tumor area, but does not act in the peritumoral environment.

1.3 Expression of VEGF a and VEGF165

Angiogenesis is a continuous, multistage process under the direct influence of several growth factors. In the previous chapters we presented the peculiarities of blood vessels associated with kidney tumors, insisting on their activated and proliferative character. For this process to be leading to the permanent formation of new blood vessels, it is necessary the intervention of growth factors with specific action on endothelial cells, both normal and associated with the tumor, of which the best currently characterized is vascular endothelial growth factor (VEGF).

VEGF has been reported by several studies with special reference to kidney tumors. In fact, renal parenchyma is a well-known positive control for reaction to VEGF-A (228). Based on clinical-pathological observations, FDA has in recent years approved the use of several molecules for the treatment of RCC, such as sorafenib, sunitinib, pazopanib and others. Many are inhibitors of VEGFR2.

VEGF 165 b is the major anti-angiogenic isoform, the first discovered in the VEGF xxx b group and the most studied, until now. The mRNA of $VEGF_{165}b$ was originally isolated from the human renal cortex, and the proportion of inhibitory forms is predominant compared to pro-angiogenic forms.

In renal tumors included in the study (n=90), the reaction for VEGF-A was positive in tumoral cells, without other elements in the tumor microenvironment, including blood vessels.

Regarding conventional clear cell RCC, most positive cases showed moderate intensity in tumor cells. In the form with microcysts, most positive cells delimit pseudo lumens and justify one of the main functions of this growth factor, namely the per permeabilization of the endothelium. With clear cells RCC, we could observe, in the same case, aspects that fall to +1, +2 or +3 and alternate with negative areas. The component that occupied more than 10% of the examined area was considered for reporting.

In cases of papillary carcinoma, the final reaction product was constantly arranged homogeneous in the cytoplasm, finely granular. In sarcomatous carcinoma, the positive reaction was mostly heterogeneous, and the full-intensity-stained tumor cells were distributed aleatory-wide throughout the tumor area, with many tumor cells isolated or arranged in intensely positive small groups. Only two cases with G4 differentiation showed almost all intensely positive tumor cells.

From the whole 90 cases included in the study, 62 (68.88%) tested positive for VEGF. Most were assigned +1 and +2. We did not obtain any statistically significant correlation with clinical-pathological prognostic factors, nor with microvascular density.

VEGF165b was performed immunohistochemically following a technique like that for VEGF-A. The same evaluation score was applied, as was applied for VEGF-A. In the tumor stroma, we constantly noticed the presence of medium size cells, isolated or in clumps, in some of them being visible the granular character of the final reaction product. These cells were predominantly observed in cases where the reaction was negative in tumor cells and in areas where inflammatory lymphoplasmacyte infiltrate is present. These cells are also present in full moral rangeamong malignant cells. In specialist literature, we did not find any data regarding the expression VEGF165b in stromal cells, nor in elements of the tumor microenvironment of renal cell carcinoma.

From the whole 90 cases included in the study, 17 were positive (18.88%) for VEGF165b. In all positive tumors, in tumor cells, the intensity of the reaction was scored by +1, significantly weaker than for VEGF-A. This signals the disbalance between angiogenic and angiogenesis inhibitory factors during tumor obliteration, leading to blood vessel proliferation. All cases of clear e-cell carcinoma were characterized by heterogeneous appearance, unlike papillary tumors, in which cells were poorly and homogeneously stained. We obtained statistically significant correlation with microvascular density in papillary carcinoma ($p<0.0023$) and inverse correlation with MVD in clear cell carcinoma ($p<0.0017$).

Conclusions

Immunohistochemical expression of VEGF-A was identified in 62 (68.88%) cases, from the whole 90 cases included in the study. We found no statistically significant correlation between VEGF-A expression, clinical-pathological prognostic factors and microvascular density. We report marked reduction in VEGF165b expression in all histopathological forms of CCyR and inverse correlation with microvascular density. We describe for the first time the existence of cells from the tumor microenvironment that are intensely positive for VEGF165b.

1.4 The PDGF heterogeneity molecular pathway defines three distinct subgroups of renal cell carcinomas

Within the heterogeneity of the renal cell carcinoma microenvironment, two main components appear to be responsible for the rapid development of resistance to therapy: tumor breast vessels and immunogenic profile. The vascular and immunogenic compartments of ccRC are the most "targeted" compartments in ccRCC, with antiangiogenic therapies and immune checkpoint inhibitors currently being the gold standard for kidney cancer treatment.

Because of discrepancies between the large number and unusual morphology of blood vessels in ccRCC (despite a low rate of endothelial cell proliferation) and the very mature location of these tumor blood vessels, we considered that the study of the PDGF-B /PDGFRβ axis is mandatory in ccRCC correlated with vascular endothelial growth factor (VEGF) inhibitory isoform, VEGF 165b.

Initial microscopic evaluation of each ccRCC sample included determination of tumor grade. Eight cases were marked as having grade 2 tumors, and 4 cases with grade 3 tumors. We detected an angiogenic process raised inside the tumor mass, with a high density of tumor

blood vessels identified at all stages of the angiogenic process (buds, cords, tubule-like structures, infused vessels).

All cases had stable tumor blood vessels evidenced by CD34/SMAct immunostaining throughout the area or only in isolated areas in the tumor mass. Most tumor blood vessels showed a network of upsized perivascular cells evidenced by SMA positive staining. Also, the arrangement of SMA-positive perivascular cells was not normal (tightly attached to the outer circumference of the vessel wall), being partially detached or being distributed as bridge-like structures between loops of small tumor blood vessels.

Based on these preliminary microscopic observations, together with a high immunohistochemical expression of PDGF-BB previously reported by our team, and given that perivascular cell acquisition is driven by the PDGF-B/PDGFR pathway, we performed the TaqMan Array to highlight the peculiarities of the PDGF pathway gene expression profile in order to identify links between gene over expression and early presence, unusual, morphology and distribution of SMAct-positive perivascular cells.

Immunohistochemical evaluation of PDGF-B/PDGFRβ showed that all 12 cases of ccRCC were positive for PDGF-BB by immunohistochemistry and RNA, of which 91.6% were confirmed by RT-PCR. The gene expression profile of the PDGF pathway was evaluated in correlation with our previous immunohistochemical and RNA expression results of VEGF and with our previous classification stratifying tumor blood vessel types in ccRCC into 4 categories: reticular, diffuse, fascicular and trabecular.

Based on the analysis of TaqMan Array results, a gene expression profile of the PDGF (HeatMap) pathway was automatically created by the Data Assist. analysis software. The HeatMap analysis revealed genes involved in several pathways of PDGF overexpression. The genes with the highest overexpression were: PIK3C3 (VsP34), SLC9A3, STAT1, JAK2, SHC2, SRF and CHUK.

Based on genetic analysis of the PDGF pathway in ccRCC, correlated with VEGF expression and tumor blood vessel types, we defined three subgroups of renal cell carcinomas that may have three different therapeutic options. The first is the VEGF-high subgroup showing overexpression PIK3C3 (VPS34) and SLC9A3. This group includes both reticular and diffuse vascular patterns and could be eligible for a combined therapeutic option of anti-VEGF agents and PIK3C3 inhibitors. The second group can be defined as VEGF-low showing similar overexpression of PIK3C3 (VPS34) and SLC9A3. For this group, anti-PDGF-PDGFR therapy may be suitable. The third subgroup we called the JAK/STAT subgroup, is characterized by overexpression of 5 genes, suitable for anti-JAK/STAT therapy associated with PIK3C3 and IKK inhibitors.

Conclusions

We demonstrated here the heterogeneity of PDGF expression profile in ccRCC, related to vascular patterns, tumor invasion, and tumor grade. By correlating PDGF pathway expression gene profile and vascular patterns in ccRCC, we defined 3 distinct ccRCC subgroups that may potentially impact the choice of the best therapeutic strategy for each patient. Further studies will be needed to elucidate the origin of perivascular cells around tumor vessels and to

evaluate molecular mechanisms responsible for early and rapid maturation of tumor blood vessels in ccRCC.

1.5 Chlorine transmembrane channel protein(CLIC1) expression in tumor blood vessels of clear renal cell carcinomas (cc CRC)

The discovery and validation of new endothelial markers is imperative for the development of new targeted therapies in renal cell carcinomas.

Among future candidate markers, intracellular chloride channel 1 (CLIC1) appears to be a promising therapeutic target for both tumor and endothelial cells from malignant pathologies.

Based on recently reported data in the literature on vesicular transfer of micro vesicles from glioblastoma malignant cells to microvascular endothelial cells, we found it interesting to evaluate CLIC1 expression in neoformation vessel tumor endothelium in ccRCC and also to discuss its impact on tumor progression.

CLIC1 expression was quantified in tumor cells (ccRCC-TC) but also in tumor blood vessel endothelium as CLIC1 microvascular density (ccRCC-TBvsE). 87.5% of ccRCC cases had CLIC1-positive tumor cell areas with homogeneous or heterogeneous distribution in the tumor site. The presence of CLIC1 at the endothelial level was recorded in approximately 65% of all cases, and 59% of cases had CLIC1 co-localization in both tumor cells and endothelium of tumor blood vessels.

CLIC1 expression in tumor cells, divided ccRCC cases into 4 classes. Twelve percent of all cases were included in Clet 0. For this class, no significant correlation was found between CLIC1-MVD micro density and TNM p-parameters(p-value = 0,543 for T, *p-value* = 0,862 for N, and *p-value* = 0,862).

Class 1 included the lowest number of cases, but all had CLIC 1 tumor-positive blood vessels with a density of 10 to 17 microscopic vessels/field X 20. Based on statistical analysis, it appears that for class 1, the presence of CLIC1 did not influence TNM staging (p-value = *0.614* for T-value and *p-value* = *0.386* for N).

For Class 2 cases, CLIC 1 expression in tumor blood vessels was significantly inversely correlated with parameter N in the TNM staging system (p-value = 0.004).

The increased CLIC 1-MVD micro density for cases in Class 3 had impact on parameters T and M (p value = 0.007 for T value and *p = 0.006 for* M). Significant correlations were also observed between TNM staging parameters. Parameter T was significantly correlated with parameter N (p-value = 0.033), but a strong correlation was observed between parameters T and M (p-value 0.001).

CLIC1 is known for its high translocation capacity between its three main cell compartments (nucleus, cytoplasm, and membrane). We evaluated this variability in relation to CLIC1-MVD micro density and TNM parameters .

Twenty-four percent of the cases expressed CLIC1 in all three cellular compartments of tumor cells (nuclear-N, cytoplasmic-C, and membrane-M). We defined this as the NMC subgroup. Within this group, more than half of the cases were categorized as pT2b, 3a, 3b

and 4. For the case categorized pT4 in the NMC group, extensive lymph vascular invasion was observed. Lymphatic and vascular emboli were NMC-CLIC1 positive, like cells in the tumor area.

This finding suggests that CLIC1 translocation is induced not only in tumor cells, but also in the endothelium of tumor blood vessels.

Away from the tumor, in the subcapsular area, tumor positive emboli were observed at CLIC1-NMC, inside blood vessels with endothelium positive at CLIC1.

The statistical analysis applied to the CLIC1-NMC subgroup, revealed a significant correlation between the CLIC1-MVD parameter and M (*p-value 0,001).* A significant correlation was also observed between parameter T and N (*p-value = 0,021) and M (p-value = 0,032).*

We did not identify significant correlations between CLIC-MVD and TNM parameters in any of the other patterns (NC, NM, C, N, M).

We looked at CLIC1 expression (low or high) in cases of ccRCC with possible impact on patient survival based on TNM staging. We identified a total of 528 cases with ccRCC from the TCGA database. The survival analysis was based on decreased or increased degree of CLIC1 mRNA expression in relation to tumor stage.

The global analysis of all 528 cases in the TCGA database showed that 35.7% of them had an increased CLIC1 mRNA expression. Significant differences in survival were identified between cases with increased or decreased CLIC1 expression, depending on tumor stage and sex.

Conclusions

CLIC1 exhibits heterogeneous expression in tumor cells and endothelium of tumor vessels within ccRCC and influences tumor stage, disease progression, and distant metastatic ganglion potential. Based on previous experimental data and data from the present study, CLIC1 released by tumor cells and intravascular tumor embolisms shows a strong angiogenic effect, an effect supported by the intratumorally and peritumoral presence of small and well-defined vessels, lined with CLIC1-positive endothelium and with a highly suggestive morphology for an intense angiogenic process.

The expression of CLIC1 in both tumor and endothelial cells, makes it a possible dual target for antibody-based therapies, therapies proven effective in experimental models of ccRCC.

General conclusions

1. CCR heterogeneity is far from being elucidated at present, both morphologically and molecularly and in terms of clinical behavior, the response to therapy and the

development of resistance to personalized treatments being a real challenge for the global scientific community.

2. The results in this paper are based on fundamental microscopic morphological and immunohistochemical observations, that described vascular heterogeneity and angiogenic factor expression in ccRCC.
3. Conventional evaluation should be accompanied by gene analysis of VEGF and PDGF angiogenic pathways already described in the literature and recognized as part of the CC RCC angiogenic process, because it can define distinct subclasses and identify patients who will develop subsequent resistance to personalized therapy already used in clinical practice.
4. Gene analysis of VEGF and PDGF pathways
5. Vascular micro density is not sufficient to assess the angiogenic microenvironment of kidney tumors, due to discrepancies between expression of proangiogenic growth factors and vascular micro density values
6. The evaluation of tumor vessel types and their ability to acquire perivascular cells defined the existence of three morphological types of different vessels, as growth and maturation patterns.
7. TAqMan analysis of VEGF and PDGF conditions, which derived from previousmicroscopic observation, identified three molecular subgroups of ccRCCs characterised by particular gene expression that define them and recommend the applicationof differentiated personalised therapyThere you go.
8. Idenitification of CLIC1 expression and its characterization in tumor cells, but also in endothelial cells in tumor vessels, identified a risk subgroup with impact on progression andmetastasis.
9. Thedual expression of CLIC1 in tumor cells and endothelium of tumor vessels, Irecommend it as an int for personalized therapy aimed at the sametime, both in the compartment tumor and vascular stromal compartment.
1. The three subgroups of CCRC defined by gene analysis determine the differentiation of antiangiogenic/antivascular therapy depending on the type of overexpended genes, which results in an optimally personalized response to this type of treatment.