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Synergic effect of microRNAs and metalloproteinases derived from extracellular vesicles in the establishment of the metastatic niche in renal cell carcinoma patients

Francisca Carvalho Dias

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Instituto Ciências Biomédicas Abel Salazar

FRANCISCA GUILHERME CARVALHO DIAS

SYNERGIC EFFECT OF MICRORNAS AND METALLOPROTEINASES DERIVED FROM EXTRACELLULAR VESICLES IN THE ESTABLISHMENT OF THE METASTATIC NICHE IN RENAL CELL CARCINOMA PATIENTS

Tese de Candidatura ao Grau de Doutor em Ciências Biomédicas;
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“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.”

Louis Pasteur

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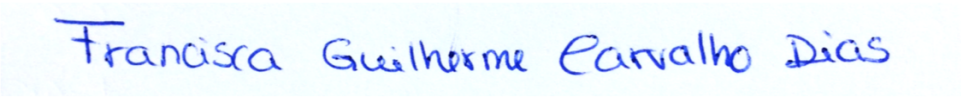

(Francisca Guilherme Carvalho Dias)

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De acordo com o disposto no ponto n.º 2 do Art.º 31º do Decreto-Lei n.º 74/2006, de 24 de Março, aditado pelo Decreto-Lei n.º 230/2009, de 14 de Setembro, o autor declara que na elaboração desta tese foram incluídos dados das publicações abaixo indicadas. O autor participou ativamente na conceção e execução dos trabalhos que estiveram na origem dos mesmos, assim como na sua interpretação, discussão e redação.

According to the relevant national legislation, the author declares that this thesis includes data from the publications indicated below. The author participated actively in the conception and execution of the work that originated that data, as well as in their interpretation, discussion and writing.

The following articles wered used to write this thesis:

- **Dias, F**., Teixeira, A.L, Ferreira, F., Adem, B., Bastos, N., Vieira, J., Fernandes, M., Sequeira, M.I., Maurício, J., Lobo, F., Morais, A., Oliveira, J., Kok, K. and Medeiros, R. (2017). Plasmatic miR-210, miR-221 and miR-1233 profile: potential liquid biospsies for Renal Cell Carcinoma. *Oncotarget*, 8: 103315-103326 (DOI:10.18632/oncotarget.21733)

- **Dias, F**., Teixeira, A.L., Nogueira, I., Morais, M., Maia, J., Bodo, C., Ferreira, M., Vieira, I., Silva, J., Lobo, J., Sequeira, J.P., Maurício, J., Oliveira., J., Kok, K., Costa-Silva, B. and Medeiros, R. (2020). Clear cell renal cell carcinoma extracellular vesicles-derived TIMP-1 mRNA as prognostic biomarker: a pilot study (*submitted manuscript*)

- **Dias, F**., Teixeira, A.L., Nogueira, I., Morais, M., Maia, J., Bodo, C., Ferreira, M., Silva, A., Vilhena, M., Lobo, J., Sequeira, J.P., Maurício, J., Oliveira., J., Kok, K., Costa-Silva, B. and Medeiros, R. (2020). Extracellular vesicles enriched in hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p dynamics in ccRCC patients: potential impacto n PI3K/Akt pathway activation (*submitted manuscript*)

Other articles published during my PhD:

- Morais, M*., **Dias, F*.**, Teixeira A.L., and Medeiros, R. (2020). Telomere Length in Renal Cell Carcinoma: The Jekyll and Hyde Biomarker of Ageing of the Kidney. *Cancer Manag Res.* 12: 1669-1679. (DOI: 10.2147/CMAR.S211225)

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Abstract

Renal Cell Carcinoma (RCC) is the most common solid cancer of the adult kidney and remains one of the most letal urologic malignancies. The most common and aggressive subtype is the clear cell RCC (ccRCC), which accounts for approximately 80% of all cases. CcRCC is characterized by a difficult diagnosis and a high metastatic potential. About 20 to 30% of all patients present metastatic disease a the time of diagnosis and up to 40% of the patients with localized disease submitted to surgery will relapse in distant sites. When metastatic disease sets in, patients present a 5-year survival lower than 10%. Despite metastatic ccRCC remains incurable, the prognosis of recurrent ccRCC is variable and the detection of early relapse could allow patients' stratification according to their prognosis, therefore contributing for a personalized medicine approach for these patients. There have been innumerous attempts of biomarkers implementation and, despite the promising results, none have made it to the clinical practice so far. This raises the question that additional biomarkers, aside from the classical genetic alterations that are commonly studied, could be the missing puzzle piece that could allow a better understanding of this complex disease. Since cancer was recently defined as an ecosystem due to its clonal heterogeneity and microenvironment of its on, the scientific community is now focusing on the understanding of the networks established among cancer cells and also between the tumor and its host. One of the key players of the complex communication networks established between the tumor, its environment, and the host are extracellular vesicles (EVs). EVs are a recent and attractive field of study since they are able to shuttle bioactive molecules between cells with an impact on the recipient cell phenotype. Thus, the aim of this study was the definition of an EV-derived microRNA and matrix metalloproteinases (MMPs) profile associated with progression and metastatic potential in ccRCC patients. In addition to that, we also compared the applicability of circulating EV-free miRNAs *versus* EV-derived miRNAs as potential biomarkers. We observed that patients with localized ccRCC tumors had EVs enriched in hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p, and that enrichment decreased after tumor removal. On the other hand, patients with metastatic disease had EVs enriched in hsa-miR-301a-3p and TIMP-1 mRNA, being the later associaded with a lower overall survival. Both the miRNA profile and TIMP-1 are able to activate the PI3K/Akt pathway, suggesting a new mechanism by which the tumor impacts its host. We also observed that hsa-miR-210-3p, hsa-miR-221 and hsa-miR-1233 EV-free plasma levels could differentiate ccRCC patients from healthy individuals and their expression was also related to clinicalpathological features such as higher tumor size

and metastasis. Higher plasmatic levels of these EV-free miRNAs were also associated with a lower cancer-specific survival and increased the *c* index of the predictive model of death by RCC from 0.744 to 0.828, when compared to the current variables used by clinicians. Thus, both EV-free and EV-derived miRNAs have great potential as cancer biomarkers and can be used in a complementary way. The EV-derived miRNAs may exert a biological effect on the host, while EV-free could be remnants of cellular activity implicated on tumor development. Future studies should include more miRNAs and mRNAs related to the PI3K/Akt pathway and use larger cohorts of patients with larger follow-up periods for a better understanding of the EVs network dynamics in the host.

Keywords: clear cell Renal Cell Carcinoma; Biomarkers; Extracellular vesicles; microRNAs; TIMP-1.

Resumo

O Carcinoma de Células Renais (CCR) é o tumor sólido mais frequente no rim adulto e constitui umas das neoplasias urológicas mais letais. O subtipo mais comum, e mais agressivo, é o CCR de células claras (CCRcc) que representa cerca de 80% de todos os casos. O CCRcc é caracterizado por um diagnóstico difícil e um elevado potencial metastático. Cerca 20 a 30% dos doentes apresenta doença metastática no momento do diagnóstico e cerca de 40% dos doentes com doença localizada, e submetidos a cirurgia, irão desenvolver metastases à distância. Quando a doença metastática se desenvolve, os doentes apresentam uma sobrevida a 5 anos anos inferior a 10%. Apesar da doença metastática permanecer incurável, o prognóstico do CCRcc recurrente é variável e a deteção de recorrência precoce permitiria a estratificação dos doentes de acordo com o seu prognóstico, contribuindo assim para uma medicina mais personalizada. Existem inúmeras tentativas de implementação de novos de biomarcores de diagnóstico e prognóstico *mas*, apesar dos resultados promissores, de momento ainda nenhum foi implementado na prática clínica. Este cenário levanta a questão de que biomarcadores adicionais, à parte das alterações genéticas clássicas que englobam a maior parte dos estudos, podem ser a peça em falta no *puzzle* que permitirá uma melhor compreensão desta doença complexa. Uma vez que o cancro foi recentemente definido como um ecossistema devido à sua heterogenidade clonal e microambiente próprio, a comunidade científica está agora focada no estudo das redes de comunicação estabelecidas entre as células tumorais bem como nas redes estabelecidas entre o tumor e o hospedeiro. Um dos agentes mais importantes nas complexas redes de comunicação celular são as vesículas extracelulares (VEs). As VEs são uma área de estudo recente e bastante atrativa uma vez que são capazes de transferir moléculas bioativas entre células, com um impacto no fenótipo da célula recetora. Deste modo, o objetivo principal deste estudo foi a definição de um perfil de microRNAs e metaloproteinases de matriz derivado de VEs associado à progressão e potencial metastático em doentes com CCRcc. Adicionalmente, também foi comparado o impacto de microRNAs circulantes livres relativamente a microRNAs derivados de VEs. Observamos que doentes com CCRcc localizado apresentavam VEs enriquecidas em hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p e hsa-miR-301a-3p, e que esse enriquecimento diminuia após a remoção do tumor. Por outro lado, doentes com CCRcc metastático apresentaram VEs enriquecidas em hsa-miR-301a-3p e mRNA de TIMP-1, sendo este último associado a uma menor sobrevida global. Uma vez que tanto o perfil de microRNAs como a TIMP-1 são capazes de ativar a via PI3K/Akt, estes resultados sugerem um novo mecanismo

através do qual o tumor é capaz de influenciar o hospedeiro. Por outro lado, observamos que os níveis plasmáticos livres de hsa--miR-210-3p, hsa-miR-221 e hsa-miR-1233 permitiam diferenciar doentes com CCRcc de indivíduos saudáveis e que a sua expressão estava relacionada com variáveis clínicopatológicas, tais como o tamanho do tumor e a presença de metástases. Observamos também que níveis plasmáticos mais elevados destes 3 microRNAs estavam associados a uma menor sobrevivência livre de doença e eram capazes de o índice *c* do modelo preditivo de morte por CCR de 0.744 para 0.828, quando comparados com as váriaveis clínicas atualmente utilizadas. Deste modo, tanto os microRNAs livres como os microRNAs derivados de VEs apresentam potencial como biomarcadores de cancro, podendo ser utilizados de forma complementar. Os microRNAs derivados de VEs são capazes de exercer um efeito biológico no hospedeiro, enquanto os microRNAs livres poderão ser remanescentes de processos de atividade celular implicada no desenvolvimento tumoral. Estudos futuros deverão incluir mais microRNAs e mRNAs relacionados com a via PI3K/Akt e recorrer a coortes maiores de doentes, com períodos de follow-up mais alargados, de modo a permitir uma melhor compreensão da dinâmica das VEs no hospedeiro.

Palavras-chave: Carcinoma de Células Renais de células claras; Biomarcadores; Vesículas extracelulares; microRNAs; TIMP-1

List of Abreviations

A

AGO2 – Argonout RISC catalytic component 2

AJCC – American Joint Committee on Cancer

AKT – AKT serine/threonine kinase

B

BCL-2 – B-cell lymphoma 2

BCL2L11 – *BCL-2 like 11*

C

CAIX – *Carbonic Anhydrase IX*

CCL2 – C-C motif chemokine ligand 2

ccRCC – clear cell Renal Cell Carcinoma

CFSE – Carboxyfluorescein succinimidyl ester

$CoCl_2$ – Cobalt (II) chloride

Cq – Cycle quantification

CSCs – Cancer Stem Cells

CT – Computed tumography

CXCR4 – *C-X-C chemokine receptor type 4*

D

DNA – Deoxyribonucleic Acid

E

EDTA - Ethylenediamine tetraacetic acid

EMT – Epithelial mesenchymal transition

EVs – Extracellular vesicles

F

FDA – Food and Drug Administration

FGFR – Fibroblast Growth Factor Receptor

fPBS – filtered Phosphate-Buffered Saline

G

GLUT1 – *Glucose Transporter 1*

GLUT4 – *Glucose Transporter 4*

GO – Gene Ontology

GW182 – protein coded by *Trinucleotide Repeat Containing Adaptor 6A* gene

H

HDL – High-density lipoprotein

HIF-1α – *Hypoxia Inducible Factor 1 alpha*

HIF-2α – *Hypoxia Inducible Factor 2 alpha*

HIF-β – *Hypoxia Inducible Factor beta*

I

IARC – International Agency for Research on Cancer

IL-2 – Interleukin 2

INF-α – Interferon alpha

ISEV – International Society of Extracellular Vesicles

ISUP – International Society of Urological Society

ITH – Intratumor Heterogeneity

K

KEGG – Kyoto Encyclopedia of Genes and Genomes

KIT – *KIT proto-oncogene*

L

LALS – Large Angle Light Scatter

M

MALS – Middle Angle Light Scatter

MET – *MET proto onco-gene*

miRNAs – microRNAs

MMP1 – Matrix metalloproteinase 1

mRNA – messenger Ribonucleic Acid

mTOR – mechanistic target of rapamicyn kinase

MVBs – Multi-vesicular bodies

N

NGS – Next Generation Sequencing

NK – Natural Killer

NPM1 – Nucleophosmin 1

NTA – Nanoparticle Tracking Analysis

O

OS – Overall Survival

P

PBS – Phosphate-Buffered Saline

PD-1 – Programmed cell death 1

PD-L1 – Programmed death ligand 1

PDGF – Platelet derived growth factor

PDGFR – Platelet derived growth factor receptor

PFP – Platelet-free plasma

PI3K – Phosphatidylinositol 3-kinase

PMT – Photomultiplies tubes

PTEN – Phosphatase and tensin homolog

R

RCC – Renal Cell Carcinoma

RET – *RET proto-oncogene*

RISC – RNA-induced Silencing Complex

RNA – Ribonucleic Acid

S

SALS – Small Angle Light Scatter

T

TCGA – The Cancer Genome Atlas

TEM – Transmission Electron Microscopy

TGF-α – *Transforming Growth Factor alpha*

TIMP-1 – Tissue inhibitor of matrix metalloprotease 1

TIMP-2 – Tissue inhibitor of matrix metalloprotease 2

TKI – Tyrosine Kinase Inhibitor

TNM – Tumor-node-metastasis

U

US – Ultrasound

V

VEGF – Vascular Endothelial Growth Factor

VEGFR – Vascular Endothelial Growth Factor Receptor

VHL – von Hippel Lindau

W

WHO – World Health Organization

INTRODUCTION

Renal Cell Carcinoma

1.1. Epidemiologic and clinical aspects

Renal cell carcinoma (RCC) is the most common solid cancer of the adult kidney and remains one of the most lethal urologic malignancies, with the highest incidences occuring in developed regions, namely North America, Europe and Australia/New Zealand (1). During the past two decades there has been an anual increase of 2% in kidney cancer incidence both worldwide and in Europe. In fact, kidney cancer was responsible for 403 000 new cases and 175 000 deaths worldwide and 99 200 new cases and 39 100 deaths within the European Union in 2018 (2,3). According to the most recent data from GLOBOCAN 2018, the International Agency for Research on Cancer (IARC) online database, there were an estimated 1301 new kidney cancer cases and 507 kidney cancer-related deaths in 2018 in Portugal (4).

RCC accounts for 90% of all kidney cancers and comprises a heterogeneous group of cancers that develop in the renal parenchyma and presents different subtypes, each derived from a different part of the nephron, with different genetic and molecular alterations, histological features and therapeutic implications (5,6). Morover, there is a 1.5:1 predominance in men over women, with a peak incidence at 60-70 years of age and the established risk factors include cigarette smoking, obesity, hypertension and chronic kidney disease (1). Thus, the current knowledge highlight that the reduction of the obesity and the cigarette smoking cessation are the most effective prophylaxis measures (7).

Although the vast majority of RCCs are sporadic, 2-3% are hereditary types and several associated rare autosomal dominant syndromes are described, being the most common the von Hippel-Lindau (VHL) disease, which is associated with a very small percentage of cases of the clear cell RCC (ccRCC) subtype (8). The ccRCC subtype arises from the proximal tubular epithelial cells of the nephron and accounts for approximately 80% of all RCC cases (8). The ccRCC is histologically characterized by a high cellular lipid content and a richly vascularized tumor stroma and, in addition to being the most common subtype of RCC, it is also the most aggressive due to its high metastatic potential (6). The latest edition of the World Health Organization (WHO) histological classification of renal tumors was published in 2016 and it was based on tumor histology, chromosomal alterations and deregulated molecular pathways (6)

Due to the kidneys anatomical location, many tumors remain asymptomatic until the late stages of the disease. However, the increased use of routine imaging in the past decades led to the incidental detection of more than 60% of RCCs in early stages as a consequence of the use of ultrasound (US) or computed tomography (CT) for other clinical reasons (8). Thus, only 30% of patients are diagnosed based on the classical

symptoms, wich include palpable abdominal mass, flank pain and haematuria and are usually associated with a worse prognosis. In addition to that, locally advanced disease continues to be diagnosed in a considerable porportion of patients, with up to 17% of patients harbouring distant metastasis at the time of diagnosis (9). Although radical nephrectomy was historically the standard of care for management of localized renal tumors, the detection of small renal masses and accumulating evidence that surgically induced kidney diseases can increase patients' morbidity have led to more conservative approaches, such as active surveillance and nephron sparing surgery (10,11). The increase in active surveillance approach for small renal masses and the use of targeted therapy for metastatic patients have led the scientific community to improve the expertise in biopsy performance and pathological interpretation. However, biopsy accuracy ranges from 38% to 100% which may be related to the intratumor heterogeneity (ITH) characteristic of ccRCC (12).

Several authors already demonstrated the limitations of biopsies to define histologic type, tumor grade and sarcomatoid changes in ccRCC (13,14). A growing body of evidence takes into consideration that incomplete tumor sampling and the underestimated representativeness of the tissue samples obtained in the daily practice is responsible for the lack of accuracy of conventional biopsies. Potential solutions to solve these limitatons could include the performance of multiple biopsies, which would raise ethical concerns, or total tumor sampling which is not sustainable because several tumors are too large to be analyzed in their entirety (15,16). At the present time, one of the challenges faced by the clinicians is that the outcomes of patients with similar clinical and histological features cannot be appropriately distinguished due to the fact that there are no molecular biomarkers of progression robust enough to be implemented in the clinical practice. Thus, overcoming this limitation is of paramount importance since it could provide valuable help in decision-making, namely in the stratification of the best candidates for active surveillance and also in the choice of the best therapeutic approach for the remaining patients.

1.2. Molecular biology of ccRCC

Despite intensive efforts and the recent advances in techniques such as Next Generation Sequencing (NGS), the molecular mechanisms involved in ccRCC development and progression remain not completely understood (17). According to The Cancer Genome Atlas (TCGA) the molecular alterations underlying ccRCC occur mostly in genes involved on the control of cellular oxygen sensing and, in a less extent, in genes

involved in chromatin remodeling (18). In addition to that, it was also observed that aggressive high grade ccRCC was associated with a metabolic shift consistent with the Warburg effect (18).

One of the most important and studied signaling pathways involved in the early events that give rise to ccRCC is the deregulation of von Hippel-Lindau (VHL) pathway, which is involved in the control of oxygen sensing (19). In normoxic conditions, pVHL (the protein encoded by the *VHL* gene) serves as recognition site for the regulatory subunits of *Hypoxia Inducible Factor* (HIF) – HIF-1α and HIF-2α - targeting them to degradation. Under hypoxic conditions, or in the absence of a functional pVHL, HIF-α subunits escape proteosomal degradation and heterodimerize with HIF-β, migrating to the cell nucleus in order to initiate the transcription of various hypoxia responsive genes (20). Once activated, these genes are responsible for activating several cellular pathways, namely involved in the development of blood vessels (*Vascular endothelial Growth Factor* – VEGF; *Vascular endothelial Growth Factor Receptor* – VEGFR), cell proliferation (*Transforming Growth Factor alpha* – TGF-α), glucose metabolism (*Glucose Transporter Type 1* – GLUT1*; and Glucose Transporter Type 4* – GLUT4), pH regulation (*Carbonic Anhydrase IX* - CAIX) and cell migration (*C-X-C motif chemokine receptor 4* - CXCR4), among others (19,21). The majority of sporadic ccRCCs present somatic inactivation of both *VHL* alleles with loss of function of the tumor suppressor protein pVHL. Approximately 90% of ccRCC cases display loss-of-function coding mutations in the *VHL* gene, chromosomal aberrations on chromosome 3p25 that affect the *VHL* locus, or hypermethylation of the *VHL* promoter (21). The loss-of-function/inactivation of the *VHL* gene in ccRCC results in a hypoxic response from the cells in non-hypoxic conditions, making this cancer subtype extremely aggressive and with a highly angionenic and metastatic potential. Another consequence of *VHL* loss/inactivation is aberrant activation of PI3K/AKT/mTOR signalling pathway since *VHL* inactivation subsequently activates mTOR, which in turn will stimulate cellular proliferation (22). In addition to that, the PI3K/AKT pathway is recurrently mutated in ccRCC, which will further accentuate mTOR activation (18).

Taken together, the knowledge of the molecular biology of ccRCC lead to the development of targeted therapies directed to some of the key players of these pathways in order to give the patients a more effective and personalized treatment.

1.3. Metastatic ccRCC management

Apart from the patients that present metastatic disease at the time of diagnosis, up to 40% of patients submitted to surgery with a curative intent will also relapse within a 5-year period (23,24). The most frequent sites of distant metastasis are the lungs, bone and brain but the adrenal glands, contralateral kidney and liver might also be involved (25). Due to the hypoxic microenvironment it generates, ccRCC is traditionally resistant do chemo- and radiotherapy, leaving the targeted therapies as the only agents available to manage metastatic patients' (26,27).

For more than 20 years, interferon alpha (IFN-α) and high-dose interleukin 2 (IL-2) were the treatment options for metastatic RCC (mRCC) patients, but unfortunately the response rates to these immunotherapies only ranged between 15 to 25% (28). Due to the advances in the study of the molecular biology of ccRCC, the targeted therapies evolved and became mainly focused on the tumor neoangiogenesis provoked by the downstream alterations caused by the disruption of *VHL* pathway (29,30). In total, there are 11 FDA approved agents for ccRCC, focused on three main therapeutic approaches: vascular endothelial growth factor (VEGF) pathway inhibition, mTOR pathway inhibition and immunotherapy. The therapeutic agents available for the first two approaches include diverse tirosine kinase inhibitors (TKIs) against Vascular Endothelial Growth Factor Receptor (VEGFR), Platelet-derived Growth Factor Receptor (PDGFR), Mesenchymal Epitelial Transition (MET), AXL, Fibroblast Growth Factor Receptor (FGFR), RET and KIT tyrosine kinase receptors; monclonal antibodies against VEGF and mTOR inhibitors (31,32). However, despite the diversity of agents available, and the demonstrated benefit in patient overall survival (OS), development of resistance to this agents remains inevitable (33-35). In addition to that, these therapeutic agents are expensive, with significant side effects and an objective response rate as high as 45%, which may be due to the fact that they don't target directly the tumor cell, allowing the potential for disease progression despite treatment (36). In fact, a subset of patients (~25%) do not seem to experience any clinical benefit from these targeted therapies, while in the majority of cases, patients respond to therapy initially but later develop resistance to it and enter in the phase of disease progression (37). Usually, resistance to the targeted agents in ccRCC patients has been shown to develop after a median of 5-11 months of treatment (38). Thus, in the last few years a new hope as emerged with the introduction of immune checkpoint inhibitors against PD-1 and PD-L1 as a new approach for mRCC treatment, since the response rates to these agentes in clinical trials were are encouraging (39). Unfortunately, only a subset of patients seems to benefit from this therapeutic approach, leaving the clinicians, once again, to deal with the challenge of therapy resistance (40).

Resistance to targeted agents can be classified into primary (intrinsic) and secondary (acquired) resistance. Primary resistance can be classified as an immediate inefficacy of therapeutic agents and can be attributed to the presence of resistant tumor clones prior to therapy due to inherited resistance or evolutionary clonal selection. Secondary resistance, on the other hand, is characterized by tumor growth after initial tumor regression while the patient is still receiving therapeutic treatment (41). In an attempt to overcome this problem, oncologists administrate these agents in a sequence, according to established guidelines, in order to maximize the patients' outcome in terms of OS (42). During this therapeutic approach, when a patient develops toxicity or stops responding to an agent, the oncologists "attack" with another targeted agent until there aren´t more options available. Despite metastatic ccRCC remains incurable, the prognosis of recurrent ccRCC is variable and the detection of early relapse could improve the patients' prognosis and could also help in pateints' stratification according to their metastatic risk (43). There have been innumerous efforts from the scientific community in order to find reliable biomarkers to solve this issue but so far none has made it into the clinical practice.

2. Tumor microenvironment: implications in metastatic dissemination

2.1. The role of intratumor heterogeneity

Tumor evolution begins when a single cell in a normal tissue transforms and proliferates in order to form a tumor mass. During this complex biological process clonal lineages diverge and form distinct subpopulations, which will result in intratumor heterogeneity (ITH) (15). ITH is known to be modulated genetically and epigentically through interactions of the tumor cells with the tumor microenvironment (44). In fact, over the past decades it has been shown that tumor cells encounter selective pressures in their microenvironment, such as nutrient deprivation, geographic barriers, pH changes, chemotherapy, radiation and the immune system (45). The adaptative mechanisms of tumor cells to their ever-changing habitat are a crucial issue to survive, grow and proliferate. An important aspect that contributes to the adaptative mechanim of tumor cell survival is the community-type behavior demonstrated by the cancer cells, also known as swarming, which is defined by dynamic interactions between cells that lead to a colective behavior without any centralized guidance or government (46).

Four different models of tumor evolution have been proposed: linear, branched, neutral and punctuated (15). Regarding evolutionary patterns, it was reported very recently that most ccRCC follow the branched or the punctuated model, which will translate into different patterns of metastatic disease (Figure 1) (47). In the branched

model the amount of ITH will fluctuate during tumor progression and different clones are expected to be present at the times of clinical sampling, which will have an impact in the diagnosis accuracy since those subclones will give rise to different sub-regions within the regions of the tumor (48). This model is associated with low chromosomal complexity and high ITH and is associated with a higher time to disease progression (Figure 1A) (17).

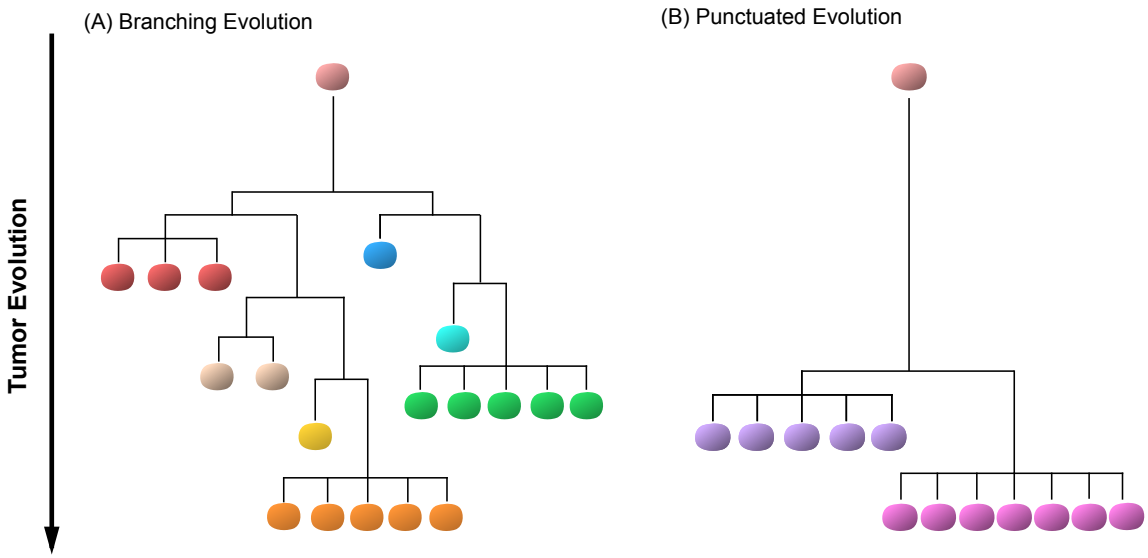

Figure 1 – Phylogenetic trees expected from the (A) Branched and (B) Punctuated models of tumor evolution.

On the other hand, in the punctuated model, a large number of genomic alterations occur during short periods of time during the early stages of tumor initiation, resulting in a few dominant clones that stably expand to form the tumor mass (15). This model, also known as the "The Big Bang" of tumor evolution, is associated with high chromosomal complexity and low ITH and is associated with and aggressive clinical behaviour which translates into a rapid disease progression (Figure 1B) (15).

The co-existence of multiple clones and subclones in both models raises several interesting questions about the degree of their interaction, namely if they cooperate or compete in order to sustain tumor development and, most importantly, how are their communications networks established. The better understanding of these networks could shed some insight on the mechanisms behind tumor evolution and help in define new tumor management strategies, namely in the definition of more accurate prognosis biomarkers and also in the development of new treatment options.

Traditional cell-cell communication is made through gap junctions or cell signalling transduction such as cytokines, hormones or neurotransmiters. However, another mode of

cell communication as emerged in ther last years and it is centered on microRNA signalling. MicroRNAs (miRNAs) are small non-coding RNAs (~19-25 nucleotides in length) that regulate gene expression by sequence-selective targeting of mRNAs, leading to their degradation or blockade at the post-transcriptional level, depending on the degree of complementarity between the miRNAs and the target mRNA (49,50). Thousands of human protein-coding genes are regulated by miRNAs, indicating that miRNAs are master regulators of diverse biological systems, with an impact in the body physiological responses. MiRNAs have been shown to regulate every aspect of cellular activity, including differentiation and development, metabolism, proliferation and apoptosis (51). The intensive research of the past years have demonstrated that miRNAs are secreted in various body fluids (e.g. serum, plasma, saliva and urine) routinely examined in patients (52). In fact, accumulating evidence demonstrated that circulating levels of miRNAs are associated with cancer development and outcome in several tumor models and their altered expression may also be observed before clinical symptoms or medical examination (53). CcRCC is no exception, and there are several studies associating circulating miRNA profiles with clinical pathological characteristics, such as histology and and staging, and also with clinical endpoints (54-57).

MiRNAs are able to circulate in biofluids through two different ways: incorporated into protein complexes or inside extracellular vesicles (EVs), reflecting different mechanisms of excretion and, possibly, different purposes (58). Both mechanisms protect miRNAs from RNAse degradation which makes them highly stable and resistant to adverse conditions, such as temperature or pH alterations (59). Regarding miRNA transportation via protein complexes, argonoute 2 (AGO2) is the most important, followed by GW182, nucleophosmin 1 (NPM1) and high-density lipoprotein (HDL) that are also capable of carrying miRNAs (58). However, its the miRNA transportation via EVs that has been gaining a lot of attention from the scientific community, since this type of communication relies on the exchange of vesicles between cells with an impact on the recipient cell phenotype, which makes this type of communication more appelaling for studies aiming to understand the tumor microenvironment dynamics.

2.2. The role of extracellular vesicles

Despite being an object of great interest for the scientific community during the past few years, the history of extracellular vesicles (EVs) goes way back to the middle of the XX century. The first suspicion of EVs existence was in 1946, when Chargaff and West demonstrated that the platelet-free plasma (PFP) fraction maintained clotting properties, and that those were diminished after a high-speed ultracentrifugation that

pelleted a particulate fraction (60). Twenty one years later, in 1967, Peter Wolf studied that particulate fraction by electron microscopy and baptized those lipid particles as “platelet-dust” (61). In 1987, Johnstone and colleagues introduced for the first time the term “exosomes” to describe these lipid vesicles, but they were still seen as “waste” vesicles used by cells to excrete metabolites or other components (62). It was only in 1998 that EV-mediated cell-cell communication was first proposed, in a study by Zitvogel and colleagues, were they suggested that EVs had a role in the communication between the cells of the immune system (63). These achievements set the beginning of EVs research field and, from that point on, the interest for this field has been continuously growing until the present days.

In the light of the present knowledge, EVs are small vescicles composed by a lipid bilayer and are released by pratically all types of cells of a multicellular organism and also by microorganisms (64-66). These vesicles are able to carry biologically active molecules such as proteins, lipids, DNAs, RNAs, microRNAs and other non-coding RNAs derived from a donor cell and deliver them to a recipient cell, with an impact on the recipient cell phenotype (67-69).

Since it is a recent field, it still faces some basic challenges, namely in terms of EVs nomenclature, isolation procedures and characterization methods (70). With the aim of achieving consensus in the field, in 2014 the International Society for Extracellular Vesicles (ISEV) published guidelines regarding the experimental methods and minimal information that should be included in studies involving EVs, with focus on three key points: 1) EV isolation/purification, 2) EV characterization and 3) EV functional studies (71,72). However, it is important to note that even the most recent isolation protocols have flaws since they aren’t able to discriminate EVs according to their origin and, despite providing enriched EV fractions, they aren’t completely pure since they often result in a mixture of different EV subpopulations and may also contain other contaminants such as proteins (70). Another issue to take into account is the fact that various nomenclatures have been used to describe EVs over the years, and this lack of accurate annotation is still an ongoing problem that may generate some confusion when looking to some EVs studies (73).

There are various types of EVs and they are mainly classified relatively to size and origin (Figure 2) (73). Exosomes are the smallest class of EVs (~30 to 150 nm) and are formed through the inward budding of the endossomal membrane, giving rise to multivesicular bodies (MVBs) that will fuse with the plasma membrane and release them to the microenvironment (74,75). Next we have the microvesicles that usually present a higher diameter (~100-1000 nm) and are formed by direct budding of the plasma membrane of the cell. Lastly, we have the apoptotic bodies (~50 to 5000 nm) that are

released upon programmed cell death through membrane blebbing (75). However, due to the overlap in EVs sizes, composition similarities and lack of differentiating markers, it remains a challenge to assign individual EVs to one of the biogenesis pathways.

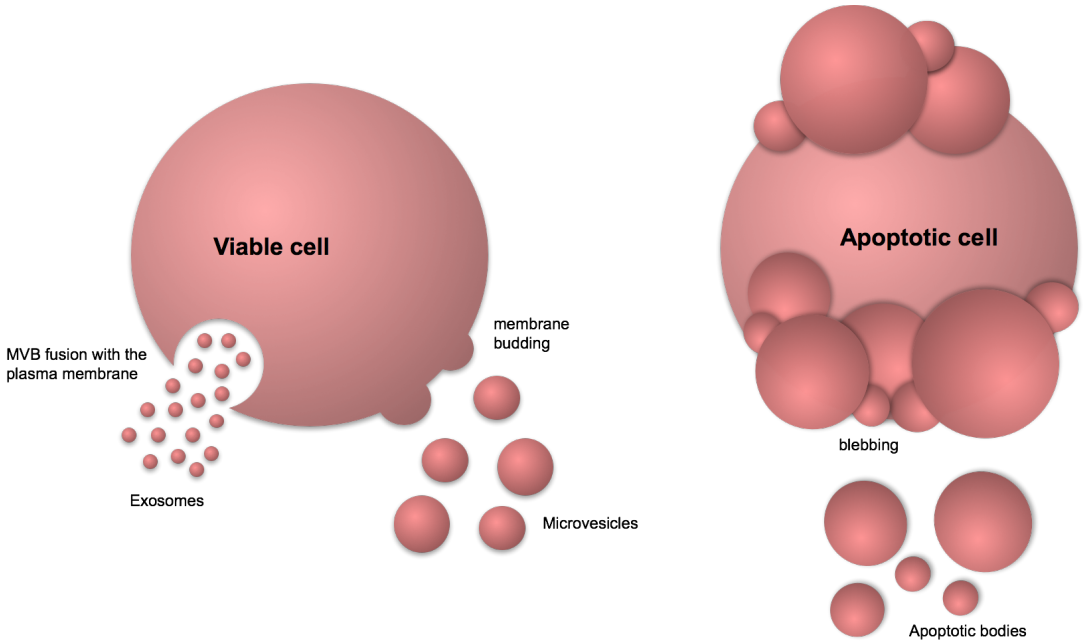

Figure 2 – Schematic representation of the major pathways for the origin and secretion of EVs.

Regardless of the challenges the field faces due to its novelty, the importance of EVs to cellular communication is well established (76). Multicellular organisms function as ecosystems and the coexistence of the diverse cell types requires a high level of coordination which was previously though to be achieved through the secretion of soluble protein-based factors, such as cytokines and growth factors, but in the light of recent discoveries we know now that the EVs are also part of this equation (64,69). In addition to that, cancer is now seen as an entity with ecosystem features due to its clonal heterogeneity and microenvironment of its own (77). Cancer microenvironment is composed by diverse cell types, such as epithelial cells, endothelial cells, stromal cells, mesenchymal stem cells and cells from the immune system (78) The complex communication networks established by these cell populations sustain tumor growth and development through the modulation of several of the cancer hallmarks proposed by Hanahan and Weinberg (79,80). Depending on the distance between the donor cell and the target cell, there are different types of intercellular communication: 1) autocrine, if the secreting and target cell are the same; 2) paracrine, if the secreting cell is in close proximity with the target cell; and 3) endocrine/systemic, if the target cell is distant and the secreted factors need to travel great distances through the blood stream (81). EVs have been proven to act both locally and at distant sites through their ability to modulate tumor

growth, invasion, angiogenesis, immune inhibition and establishment of pre-metastatic niches (Figure 3) (76).

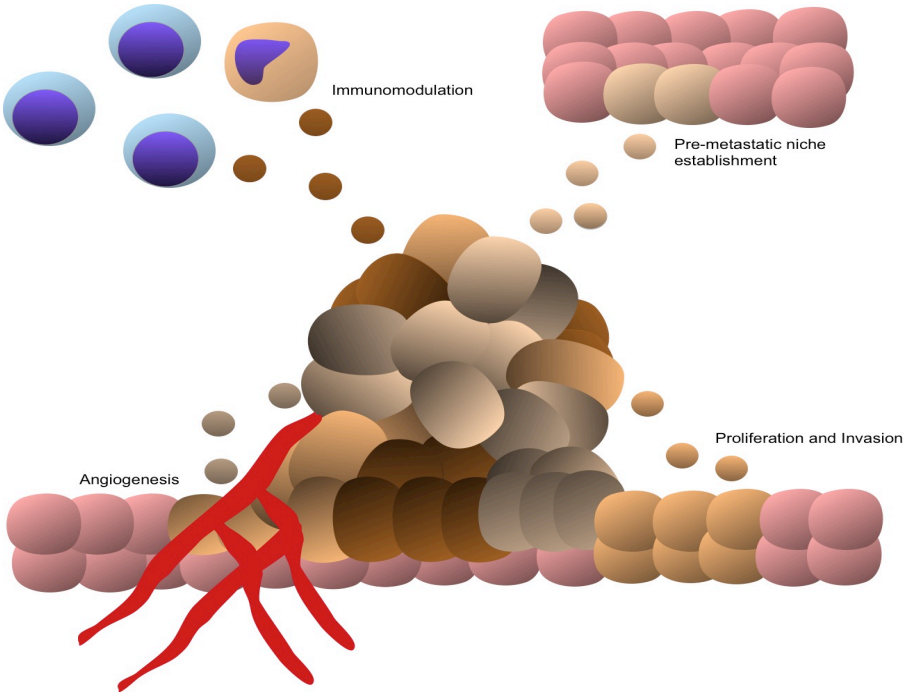

Figure 3 – Schematic representation of the role of tumor-derived EVs in the local microenvironment and in the pre-metastatic niche stablisment. The EVs are derived from different clones from the tumor and have a different target cell, which reflects the impact of tumor heterogeinity in cancer development.

The stability in body fluids, due to their lipidic composition, and the ability to shutlte bioactive molecules between cells launched EVs as promising biomarker candidates for the oncology field, especially in terms of application in the “liquid byospies” context. Similar to other tumor models, several studies have been performed in RCC with the aim of elucidating the impact of EVs in RCC biology and their potential applications. During the last 5 years, 19 studies were published on this matter with special interest on the EVs cargo.

MiRNAs are the most studied EV content in RCC and seem to be implicated in tumor growth, EMT, invasion, pre-metastatic niche establishment and targeted therapy resistance, namely to sunitinib (table 1). Interestingly, lncARSR, another non-conding RNA class, was also found in RCC-derived EVs and was also implicated in sorafenib resistance, which is another targeted therapy agent used in RCC (82).

Table 1. List of RCC derived EVs non-coding RNAs and their impact in RCC landscape.

miRNAs	Impact in RCC	EV's source	Reference
hsa-miR-31-5p	Sorafenib resistance	786-O and ACHN cell lines	He *et al.* 2019 (83)
hsa-miR-204-5p	Biomarker of Xp11.2 Translocation in RCC	Urine of an RCC xenograft model	Kurahashi *et al.* 2019 (84)
hsa-miR-19b-3p	EMT promotion	ccRCC patients CSCs	Wang *et al.* 2019 (85)
hsa-miR-30c-5p	Tumor proliferation	Urine of ccRCC patients	Song *et al.* 2019 (86)
hsa-miR-210-3p hsa-miR-1233	Hypoxia biomarkers	Serum of ccRCC patients	Zhang *et al*. 2018 (87)
hsa-miR-126-5p hsa-miR-17-5p hsa-miR-21-3p	Therapeutic monitoring biomarkers after tumor cryoablation	Serum of RCC mouse model	Zhang *et al*. 2018 (88)
hsa-miR-205	Diagnostic biomarker of ccRCC	786-O and HK-2 cell lines	Crentsil *et al.* 2018 (89)
hsa-miR-210-3p	Hypoxia biomarker	Serum of ccRCC patients	Wang *et al.* 2018 (90)
hsa-miR-224	Invasion and metastasis	Serum of ccRCC patients	Fujii *et al.* 2017 (91)
hsa-miR-34b-5p hsa-miR-17-5p hsa-miR-21-5p hsa-miR-25-3p	Exosomal miRNAs involved in cell communication in RCC	786-O, ACHN and Caki-2 cell lines	Butz *et al.* 2016 (92)
hsa-miR-200c hsa-miR-146a hsa-miR-184 hsa-miR-335 hsa-miR-646 hsa-miR-449b hsa-miR-650 hsa-miR-141 hsa-miR-183 hsa-miR-19b hsa-miR-29c hsa-miR-182 hsa-miR-19a hsa-miR-92 hsa-miR-301 hsa-miR-151 hsa-miR-130b hsa-miR-29a hsa-miR-22 hsa-miR-186 hsa-miR-let7g hsa-miR-140 hsa-miR-486 hsa-miR-26b	Enriched in $CD105^{+}$ MVs Potentialy involved in the lung pre-metastatic niche estasblishment	RCC CSCs	Grange *et al.* 2011 (93)
lncRNA			
lncARSR	Sunitinib resistance	RCC patients plasma, 786-0 and HK-2 cell lines and xenograft model	Qu *et al.* 2016 (82)

Another class of RNA found in RCC-derived EVs is the mRNAs. Despite the limited number of studies, the majority of the mRNAs reported in RCC EVs are associated with EMT and angiogenesis, with an implication on the pre-metastatic niche establishment (Table 2). MRNAs represent a very attractive target to study in EVs, since they are large unstable molecules that are subject to degradation by RNase activity and the incorporation into EVs protects them, allowing their circulation in body fluids (94).

Table 2. List of RCC derived EVs mRNAs and their impact in RCC landscape.

mRNAs	Impact in RCC	EV's source	Reference
PTRF	Diagnosis biomarker	ccRCC patients urine	Zhao *et al.* 2019 (95)
VEGF FGF ANGPT1 EFNA3 MMP2 MMP9	Enriched in $CD105^+$ MVs Potentialy involved in the lung pre-metastatic niche estasblishment	RCC CSCs	Grange *et al.* 2011 (93)

In terms of RCC EV-derived proteins, their impact is associated with the immune system modulation and also with angiogenesis promotion (Table 3). In fact, from all the RCC EV-derived cargo analyzed, EV-derived proteins were the only EV content described so far capable of modulating the immune system, with an impact on $CD8^+$ T cells, NK cells and monocytes.

Table 3. List of RCC derived EVs proteins and their impact in RCC landscape.

Proteins	Impact in RCC	EV's source	Reference
LAIR1	Accelerated cell proliferation and tumor growth in and poor PFS	ccRCC tissue-exudate	Jingushi *et al.* 2019 (96)
FasL	$CD8^+$ T cell suppression	RenCa cell line	Xu *et al.* 2019 (97)
AZU1	morphology disruption of vascular endotelial cells	ccRCC tissue-exudate	Jingushi *et al.* 2018 (98)
CA9	Potentialy involved in angiogenesis promotion	Caki-1, OSRC-2, KMRC-1 and 786-O cell lines	Horie *et al.* 2017 (99)
TGFB1	Inhibition of NK cells cytotoxicity	ccRCC tissue, patients' NK cells, HK-2 and 786-O cell lines	Xia *et al.* 2017 (100)
HLA-G	Inhibition of monocyte-derived dendritic cell differentiation	RCC CSCs and healthy donors PBMCs	Grange *et al.* 2015 (101)

Tumor derived EVs are therefore seen as a promising source for biomarkers for disease diagnosis and monitoring, as they reflect the cell of origin in terms of content and are able to travel great distances in the body which potentiates their use as “liquid biopsies” that can be collected through minimally invasive methods. Due to the metastatic potential of RCC, it would be interesting to deepen our knowledge regarding the tumor-derived EVs network in these patients, namely with respect to the EV content that has an impact in the pre-mestastatic niche establishment since even the patients that undergo surgery present a high risk of developing metastasis in the following years.

In conclusion, the better understanding of RCC-derived EVs networks could be an important tool for the establishment of surveillance schedules for patients following surgery, stratification of metastatic patients in terms of more or less aggressive disease and also in the context of active surveillance of patients with small renal masses.

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OBJECTIVES

The aim of this work is the definition of an informative and accurate EV-derived miRNAs and matrix metalloproteinases (MMPs) profile associated with ccRCC progression and metastatic potential. Both miRNAs and MMPs are attractive for the biomarker discovery field since miRNAs are important regulators of gene expression at a post-transcriptional level and MMPs are key regulators of extracellular matrix remodeling, with a known impact on cancer metastization.

We intend to analyze this molecular profile in EVs isolated from patients' plasma, in order to establish a molecular model to predict disease outcome using a less invasive follow-up approach. In addition to that, we also intend to compare the impact of circulating miRNAs *versus* EV-derived miRNAs to compare the potential of these two approaches.

The identification of which patients have a greater probability to relapse will allow us in the future to individualize different treatments and submit the patients to therapies according to their expression profile and tumor aggressiveness. This approach could help to achieve one of the main goals of precise medicine.

Specific objectives

- Analysis of a profile of free circulating miRNAs in plasma samples of healthy individuals and RCC patients' and evaluation of their prognosis capacity as molecular biomarkers;

- Analysis of an EV-derived miRNAs and MMPs profile in plasma samples of ccRCC patients in different clinical time points and evaluation of their association with the disease phase and their capacity as molecular biomarkers;

- Evaluation of the association of the circulating EV-derived miRNAs and MMPs profile with clinical outcomes.

RESULTS

In order to answer to the proposed objectives, this section will be divided into three topics:

1. **Plasmatic miR-210, miR-221 and miR-1233 profile: potential liquid biopsies candidates for renal cell carcinoma**
    - This subchapter will focus on the EV-free circulating microRNAs biomarker potential to predict RCC patients' prognosis;

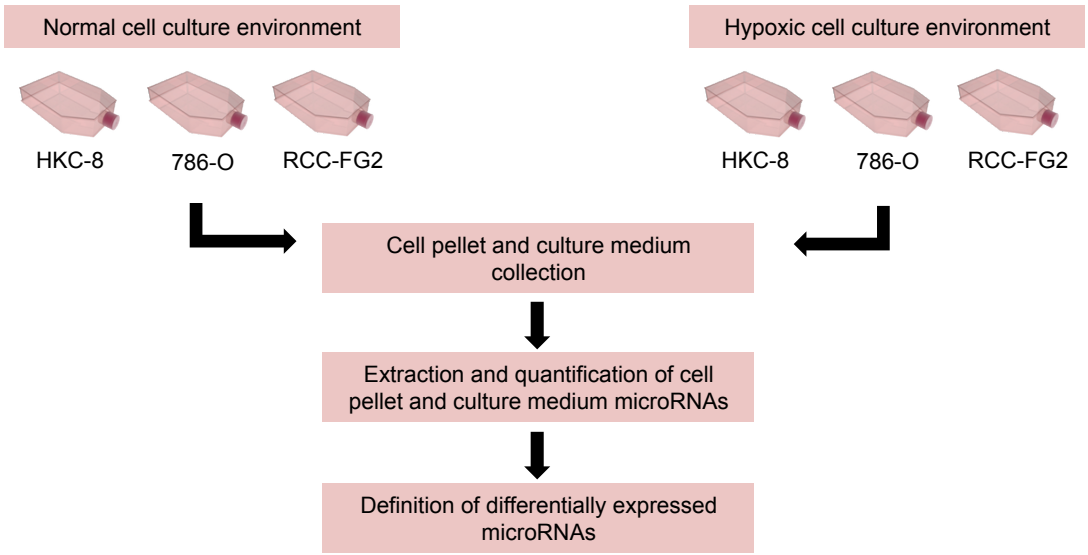
2. **Clear cell renal cell carcinoma extracellular vesicles-derived TIMP-1 mRNA as a prognostic biomarker: a pilot study**
    - This subchapter is focused on the analysis of EV-derived MMP profile in plasma samples of ccRCC patients in different clinical time points and evaluation of its association with patient prognosis;

3. **Extracellular vesicles enriched in hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p dynamics in ccRCC patients: potential impact on PI3K/Akt pathway activation**
    - This subchapter is focused on the analysis of and EV-derived microRNA profile in plasma samples of ccRCC patients in different clinical time points and evaluation of its association with patient prognosis.

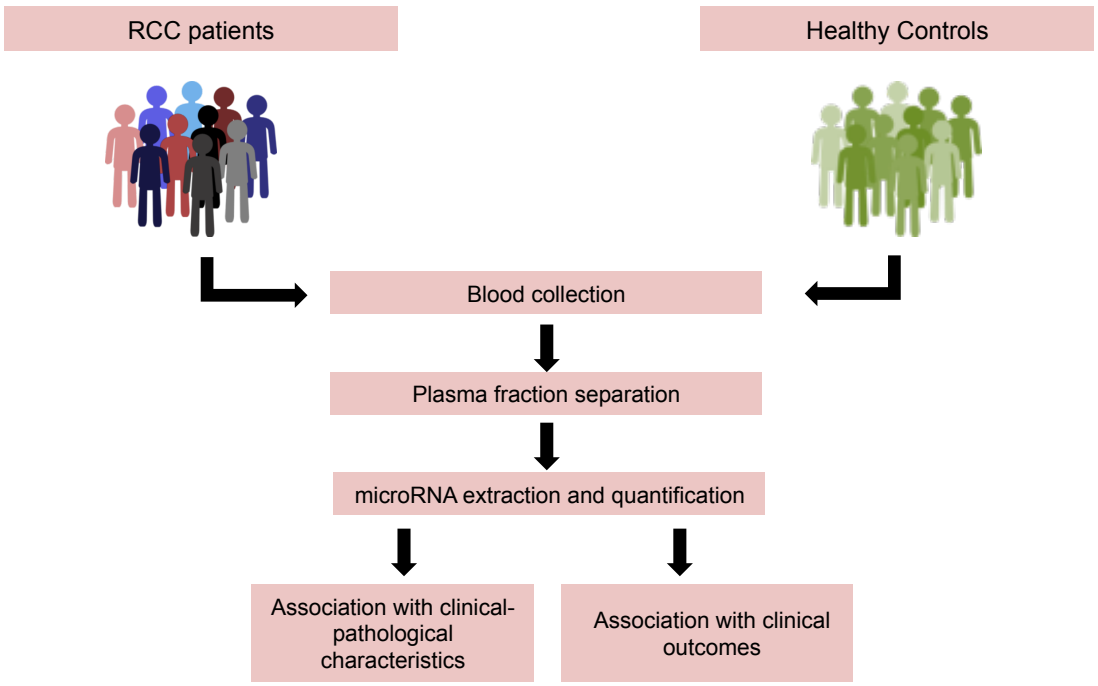
1. Plasmatic miR-210, miR-221 and miR-1233 profile: potential liquid biopsies candidates for renal cell carcinoma

1.1. Study design

Phase I: *In vitro* Study



Phase II: Validation study



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Plasmatic miR-210, miR-221 and miR-1233 profile: potential liquid biopsies candidates for renal cell carcinoma

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ABSTRACT

Renal cell carcinoma (RCC) represents a challenge for clinicians since the nonexistence of screening and monitoring tests contributes to the fact that one-third of patients are diagnosed with metastatic disease and 20–40% of the remaining patients will also develop metastasis. Modern medicine is now trying to establish circulating biomolecules as the gold standard of biomarkers. Among the molecules that can be released from tumor cells we can find microRNAs. The aim of this study was to evaluate the applicability of cancer-related miR-210, miR-218, miR-221 and miR-1233 as prognostic biomarkers for RCC. Patients with higher levels of miR-210, miR-221 and miR-1233 presented a higher risk of specific death by RCC and a lower cancer-specific survival. The addition of miR-210, miR-221 and miR-1233 plasma levels information improved the capacity to predict death by cancer in 8, 4% when compared to the current variables used by clinicians. We also verified that hypoxia stimulates the release of miR-210 and miR-1233 from HKC-8, RCC-FG2 and 786-O cell lines. These results support the addition of circulating microRNAs as prognostic biomarkers for RCC.

INTRODUCTION

Renal cell carcinoma (RCC) is the most common solid cancer of the adult kidney, accounting for approximately 90% of kidney neoplasms and 3% of all adult malignancies [1]. The most common histological RCC type is the clear cell RCC (ccRCC), which accounts for 80–90% of all RCCs. Worldwide RCC mortality currently exceeds 100.000 patients each year, with the incidence and mortality rates increasing by 2–3% per decade [2]. This reality and the nonexistence of screening and monitoring tests, contributes to the fact that one-third

of patients are diagnosed with metastatic disease and 20–40% of the RCC patient's submitted to nephrectomy will also develop metastasis [3]. Metastatic ccRCC remains incurable, but the prognosis for recurrent ccRCC varies widely and it has been reported that detecting early relapse can improve a patient's prognosis [4].

The current gold standard of cancer diagnosis is the histological examination of tissue, mainly obtained by biopsy. However this procedure is invasive, expensive and present risks for the patient, which emphasizes the need for alternative diagnostic techniques. Liquid Biopsies hold great clinical promise, as their non-invasive nature allows for rapid, economical and multiple sampling. These features allow their use in screening programs and the close monitoring of disease progression and treatment response, allowing earlier intervention and a dynamic treatment management [5]. Among the possible non-invasive biomarkers that have been studied in RCC, the ones that seem more promising are the microRNAs (miRNAs), since they can be detected using non-invasive procedures and are easier to quantify when compared to other molecules [6]. MiRNAs are small (18–24 nucleotides) non-coding RNAs that are responsible for the regulation of gene expression at a post-transcriptional level and have been widely studied in oncology since they are potent modulators of cellular behaviour and tumoral microenvironment [7, 8]. As a single miRNA may target up to several hundred mRNAs, aberrant miRNA expression may affect a multitude of transcripts and profoundly influence cancer-related signaling pathways [9]. MiRNAs are also present high stability in biofluids since they can be actively secreted from cells inside exosomes and microvesicles or circulate free in complex with proteins such as RNA-binding proteins, lipoproteins, high density lipoproteins (HDLs) and argonoute proteins [10]. The multitude of ways in which miRNAs can be released into circulation gives tumor cells the power to modulate the human body's response to their own advantage [10]. There is evidence that miRNAs regulate the "hallmarks of cancer", including the hypoxic microenvironment, a well established cellular characteristic of ccRCC [5]. Hypoxia is a unique environmental stress that induces global changes in a complex regulatory network of transcription factors and signaling pathways in order to coordinate cellular adaptations in metabolism, proliferation, DNA repair, and apoptosis [11]. One of the early molecular events in the oncobiology of ccRCC is the loss of *von Hippel Lindau* (*VHL)* gene which leads to an increase of *Hypoxia Inducible Factor alpha* (HIF-α) and, consequently, triggers an hypoxic response from the cell [12, 13]. Among the miRNAs regulated by hypoxia, we can find miR-210, miR-218 and miR-1233. MiR-210 expression is induced by hypoxia, which makes this miRNA an accurate indicator of the hypoxia state [14]. This miRNA is widely studied in cancer, including RCC, however, studies of its expression in biofluids are few and present contradictory results [15–18]. MiR-218 is considered a tumor suppressor miRNA in RCC and its expression is downregulated by hypoxia [19]. The studies regarding this miRNA were only performed in cell lines and tissue samples, so it would be interesting to evaluate its behavior in patients biofluids [20–23]. HIF1-α induces the transcription of multiple proangiogenic and growth factors including the vascular endothelial growth factor (VEGF) that subsequently activates a number of downstream pathways by binding mainly to VEGFR-2 [24]. MiR-221 targets VEGFR-2, its involved in the EGFR pathway activation and its overexpression in plasma samples was associated with a lower progression free survival (PFS) and lower overall survival (OS) in RCC patients by our group [25, 26]. MiR-1233 is considered an oncomiRNA since it targets p53, inhibiting its function in RCC [18]. However, so far there is only one study regarding miR-1233 expression in in RCC and, given the importance of the relation of p53 with HIF, it is important to further study the impact of this miRNA in RCC progression.

Regarding circulating miRNAs in RCC, only a few have been suggested as potential biomarkers for diagnosis and/or prognosis [6,25]. Despite promising, the miRNAs were studied in small cohorts and few were replicated by other groups, which empathizes the need for more studies in order to replicate, validate and establish circulating miRNAs as RCC biomarkers [6]. Our aim in this study is the evaluation of the impact of plasma levels of miR-210, miR-218, miR-221 and miR-1233 in clinical endpoints and their association with clinicopathological characteristics of RCC patients.

RESULTS

MiR-210, miR-218, miR-221 and miR-1233 are released from tumor cell lines

To validate the hypothesis that RCC cells may excrete these miRNAs into circulation, we performed an *in vitro* study in which we evaluate the levels of miR-210, miR-218, miR-221 and miR-1233, intracellularly and in the culture medium, of HKC-8, RCC-FG2 and 786-O cell lines. According to our results, with exception of miR-221 (Fold-change 5, $P = 0.260$), all the miRNAs are excreted from RCC-FG2 cells into their culture medium (Fold-change: miR-210: 3, $P = 0.020$; miR-218: 302, $P = 0.002$ and miR-1233: 11, $P = 0.021$). Regarding the 786-O cells, all the miRNAs were also excreted to the medium, with exception of miR-210 that only showed a tendency for excretion to medium (Fold-change: miR-210: 3, $P = 0.058$; miR-218: 193, $P = 0.017$, miR-221: 853 363, $P < 0.001$ and miR-1233: 1260, $P < 0.001$). Finally, none of the four miRNAs were excreted from HKC-8 cells (Fold-change: miR-210: 0.5, $P = 0.744$; miR-218: 7, $P = 0.482$; miR-221: 1.4, $P = 0.838$ and miR-1233: 4, $P = 0.327$) (Figure 1).

Plasma levels of miR-210, miR-218, and miR-1233 in RCC patients and their association with clinicopathologic characteristics

According to our results, we observed a significant increase in the plasma levels of miR-210, miR-218 and miR-1233 in RCC patients, when compared to healthy individuals (Fold-change: miR-210: 5, $P < 0.001$; miR-218: 80, $P < 0.001$; and miR-1233: 52, $P < 0.001$) (Figure 2A). We did not performed this analysis for miR-221 because it was already made by our group in a previous study, were we concluded that RCC patients presented higher plasma levels of miR-221 [25].

However, we did not found any statistical differences between miR-210, miR-218 and miR-1233 levels according to gender (miR-210: $P = 0.626$, miR-218: $P = 0.577$ and miR-1233: $P = 0.822$), tumor subtype (miR-210: $P = 0.138$, miR-218: $P = 0.160$, miR-1233: $P = 0.132$) and age (miR-210: $P = 0.527$, miR-218: $P = 0.413$, miR-1233: $P = 0.377$) in the patients group.

Regarding the clinicopathologic characteristics, we observed that patients with tumors larger than 7 cm present higher levels of miR-210 and miR-1233 (Fold-change: miR-210: 3, $P = 0.022$; miR-218: 2, $P = 0.249$; miR-221: 3, $P = 0.066$ and miR-1233: 4, $P = 0.003$) (Figure 2D). When we compare the miRNAs expression levels with the Fuhrman nuclear grade, only miR-1233 was associated with higher Fuhrman grades (Fold-change: miR-210: 2, $P = 0.381$; miR-218: 4, $P = 0.051$; miR-221: 2, $P = 0.246$ and miR-1233: 5 $P = 0.004$) (Figure 2C). We also observed that higher levels of miR-210 and miR-1233 were associated with presence of metastasis at the time of diagnosis when compared with patients with localized disease (Fold-change: miR-210: 3, $P = 0.045$; miR-218: 0.7, $P = 0.579$; miR-221: 3, $P = 0.030$ and miR-1233 and miR-1233: 7, $P = 0.029$) (Figure 2B).

Higher plasmatic levels of miR-210, miR-221 and miR-1233 and cancer-specific survival

The fifty patients in the entire cohort were separated into miR-210, miR-218, miR-221 and miR-1233 high risk (higher plasma levels) and low risk (lower plasma levels) groups using *Cutoff Finder* software (http://molpath.charite.de/cutoff), to generate the optimum cut-off score for their normalized plasma expression (-ΔCq). The Kaplan-Meier analysis showed that patients with higher levels of miR-210 and miR-1233 (high risk groups) present a significantly lower cancer-specific survival ($P = 0.015$, $P = 0.003$, respectively) (Figure 3, left panel). MiR-218 plasma levels weren't associated with cancer-specific survival ($P = 0.350$) and miR-221 showed a tendency to be associated with cancer-specific survival ($P = 0.089$) Additionally, the *Cutoff Finder* software also

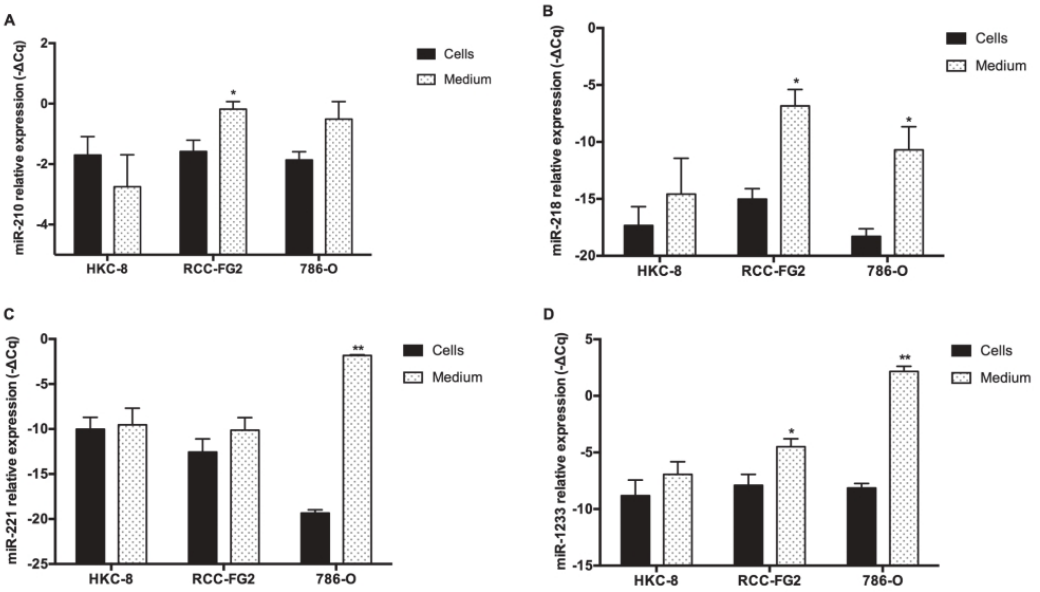

Figure 1: Intracellular and extracellular expression of miR-210, miR-218, miR-221 miR-1233 in HKC-8, 786-O and RCC-FG2 cell lines. The bars represent the –ΔCq of the miRNAs plasmatic expression normalized to RNU48. (**A**) miR-210 levels in HKC-8, RCC-FG2 and 786-O cell lines and respective medium; (**B**) miR-218 levels in HKC-8, RCC-FG2 and 786-O cell lines and respective medium; (**C**) miR-221 levels in HKC-8, RCC-FG2 and 786-O cell lines and respective medium and (**D**) miR-1233 levels in HKC-8, RCC-FG2 and 786-O cell lines and respective medium. (Mean ± Std.Error; $*P < 0.05$, $**P < 0.001$).

allowed us to apply a ROC analysis using the optimum cut-off score generated, which can be observed in Figure 3B, 3D, 3F and 3H (right panel). Regarding miR-210, the sensitivity was 60.9% and the specificity was 73.1% (AUC = 0.70); for miR-221, the sensitivity was 71.4% and the specificity was 65% (AUC = 0.62) and for miR-1233 the sensitivity was 39.1% and the specificity 92.6% (AUC 0.61). We also observed an addictive effect of the combination of the plasma levels of miR-210, miR-221 and miR-1233. In fact, the cancer-specific survival was significantly lower in patients' with higher levels of miR-210, miR-221 and miR-1233 (48 *versus* 91 months) (Figure 4). Furthermore, multivariate Cox regression model using tumor TNM stage (I /II versus III/IV), Fuhrman nuclear grade (G1/G2 versus G3/G4), Age (> 60 years) and gender as co-variants, demonstrated a higher risk of specific death by RCC in patients who presented simustaneously higher levels of miR-210, mir-221 and miR-1233 (HR = 3.02, 95%CI 1.19–7.64, P = 0.014). The concordance (c) index was used to compare the predictive ability of different prognostic variables associated with RCC overall survival; the predictive value was assessed with Harrell's concordance indexes, where a c index of 1 indicates perfect concordance [27]. Tumor TNM stage and Fuhrman nuclear grade are well-known prognostic factors for cancer progression. In our study, the predictive value of tumour TNM stage (I/II versus III/IV), Fuhrman nuclear grade (G1/G2 versus G3/G4), and age (> 60 years) for poor cancer-specific survival (death by RCC) was 0.744 (model 1). However, this predictive ability increased to 0.828 (model 2) with the addition of the information regarding miR-210, miR-221 and miR-1233 plasma levels. The addition of miR-210, miR-221 and miR-1233 plasma levels information improved the capacity to predict death by cancer in 8.4% compared with model 1 (Table 1).

Accute hypoxia exposure stimulates the release of miR-210 and miR-1233 from normal and tumor cell lines and interferes with CXCR4 mRNA expression

In an attempt to validate the hypothesis that the hypoxia is involved in the release of these miRNAs into circulation, we stimulated all the cell lines with crescent doses of cobalt chloride ($CoCl_2$), a well known hypoxia inducer, during 24h and evaluated its effects on miR-210 and miR-1233 excretion in all cell lines (Figure 5). In normal conditions, the HKC-8 cell line doesn't excrete none of the miRNAs to the cell medium. However, when we stimulate the cells with $CoCl_2$, they start to excrete miR-210 and miR-1233 (Figure 5A–5C). Interestingly, when we do the same stimulus in the RCC cell lines, we also verify an increase of the miR-210 and miR-1233

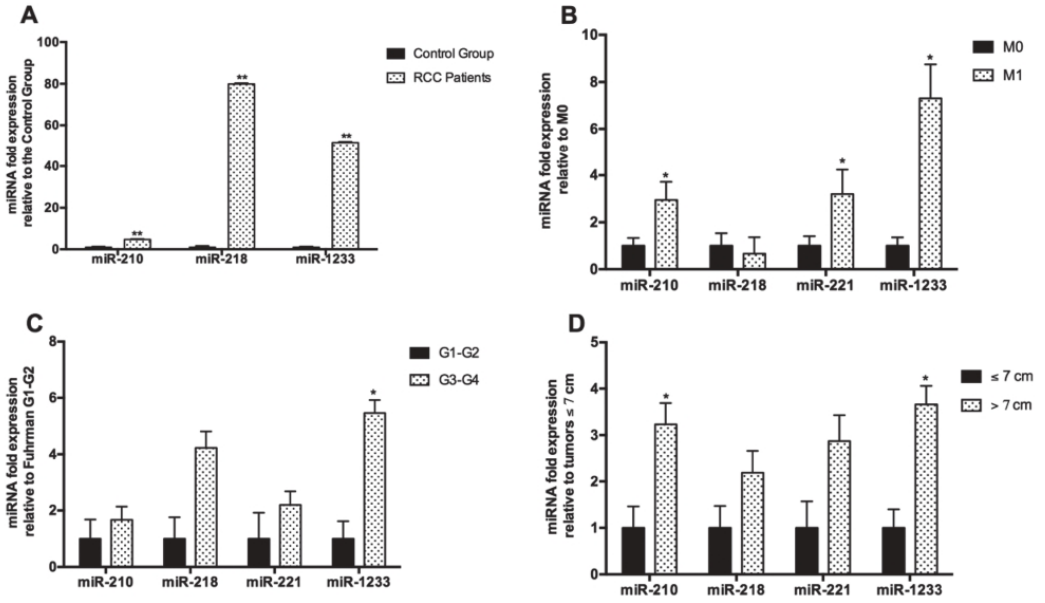

Figure 2: Fold-change of the plasmatic levels of miR-210, miR-218 and miR-1233 according to clinicopathological characteristics. The bars represent the $2^{-\Delta\Delta Cq}$ as a fold-change in miRNA plasmatic expression normalized to RNU48. Expression levels shown are means of three technical replicates for each sample. (**A**) RCC patients *vs* healthy individuals; (**B**) Tumor ≤7 cm *vs* tumors > 7 cm); (**C**) Fuhrman grade G1-G2 *vs* Fuhrman grade G3-G4); and (**D**) No presence of metastasis ate the time of diagnosis (M0) vs Presence of metastasis at the time of diagnosis (M1). (Mean ± Std.Error; *$P < 0.05$, **$P < 0.001$).

Table 1: Predictive models of death by RCC according to different prognostic factors

	HR	95% CI	*P* value	*c* index
Model 1				
Tumor TNM stage (I and II vs III and IV), Fuhrman nuclear grade (G1 and G2 vs G3 and G4), Age (> 60 years) and Gender	3.90	1.76–8.64	< 0.001	0.744
Model 2				
miR-210 + miR-221 + miR-1233 plasma expression, Tumor TNM stage (I and II vs III and IV), Fuhrman nuclear grade (G1 and G2 vs G3 and G4), Age (> 60 years) and Gender	3.89	1.26–12.01	0.018	0.828

excretion but it's not as accentuated as for the HKC-8 cell line (Figure 5D, 5E, 5G and 5H).

Additionally, we also measured the mRNA expression of CXCR4, a well known molecule involved in cancer progression and metastasis and what we observed was that, with the acute hypoxic stimulus, the HKC8 cell line started to express more CXCR4 mRNA and that expression was dependent of the $CoCl_2$ concentration (Figure 5C). However, in the 786-O cell line the CXCR4 mRNA expression diminished with the increase of $CoCl_2$ concentration and in the FG-2 cell line the CXCR4 mRNA expression was approximately the same despite the increase of $CoCl_2$ concentration (Figure 5F and 5I).

DISCUSSION

Currently, no standard approaches to biomarker sampling or analysis have been adopted for RCC since many of the potential tumor markers are still under active investigation for further validation [28]. There are several factors that must be considered when choosing miRNAs as candidate prognostic biomarkers for RCC. First, the fold-change of the miRNA should be significant enough to discriminate RCC patients from healthy individuals. Second, the biological function and carcinogenesis mechanism of each miRNA should be thoroughly investigated in RCC since a better understanding of the targeted genes of the miRNAs would advance their use in clinical settings. Last but not least, rigorous validation and demonstration of reproducibility in independent cohorts of patients are necessary to confirm the prognostic value of miRNAs [29].

Some authors state that the most miRNA enriched biofluid is the plasma and inclusively there is a study showing the superiority of plasma over serum for circulating miRNAs analysis [30]. This study is based on the release of platelets or white blood cells miRNA contents to the serum during the coagulation process, which compromise the miRNA contend in serum [30, 31]. The existing studies in circulating samples that evaluated miR-210 and miR-1233 were made in serum samples and miR-218 was never studied in biofluids [15–18]. Only miR-221 was characterized in plasma samples of RCC patients, in previous studies from our group [25, 32]. To the best of our knowledge, this is the first study that evaluates the plasma levels of miR-210, miR-218 and miR-1233 in RCC.

Our *in vitro* study demonstrated that RCC-FG2 cell line excreted miR-210, miR-218 and miR-1233 to the extracellular medium and that 786-O cell line excreted miR-218, miR-221 and miR-1233 to the medium and also a tendency to excrete miR-210. Those results were validated *in vivo* when we compared plasma samples from RCC patients with healthy controls. Additionally we observed that patients with tumors higher than 7 cm and patients that presented metastasis at the time of diagnosis presented higher levels of miR-210 and miR-1233, suggesting that these two miRNAs are related to tumor aggressiveness alongside miR-221 that we previously reported as having higher plasma concentrations in RCC patients [25]. Additionally, miR-1233 was the only miRNA associated with higher Fuhrman grades. Although we didn't find any association between miR-218 and the clinicalphatologic features, our results regarding this miRNA are interesting because it is secreted by the tumor cell line and its also higher in the plasma of the RCC patients. This suggests that miR-218 is indeed secreted by the tumor, but probably with a different purpose. Since miR-218 is described in the literature as a tumor suppressor miRNA and is down-regulated in RCC tumor samples, we hypothesize that the excretion of miR-218 is a mechanism that tumor cells use in order to prevent its tumor suppression activity [21, 22].

When we analyzed the individual impact of the expression of miR-210 and miR-1233 in clinical endpoints, we observed that they were both associated with a lower cancer-specific survival, while miR-221 only showed a tendency towards that. Subsequently, we analyzed the impact of higher levels of the three miRNAs and we observed that patients presenting higher levels of miR-210, miR-221 and miR-1233 combined presented a higher risk of specific death by RCC and a lower cancer-specific survival. Additionally we created a model of death prediction by RCC using the standard variables used in the clinic and compared it to a model were we added the expression profile of miR-210, miR-221 and miR-1233

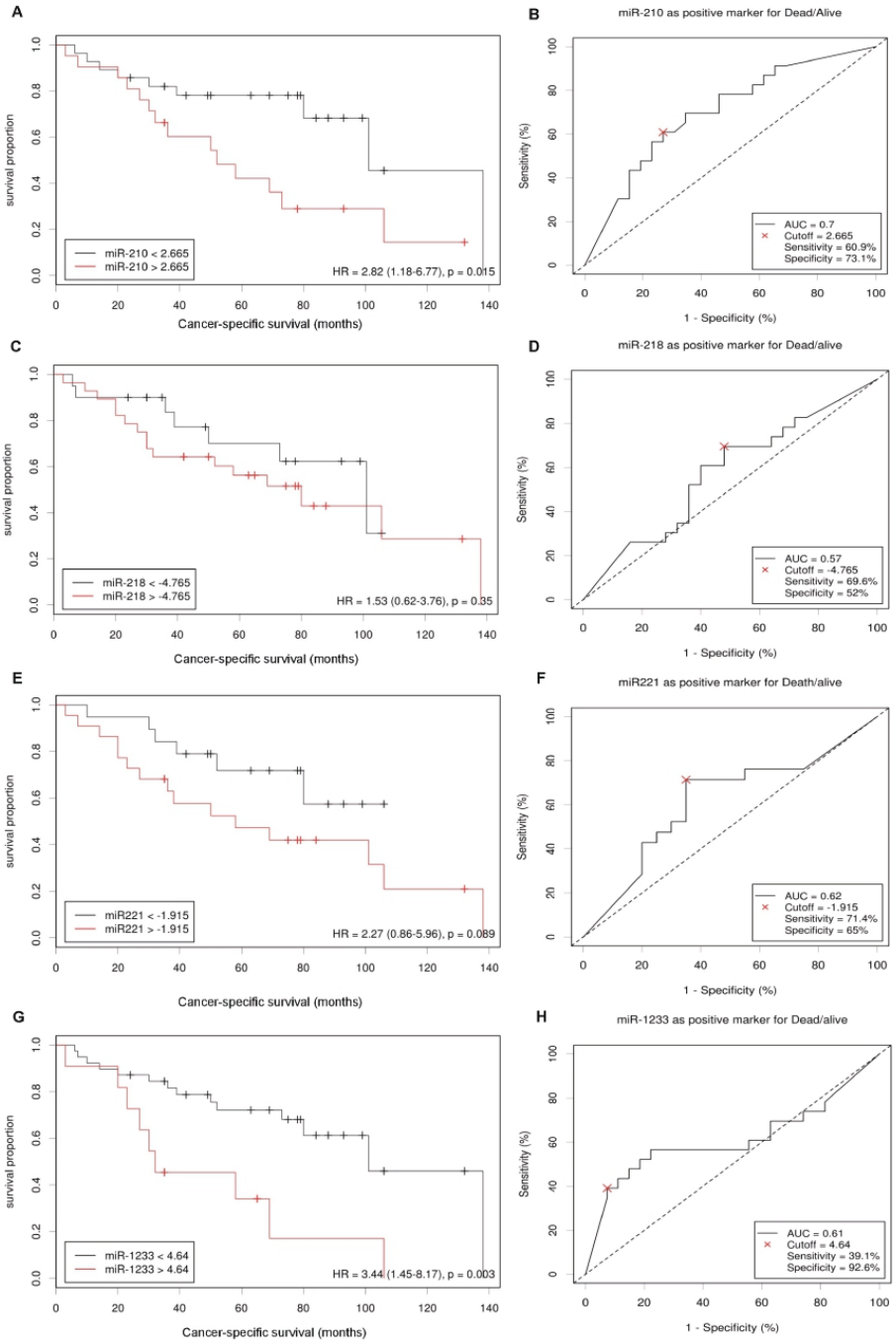

Figure 3: miR-210, miR-218, miR-221 and miR-1233 prognostic roles in patients with RCC. Cut-offs for "high" and "low" expression of miR-210 (**A**), miR-218 (**C**), miR-221 (**E**) and miR-1233 (**G**) were identified by the online web application *Cutoff Finder*. The optimal cut-off is defined as the point with the most significant (log-rank test) split and the effect of each miRNA in cancer-specific survival is presented in the corresponding Kaplan-Meier plot. The *Cutoff Finder* also allowed to assess the quality of the prediction through the construction of ROC curves using the optimal cut-off points established for miR-210 (**B**), miR-218 (**D**), miR-221 (**F**) and miR-1233 (**H**).

and we observed that the addiction of the plasma levels of miR-210, miR-221 and miR-1233 improved the capacity to predict death by RCC in when compared to the first model. These results suggest that miR-210, miR-221 and miR-1233 combined are potential prognostic profile of biomarkers for RCC and also open the door for the addition of microRNAs in predictive models of death by RCC.

Additionally, we also evaluated the impact of acute hypoxia in the two miRNAs that presented a significant individual impact in cancer-specific survival: miR-210 and miR-1233. After the addition of crescent concentrations of $CoCl_2$ to all the cell lines we observed that indeed hypoxia was part of the process by which cells excrete miRNAs into the cell medium. That effect is very marked in the HKC-8 cell line, were we observed that, in normal conditions, this cell line doesn't excretes neither miR-210 nor miR-1233 but when we stimulate then with crescent doses of hypoxia both miRNAs are excreted to the cell medium. The crescent hypoxia is also associated with crescent production of CXCR4 mRNA, a potent angiogenic and tumor progression inducer. In the ccRCC cell lines (RCC-FG2 and 786-O) we also observe that the hypoxic stimulus also increases the release of miR-210 and miR-1233 but not as markedly as in the HKC-8 cell line, which may be due to the fact that both these cell lines already present a basal excretion of theses miRNAs due to their malignancy and RCC phenotype. Interestingly, the hypoxia stiumulus as the opposite effect in 786-O and RCC-FG2 cell lines when compared to the HKC-8 cell line, which led us to the conclusion that CXCR4 may only act in the first stages of hypoxia.

In conclusion, the stimulus to hypoxia, which translates in a higher grade of cell proliferation, angiogenesis and metastatic potential, in patients with higher levels of miR-210 and miR-1233 is associated with a lower cancer specific survival, resulting in a higher risk of death by RCC. For the first time it was demonstrated that the evaluation of the combined miR-210, miR-221 and miR-1233 profile could allow a better monitorization of RCC patients' by distinguishing poor from favorable risk patients. In the future it would be interesting to improve our RCC death prediction model by studying and adding more plasma miRNAs and also to perform more functional studies to better understand the effects of hypoxia on miRNA secretion from the cells. It would also be interesting to study the mechanism by which the tumor cells excreted these miRNAs since recent studies demonstrated that tumor cells use exosomes (small vesicles) to shuttle molecules from the tumor to other locations.

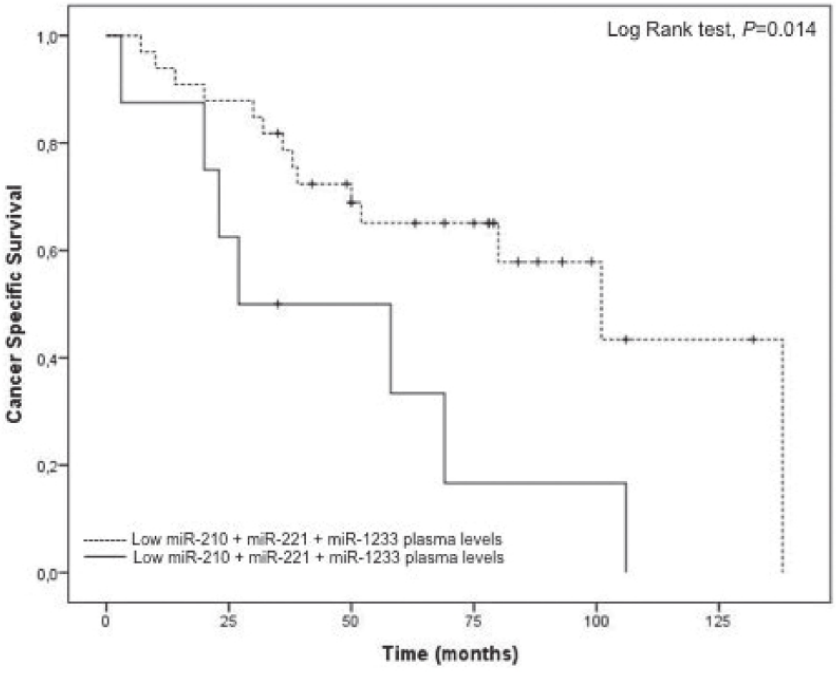

Figure 4: Cancer-specific survival according to combined expression of miR-210, miR-221 and miR-1233 plasma levels in RCC patients. Patients with higher expression of miR-210, miR-221 and miR-1233 combined present a lower cancer-specific survival (Log Rank test, $P = 0.014$).

MATERIALS AND METHODS

Cell line characterization

Three renal cell lines were used: HKC-8, RCC-FG2 and 786-O. The HKC-8 cell line is an immortalized proximal tubular epithelial renal cell line (PTEC), the RCC-FG2 is a metastatic ccRCC cell line and 786-O is a ccRCC cell line. Both HKC-8 and RCC-FG2 were kindly provided by Dr Klaas Kok (Groningem University, Netherlands) and 786-O was kindly provided by Professor Carmen Jerónimo (IPO-Porto Research Center, Portugal) [33].

Cell culture

Initially a cryopreserved vial of each cell line was thawed. The RCC-FG2 and 786-O cell lines were maintained in RPMI 1640 (1X) medium (*Gibco®*), supplemented with 10% of FBS (Fetal Bovine Serum) (*Gibco®*) and 1% of Pen-Strep (*Gibco®*). The HKC-8 cell line was kept in DMEM/F12 medium (*Gibco®*), supplemented with ITS (Insuline-transferrine-selenium) (*Sigma-Aldrich®*), Pen-Strep (*Gibco®*), EGF (Epidermal Growth Factor) (*Sigma-Aldrich®*), Hepes buffer (*Gibco®*) and Hydrocortisone (*Sigma-Aldrich®*). Both cell lines were maintained in a 5% CO_2 incubator at 37ºC.

When the desired confluence was achieved (80–90%) the medium, in which the cells were being cultured, was collected for miRNA extraction and the cells were trypsinized, using 0.05 % trypsin-EDTA (1×) (*Gibco®*) and counted using a Neubauer chamber and Tripan-Blue dye (*Gibco®*). After counting, approximately two million cells were centrifuged to form a pellet for miRNA extraction and the remaining cells were kept in culture. This procedure was repeated five times for each cell line.

Hypoxia induction

All the cell lines were harvested during the logarithmic period and counted by NanoTech cell countes. Cell suspencions, approximately 200 000 cells/well were seeded in 6-well plates (marca) and cultured

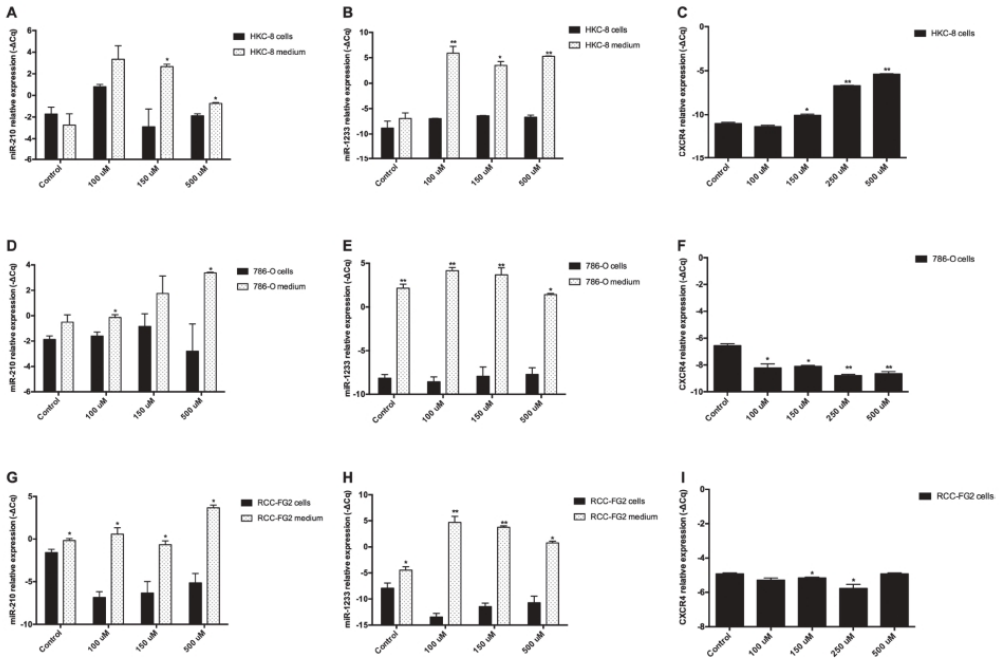

Figure 5: Extracellular expression of miR-210, miR-1233 and CXCR4 in HKC-8, 786-O and RCC-FG2 cell lines after hypoxia induction. The bars represent the –ΔCq of miR-210, miR-1233 and CXC4 after hypoxia stimulation with crescent doses of $CoCl_2$ for each cell line. (**A**) miR-210 levels in HKC-8 cell line after $CoCl_2$ stimulation; (**B**) miR-1233 levels in HKC-8 cell line after $CoCl_2$ stimulation; (**C**) CXCR4 levels in HKC-8 cell line after $CoCl_2$ stimulation; (**D**) miR-210 levels in 786-O cell line after $CoCl_2$ stimulation; (**E**) miR-1233 levels in 786-O cell line after $CoCl_2$ stimulation; (**F**) CXCR4 levels in786-O cell line after $CoCl_2$ stimulation; (**G**) miR-210 levels in RCC-FG2 cell line after $CoCl_2$ stimulation; (**H**) miR-1233 levels in RCC-FG2 cell line after $CoCl_2$ stimulation; (**I**) CXCR4 levels in RCC-FG2 cell line after $CoCl_2$ stimulation; The –ΔCq of miR-210 and miR-1233 was normalized to RNU48 and the CXCR4 –ΔCq was normalized to GUSB. (Mean ± Std.Error; $*P < 0.05$, $**P < 0.001$).

in a humidified incubator at 37ºC and 5% CO2 for 24 h. Then, $CoCl_2$ (Ref C8661, *Sigma-Aldrich®*), was added in crescent concentrations was added to each well, making final concentrations of 100 µM, 150 µM and 500 µM. The cells were incubated with $CoCl_2$ during 24h and after that period the miRNAs were extracted from the cells and respective medium and quantified using the procedures previously described. This experimente was performed two times and in duplicate for each cell line.

Study population

The validation of the circulating miRNA expression profile was made through a hospital-based study, involving a total of 104 individuals: 54 RCC patients and 50 healthy individuals. All RCC patients were Caucasian from the north of Portugal, with histopathologic diagnosis of RCC, admitted and treated at the Portuguese Oncology Institute of Porto (IPO-Porto) between 1 of September 2003 and 30 of October 2013. The mean age was 60.3 ± 12.1 years, from which 74.1% (n = 40) were male and 25.9% (n = 14) female. Patients' clinical characteristics were obtained from their medical records. Tumor classification and staging were established according to the tumor-node-metastasis (TNM) classification system of the American Joint Committee on Cancer (AJCC) 2010, 7ª edition (Table 2). For the control group, 50 healthy Caucasian individuals, from which 30% were male (n = 16) and 68% (n = 34) were female, with no history of cancer, were randomly recruited from the north of Portugal, with a mean age of 43.0 ± 15.5 years.

Sample collection and miRNA and mRNA extraction/ purification

Approximately 8 mL of peripheral blood were collected from all individuals through a standard method of intravenous collection using EDTA tubes. The blood tubes were centrifuged 5 minutes at 3000 rpm at room temperature, in order to separate the plasma fraction from the blood cells.

The miRNA isolation protocol was the same for cultured cells, cultured cells medium and plasma samples. We added an acid phenol-chloroform (5:1) solution (*Ambion®*) to the samples, which, after centrifugation at 15.000 rpm at 5ºC for 15 min, allowed the separation of the RNA/microRNA phase. MicroRNA purification was performed using the *GRS microRNA kit* (*Grisp®*), with adjustments in the manufactured protocol.

The mRNA isolation and purification of the culture cells and cultured cell medium was performed using the *GRS Total Blood & Cultures Cells kit (Grisp®)*

After isolation, RNA concentration and purity were measured at 260 and 280 nm using the *NanoDrop® ND-1000* spectrophotometer.

cDNA synthesis

The miRNA samples were used as templates for cDNA synthesis using a *Taqman®MicroRNA Reverse Transcription kit* (*Applied Biosystems®*) and sequence-specific stem-loop primers for hsa-miR-210-3p, hsa-miR-218-1-3p, hsa-miR-221, hsa-miR-1233-3p and RNU-48. We used RNU-48 as an endogenous control for data normalization since it presented a stable expression pattern among samples and was previously used for data normalization in RCC studies [34]. After protocol optimization the thermal conditions were as follows: 16ºC for 30 minutes, followed by 42ºC for 60 minutes and 85ºC for 10 minutes.

The mRNA samples served as templates for cDNA synthesis using a *High Capacity cDNA Reverse Transcription Kit* (Applied Biosystems®). The thermal conditions for PCR amplification were optimized to fby 37ºC for 120 min and 85ºC for 5 min for mRNA.

Real-time PCR relative quantification

The miRNA and mRNA expression levels were analyzed by quantitative real-time PCR. The reactions were carried out on a StepOne™qPCR Real-Time PCR machine, containing 1X Master mix (*Applied Biosystems®*), with 1X probes (*TaqMan® microRNA Expression Assays:* hsa-miR-210-3p: TM000512, hsa-miR-218-1-3p: TM-002094, hsa-miR-221: TM-002096, hsa-miR-1233-3p: TM-002768 and *TaqMan® mRNA Expression Assays:* CXCR4: Hs00607978_s1, *Applied Biosystems®*), cDNA sample (≈ 50 ng), RNU-48 endogenous control for miRNA normalization (*TaqMan® Gene Expression Assays*, TM-001006, *Applied Biosystems®*) and Human GUSB (Beta Glucoronidase) endogenous control (*Applied Biosystems®*) for mRNA normalization.

The amplification conditions were as follows: holding stage 95ºC for 20 seconds, followed by 45 cycles of 95ºC for 1 second and 60ºC for 20 seconds. Three technical replicates were made for each sample.

Data analysis was made using StepOne™ Sofware v2.2 (*Applied Biosystems®*) with the same baseline and threshold set for each plate, in order to generate quantification cycle (Cq) values for all the miRNAs in each sample.

Statistical analysis

Statistical analysis was also made using IBM®SPSS®Statistics software for Windows (Version 22.0). The $2^{-\Delta\Delta Cq}$ method, along with the Student t' test were used in order to evaluate any statistical differences in the normalized expression levels of the miRNAs here explored. We used *Cutoff Finder* web application to

Table 2: Distribution of the clinicopathological factors of the study population

	Cases (n = 54)		Control Group (n = 50)	
	n	%	*n*	%
Gender				
Male	40	74.1	16	32
Female	14	25.9	34	68
Age				
Mean ± SD	60.3 ± 12.1		43.0 ± 15.5	
Histology				
Clear Cell	39	72.2		
Others	15	27.8		
TNM Stage				
I–II	19	38		
III–IV	31	62		
T				
T1	18	33.4		
T2	5	9.30		
T3	26	48.2		
T4	5	9.30		
N				
N0	6	11.1		
N1	2	3.70		
N2	4	7.40		
Nx	42	77.8		
M				
M0	42	77.8		
M1	12	22.2		
Fuhrman Grade				
G1	1	1.90		
G2	15	27.8		
G3	16	29.6		
G4	19	35.2		
Unknown	3	5.60		

generate optimum cut-off point for the three deregulated miRNAs in the plasma samples of RCC patients. *Cutoff Finder* selects the optimum cut-off point, separating patients into high-risk and low-risk groups, by fitting Cox proportional hazard models to the dichotomized variable and the survival variable. The optimal cut-off is defined as the point with the most significant (log-rank test) slip [35]. A Cox porportional hazard model was used to analyze the patients' cancer-specific survival, considering as covariants TNM stage, Fuhrman nuclear grade and age (> 60 years). The concordance (χ) was used to compare the predictive ability of the association of well-known prognostic variables with the combined plasma levels of miR-210, miR-221 and miR-1233 with $\chi > 0.5$ being considered with a good prediction ability [27].

Abbreviations

RCC, Renal Cell Carcinoma; ccRCC, clear cell Renal Cell Carcinoma; miRNAs, microRNAs; HDL, high density lipoprotein; HIF-1α, Hypoxia Inducible Factor alpha; PTEC, proximal tubular renal cell line ; FISH, Fluorescence *in situ* Hybridization; TNM, tumor-node-metastasis; AJCC, American Joint Committee on Cancer; EFNA3, ephrin A3; PTP1B, tyrosine phosphatase non-receptor type 1; CASP8AP2, caspase 8 associated protein 2; E2F3, E2F transcription factor 3; MNT, MAX network transcriptional repressor; FGFR1, fibroblast growth factor receptor 1; EGFR, Epidermal Growth Factor Receptor; *VHL*, von Hippel Lindau tumor suppressor; *HRAS*, HRas proto-oncogene; *RASSF7*, Ras association domain family

member 7; *RAD52*, RAD52 homolog, DNA repair protein; *BLCAP*, bladder cancer associated protein.

Ethics approval and consent to participate

This study was conducted according to the principles of the Helsinki Declaration and was approved by the ethics committee at the Portuguese Oncology of Porto (Portugal) (CES 245/2013). All individuals signed a written informed consent to participate in the study.

Author contributions

FD and ALT designed and planned the study. FD did the majority of the experimental work. BA and NB helped in the *in vitro* experiments. MF helped processing the control group samples and extracting microRNAs. JV performed the FISH technique in the cell lines used. MF, MIS, JM, FM, AM and JO recruited the patients for the study and collected and organized their clinical information. FD, ALT and RM performed the stastitical analysis and interpreted the results obtained. KK provided the cell lines used in the study and contributed for the manuscript elaboration. FD wrote the manuscript. ALT and RM were also major contributors in the manuscript construction. All authors approved the final manuscript.

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CONFLICTS OF INTEREST

None.

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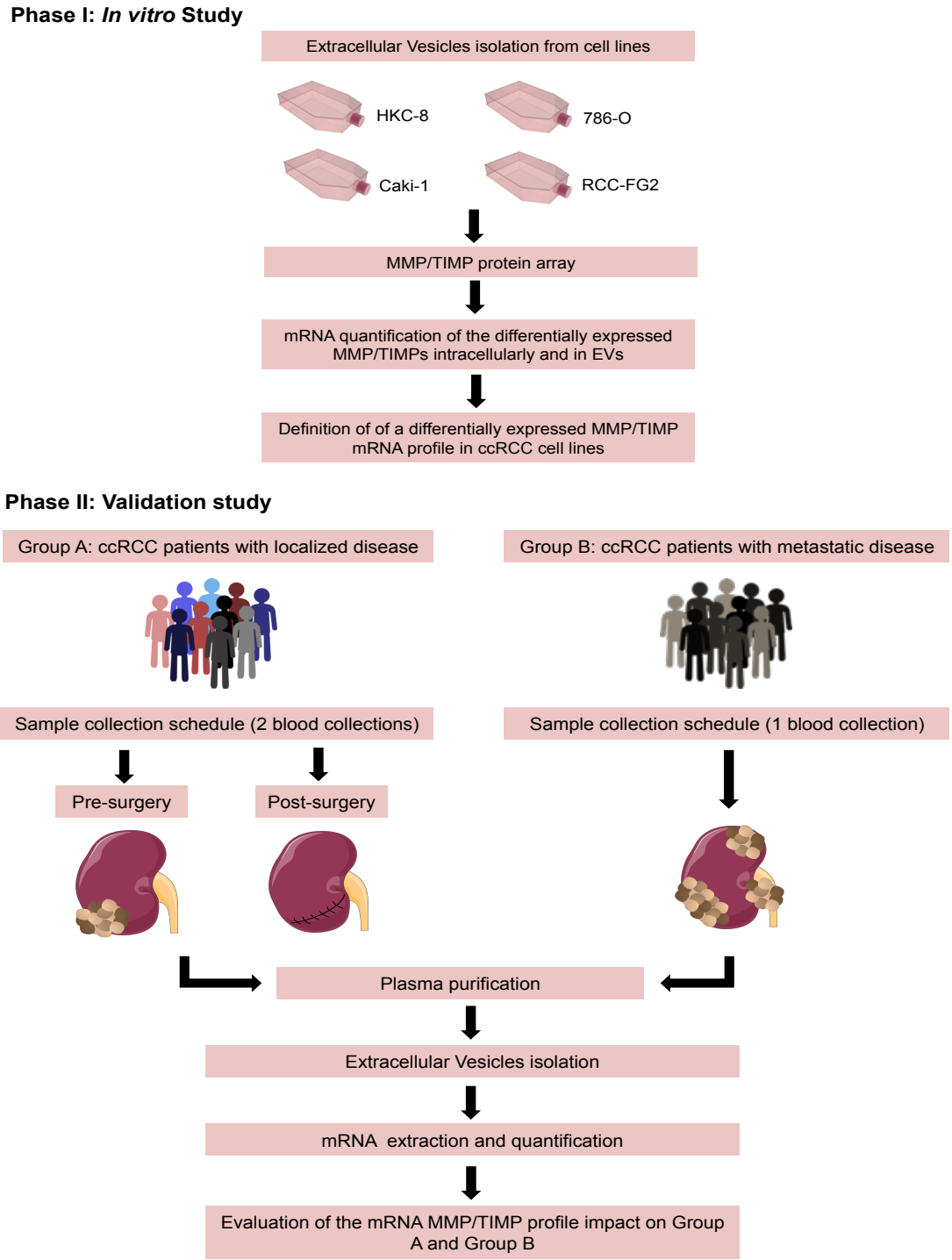
2. Clear cell renal cell carcinoma extracellular vesicles-derived TIMP-1 mRNA as a prognostic biomarker: a pilot study

2.1. Study design



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Clear cell renal cell carcinoma extracellular vesicles-derived TIMP-1 mRNA as a prognostic biomarker: a pilot study

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Abstract

The tumor microenvironment has gained a lot of attention from the scientific community since it as a proven impact in the modulation of tumor progression and metastasis. One of the key players of tumor environment modulation is extracellular vesiciles (EVs). EVs are small microvesicles sheded by cells that are able to shuttle bioactive mocules between cells and therefore have an impact on the recipient cell phenotype. Renal cell carcinoma (RCC) is one of the most lethal urological neoplasias and presents a high metastatic potential, which reinforces the need for the development more effective predictive biomarkers. Our goal was to evaluate the aplicabilty of EV-derived MMPs and TIMPs as prognostic biomarkers for clear cell RCC (ccRCC). We observed that patients with localized disease and tumors larger than 7 cm presented higher levels of plasmatic EV-derived TIMP-1 mRNA when compared with patients presenting smaller tumors (P=0.020). Moreover, patients with metastatic disease presented higher levels of EV-derived TIMP-1 mRNA when compared with patients with localized disease (P=0.002) and when we stratified those patients in high and low levels of TIMP-1 EV-derived mRNA the ones presenting higher levels had a lower overall survival (P=0.030). EV-derived TIMP-1 mRNA may be a good prognostic biomarker candidate for RCC.

Keywords: Renal cell carcinoma, Extracellular Vesicles, TIMP-1, Biomarkers

Introduction

Cancer is a heterogeneous disease, with different etiology and natural history that develops trough the interactions between environmental and genetic factors, involving the deregulation of multiple pathways responsible for the fundamental cell processes, such as proliferation, differentiation, migration and cell death [1]. However, despite the existing knowledge of several genetic factors on tumor pathophysiology, understanding the complex molecular mechanisms underlying its development remains unclear, implying that, besides the intrinsic malignant properties of tumor epithelial cells, other factors such as microenvironmental changes may modulate tumor progression, invasion and metastasis [2]. One way of microenvironment shaping is through paracrine and/or systemic signaling among cells that can be made through the release of soluble factors or through the shedding of extracellular vesicles (EVs). There are two major classes of EVs: microvesicles and exosomes. Microvesicles are formed by the outward budding of the plasma membrane of the cell and exosomes originate from multivesicular bodies and are released from cells when the multivesicular bodies fuse with the cell surface [3]. In the vast repertoire of bioactive molecules that EVs carry, we can find proteins, DNA, and several classes of RNAs including non-coding RNAs, such as microRNAs, mRNAs, rRNAs and tRNAs [4]. EVs can serve to shuttle bioactive molecules between cells and their cargo can induce pro-tumorigenic tumor microenvironments, locally or at distant sites, since EVs can travel to other areas in the body [5]. This process may preceed metastatic dissemination since it involves marked extracellular matrix (ECM) remodeling through the delivery of several molecules, including functional matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), or their mRNAs, into target cells [6-10].

MMPs are a family of 23 zinc-dependent endopeptidases that are crucial for ECM degradation and the processing of cell surface molecules [11]. On the other hand, the TIMP family, which is composed by 4 elements, is responsible for the regulation of the pericellular proteolysis of ECM and cell surface proteins through inhibition of MMPs [11]. Both MMPs and TIMPs are often deregulated in cancer with impact on patients' prognosis [12,13]. To date, 11 MMPs and 3 TIMPs have been identified in EVs derived from various cell types, including cancer cells, but the available information is still very limited [14]. The presence of proteolytic molecules in EVs can have implications in ECM remodeling and, consequently, in the modulation of the structural architecture and dynamics of the tumor microenvironment both locally and at distant sites. Thus, it is of paramount importance to increase the current knowledge on this matter.

Renal cell carcinoma (RCC) is the most common solid cancer of the adult kidney and the most lethal urological cancer [15]. The most common and aggressive subtype is the clear cell RCC (ccRCC), which accounts for approximately 80% of all RCCs [16]. The increased use of routine imaging techniques during the past decades led to the incidental dectection of early stage localized RCCs while the patients were doing exams for other medical reasons. However, due to the kidneys anatomical location, many tumors remain asymptomatic until the late stages of disease [16]. In fact, up to one third of RCC patients present metastatic disease at diagnosis and 20-40% of patients submitted to nephrectomy will present local recurrence or distant metastasis [17]. CcRCC is chemo- and radioresistant and although systemic treatment with targeted therapies may improve the survival, the development of resistance is frequent in very common in these patients [18]. Thus, there is an urgent need for the development of new effective targets and therapies, as well as the establishment of accurate prognostic and predictive biomarkers in order to improve the follow-up to these patients.

The aim of this study was the establishment of an EV-derived MMP/TIMP profile for ccRCC and evaluate its potential as a prognosis biomarker in this malignancy.

Results

EVs characterization

The NTA analysis indicated that the vast majority of isolated EVs presented a size range between 50 and 200 nm, which is consistent with the size of exosomes and small microvesicles (Figure 1A-E). Transmission Electron Microscopy (TEM) image (Figure 1F) shows the variability of sizes and morphology present in EVs from purified platelet-free-plasma (PFP). We also utilized EVs Flow Cytometry to confirm the purity of our EV isolates. This method utilizes CFSE staining to differentiate vesicular from non-vesicular particles, as this dye only becomes fluorescent when incorporated and processed within vesicles, and has been described by some authors as a pan-EV label [24-26]. In all cases, more than 80% of particles present in our isolates corresponded to $CFSE^{+}$ vesicular structures (Supplementary Fig.1).

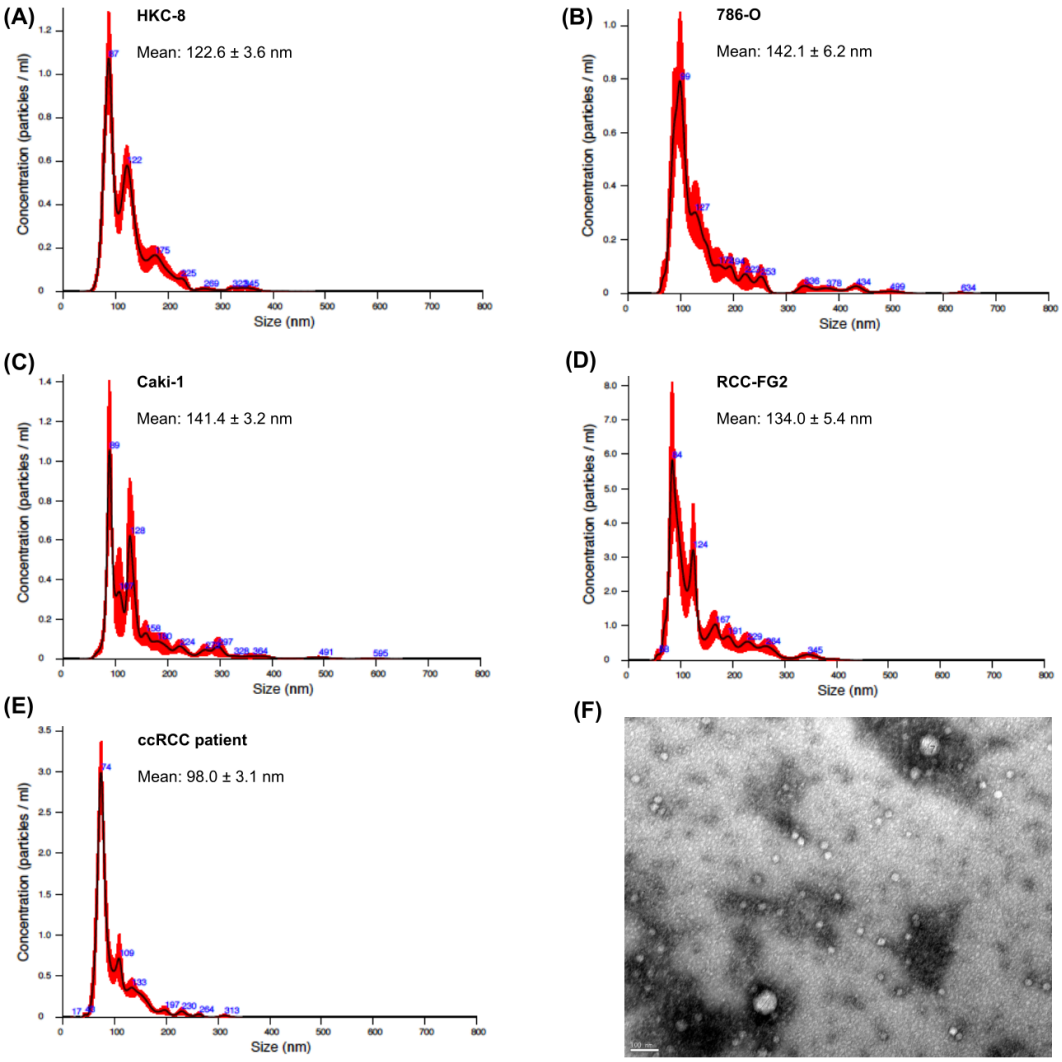
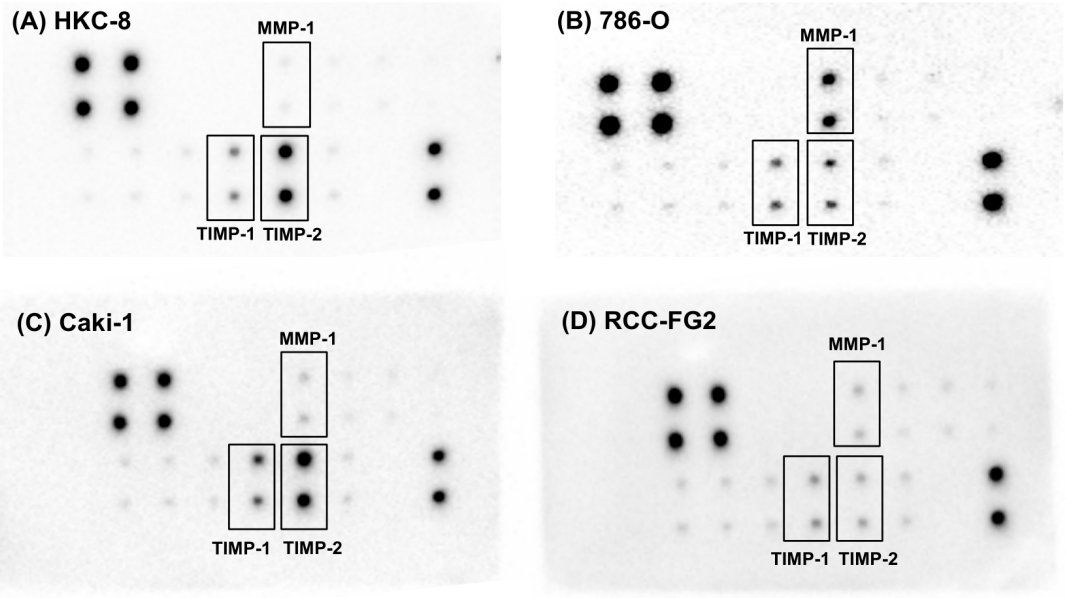


Figure 1. Nanoparticle tracking analysis (NTA) of EVs derived from (A) HKC-8, (B) 786-O, (C) Caki1 and (D) RCC-FG2 cells and (E) ccRCC patients. The red error bars indicate ± 1 standard error of the mean. (F) Transmission electron microscopy (TEM) of EVs from purified platelet-free plasma (PFP). The TEM image was acquired in the Histology and Electron Microscopy platform from I3S Porto using a Transmission Electron Microscope Jeol JEM 1400.

MMP-1, TIMP-1 and TIMP-2 present different protein levels among renal cell derived EVs

Compared to the HKC-8 immortalized normal kidney cell line (Fig.2A), all the three ccRCC-derived cell lines diverged in terms of EV protein expression: the 786-O cell line (B) presented a higher expression of MMP-1 and a lower expression of TIMP-2, the Caki-1 cell line (C) presented similar expression in terms of TIMP-2 and a slightly higher expression of TIMP-1, and the RCC-FG2 cell line (D) presented lower expression of TIMP-2 and similar expression of TIMP-1. Interestingly, the expression of MMP-1 was higher in the cell line derived from a primary ccRCC tumor (B) when compared to both metastatic ccRCC cell lines (C and D). In addition to that, TIMP-1 and TIMP-2 expression were also different in both ccRCC metastatic cell lines (C and D). Taken together, these data suggest that different ccRCC clones may present different MMP/TIMP profiles.



	A	B	C	D	E	F	G	H
1	Pos	Pos	Neg	Neg	MMP-1	MMP-2	MMP-3	MMP-8
2	Pos	Pos	Neg	Neg	MMP-1	MMP-2	MMP-3	MMP-8
3	MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4	Neg	Pos
4	MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4	Neg	Pos

Figure 2. Human MMP Antibody Array analysis in cell-derived EVs and the map for the location of MMPs and TIMPs antibodies. Membranes were probed with EV lysate from (A) HKC-8, (B) 786-O, (C) Caki-1 and (D) RCC-FG2 cells. MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinase; Pos, positive control; Neg, negative control.

MMP-1, TIMP-1 and TIMP-2 mRNA levels in renal cell lines and cell line-derived EVs

The Caki-1 cell line presented the highest intracellular TIMP-1 mRNA expression compared to the other three cell lines (Fig.3A). Regarding mRNA expression in the EVs, TIMP-1 was detected in all EV fractions. The 786-O cell line presented lower TIMP-1 mRNA expression when compared to the HKC-8, and the Caki-1 cell line presented a trend for higher TIMP-1 mRNA than the 786-O cell line (P=0.089) (Fig.3B). As for TIMP-2, HKC8 and Caki-1 had the highest intracellular mRNA levels and 786-O presented the lowest, which is in agreement with the MMP/TIMP array results (Fig.3C). TIMP-2 mRNA was detected in all EV fractions without any statistical differences in the expression level between them (Fig.3D).

The 786-O cell line presented the highest MMP-1 intracellular mRNA levels when compared to the other cell lines, which is in agreement with the results of the MMP/TIMP array (Fig.3E) However, the Caki-1 cell line also showed elevated MMP-1 mRNA expression which was not reflected in protein expression in the MMP/TIMP array. The mRNA levels of MMP-1 in cell-derived EV's are not presented since they were only detected in the EV's derived from the 786-O cell line, and absent in the EVs from the other cell lines.

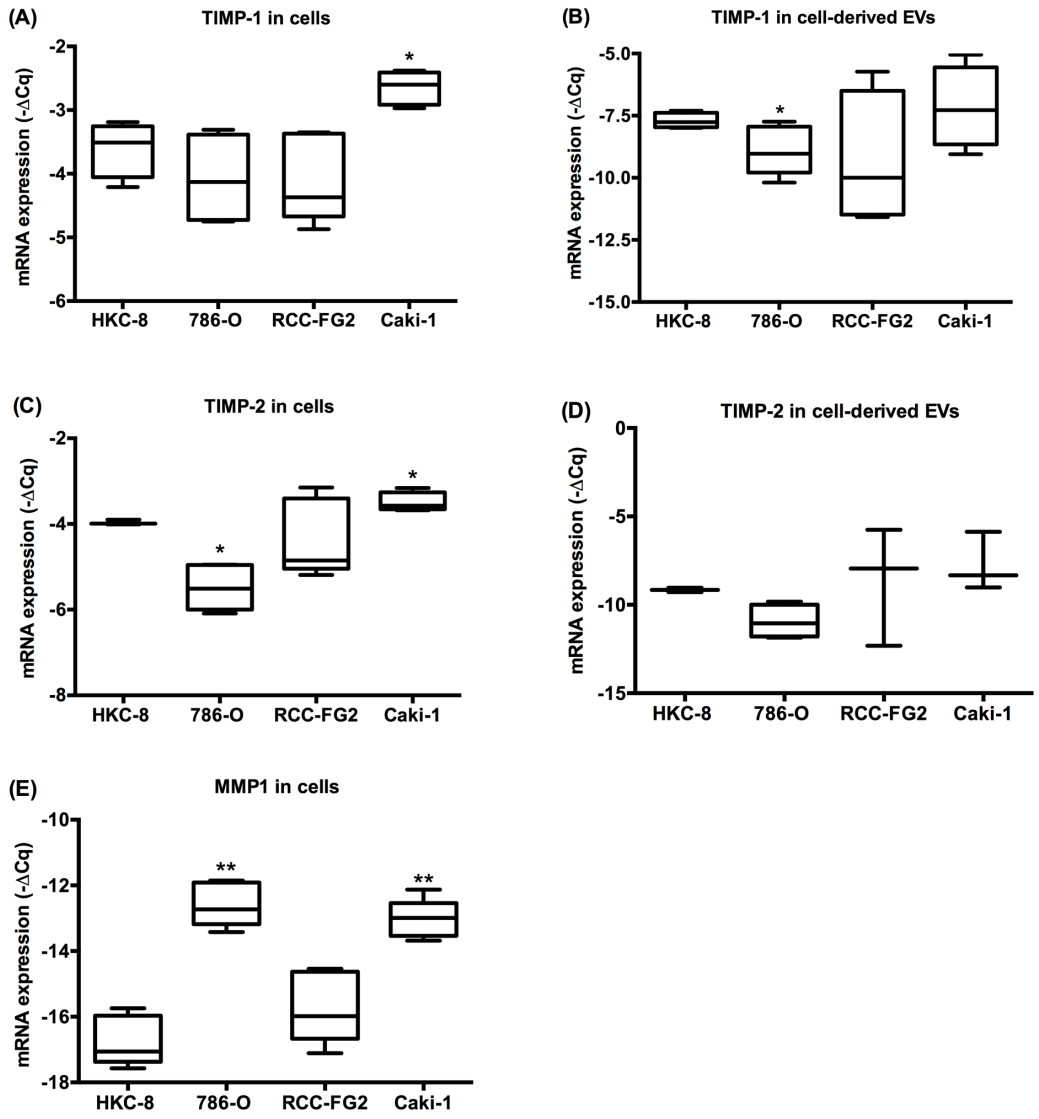


Figure 3. MMP1, TIMP1 and TIMP2 mRNA expression intracellularly (A, C and E) and in EV's (B and D) derived from HKC8, 786-O, RCC-FG2 and Caki-1 cell lines. (Mean ± Std. Error; **P*<0.05, ***P*<0.001)

TIMP-1, TIMP-2 and MMP-1 mRNA expression in ccRCC patient EVs

TIMP-1, TIMP-2 and MMP1 EV-derived mRNA expression was analyzed in two grouos of ccRCC patients: patients with localized disease, before and after surgery (Group A) and patients with metastatic disease (Group B). We observed that patients with metastatic disease presented higher TIMP-1 EV mRNA levels compared to patients with localized disease (*P*=0.002) (Fig. 4A). When we focused on the patients with localized disease, we observed that those with tumors larger than 7 cm also presented higher levels of EV-derived TIMP-1 mRNA (*P*=0.020) (Fig. 4B). Next, we divided the metastatic patients into high and low levels of TIMP-1 EV-derived mRNA using the –ΔCq mean as a cutoff value. We observed that patients with metastatic disease and higher levels of TIMP-1 EV-derived mRNA presented a lower overall survival compared to patients presenting with lower levels (Log Rank test, *P*=0.030) (Fig. 4C).

There were no statistical significant differences for TIMP-2 EV-mRNA levels between patients with localized *versus* metastatic disease (*P*= 0.338) and also not in terms of tumor size in the patients presenting localized disease (*P*=0.477) (Fig.4D and E). When we focused on the survival of metastatic patients, those presenting lower EV-derived TIMP-2 mRNA levels had a lower overall survival (Log Rank test, *P*=0.013) (Fig. 4F). However, it is important to note that TIMP-2 EV-derived mRNA was only detected in 8 samples.

MMP-1 EV-derived mRNA expression was only detected in four samples in total (1 from Group A + 3 from Group B) which didn´t allow any statistical analysis (data not shown). The percentages of TIMP-1, TIMP-2 and MMP-1 mRNA detection in patients' EVs are presented in Supplementary Fig.2.

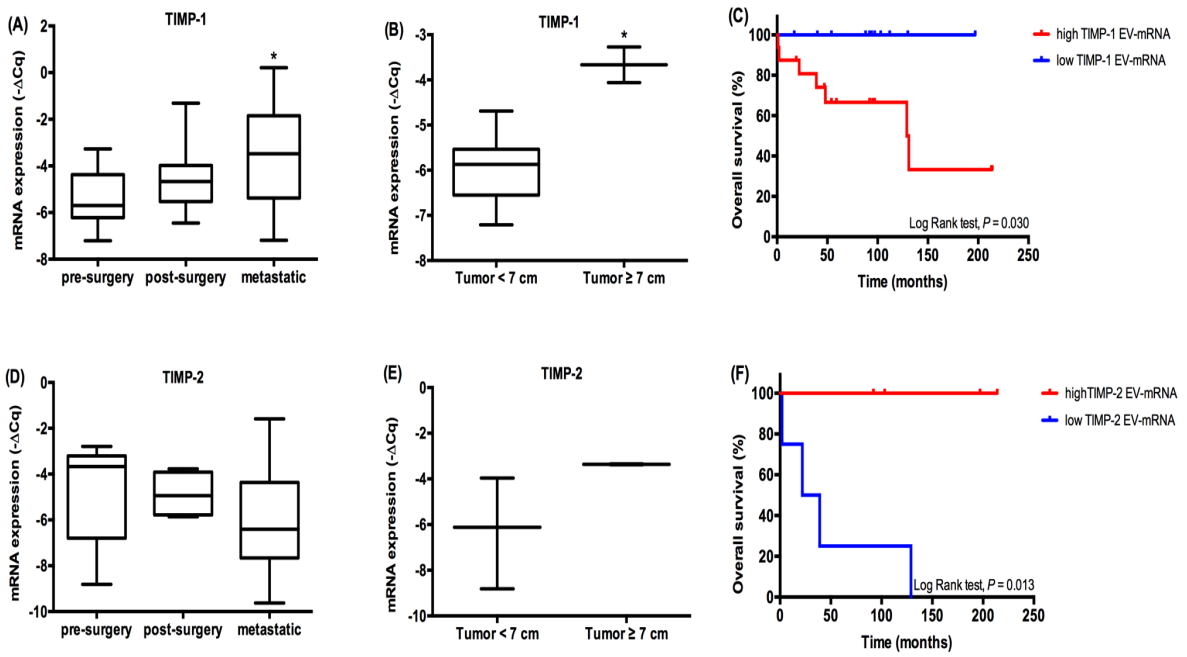


Figure 4. EV-derived TIMP1 and TIMP2 mRNA expression in ccRCC patient samples. EV-derived TIMP-1 (A) and TIMP-2 (D) mRNA expression in patients with localized disease (pre- and post-surgery) and metastatic disease. EV-derived TIMP-1 (B) and TIMP-2 (E) mRNA expression in patients with localized with tumor < 7 cm and ≥ cm. Overall survival of metastatic ccRCC patients according to EV-derived TIMP-1 (C) and TIMP-2 (F) mRNA expression (Mean ± Std. Error; **P*<0.05, ***P*<0.001).

EV-derived TIMP1 mRNA as prognostic marker in ccRCC patients: the example of two patients

As stated previously, two groups of patients were analyzed in the current study: one group with localized disease whose blood samples were collected before and after surgery and another group already with metastatic disease. From the 32 patients included in the localized disease group (Group A), two of them developed metastasis during the follow-up period. Interestingly, both of them presented high levels of EV-derived TIMP-1 mRNA both in the pre- and post-surgery samples, and both of them underwent radical nephrectomy. The patients' clinical data are presented in table 1. Given the fact that our survival analysis indicated that metastatic patients with higher EV-derived TIMP-1 mRNA present a lower overall survival and that these two patients with elevated EV-derived TIMP-1 mRNA developed metastatic disease within a short period of time, we can hypothesize that the levels of EV-derived TIMP-1 mRNA could be a prognosis biomarker, namely a metastatic disease predictor.

Taking this into account, we further investigated the clinical data of the ccRCC metastatic patients that presented high levels of TIMP1 mRNA and are currently deceased from the disease. From the seven patients that were deceased, three of them presented metastasis at the moment of diagnosis and the remaining four presented localized disease when they were diagnosed but developed metastasis and passed away during a five-year period. These four patients presented at least two recurrences each during this period, which is consistent with a more aggressive disease.

Table 1. Clinical characteristics of the two patients that present high levels of EV-derived TIMP-1 mRNA in the pre- and post-surgery samples.

	Gender	**Age**	**Tumor size**	**TNM stage**	**ISUP**	**Time to recurrence**	**Metastatic site**
Patient A	male	83	6 cm	pT3aNxM0R0	4	9 months	lung
Patient B	male	74	8 cm	pT3cN0M0R0	3	21 months	bones

Discussion

MMPs are one of the major classes of proteolytic enzymes involved in tumor invasion and metastasis establishment and their action can be modulated by TIMPs. Despite earlier studies have demonstrated that TIMPs had antimetastatic effects, during the last few years reports started to indicate a dual function of these proteins, with a positive correlation between their expression and a poorer outcome in several human cancers [27-31]. Regarding ccRCC, TIMP-1 overexpression has been reported has an independent factor associated with tumor growth and was also associated with a lower patient overall survival [32-34]. On the other hand, TIMP-2 down regulation has been reported during ccRCC disease progression, suggesting its potential role as a prognostic biomarker and its lower expression as also been associated with higher bone metastasis potential [35].

Recent studies of protein profiles have revealed the presence of TIMPs in EVs from various cell types, but their biological impact remains to be elucidated [14]. TIMP-1 and TIMP-2 proteins have been found in EVs derived from cancer cells, bone marrow mesenchymal stem cells and also in pregnant women [36-38]. In terms of their potential impact cancer, it its known that EV-derived TIMP-1 binds to CD63 and β1 integrin, which induces survival signals and promotes metastatic niche formation [11]. EV-derived TIMP-2, on the other hand, plays a crucial role in the formation of the MT1-MMP-TIMP2-pro-MMP2 ternary complex for MMP2 activation, which is well known for its impact on cancer progression [39,40].

In order to get more insight into the EV-derived MMP/TIMP profile in ccRCC, we started with the study of EVs derived from four differrent renal cell lines. All of the cell lines exhibited different EV-derived MMP/TIMPs protein profiles, being the most prominent differences in the expression of MMP-1, TIMP-1 and TIMP-2. Next, we looked into the mRNA levels of MMP-1, TIMP-1 and TIMP-2 intracellularly and in the sheded EVs from the cell lines in order to see if there was an association between them. Interestingly, the significant associations observed when comparing the intracellular mRNAs among the cell lines were not observed when comparing the cell-derived EVs.

MRNAs represent a very attractive target to study in EVs since they are unstable molecules that are subject to degradation by RNase activity and their incorporation into EVs protects them, allowing their stable circulation in body fluids without the risk of degradation [41]. We studied the mRNA expression of TIMP-1, TIMP-2 and MMP1 in plasma EVs from two groups of ccRCC patients: patients with localized disease before and after tumor removing surgery (Group A) and patients with metastatic disease (Group B). In patients with localized disease, those with larger tumors had higher expression of EV-derived TIMP-1 mRNA. When comparing both groups of patients, we observed that

patients with metastatic disease had higher expression of EV-derived TIMP-1 mRNA than patients with localized disease. In fact, metastatic patients presenting a higher expression of EV-derived TIMP-1 mRNA presented a lower overall survival. The opposite effect was observed for EV-derived TIMP-2 with lower expression levels being associated with a lower overall survival. However, TIMP-2 EV-derived mRNA was quite difficult to detect in ccRCC patients' EVs, which suggests that it may not be a good biomarker candidate and the same applies to MMP-1 mRNA.

During the course of follow-up, two of the patients with localized disease (group A) developed metastasis several months after surgery. When we looked up to the levels of EV-derived TIMP-1 mRNA we observed that both patients presented high levels before surgery and those levels kept high one month after surgery, which reinforced the hypothesis that EV-derived TIMP-1 mRNA may play a role on disease progression, even when the primary tumor is removed. It is important to note that tumor growth and invasion is also supported by non-cancerous stromal cells, which include endothelial cells, fibroblasts, pericytes and immune cells [42-44]. These cell types are an active part of the tumor microenvironment and several studies have already demonstrated that tumor-derived EVs are able to exert various effects on neighboring stromal cells [5,14,45,46].

A study performed by Cui and colleagues demonstrated that an increase of both endogenous and exogenous levels of TIMP-1 led to upregulation of miR-210 in a CD63-PI3K-AKT-HIF1-dependent pathway in lung adenocarcinoma cells. Moreover, upon the overexpression of TIMP-1, miR-210 was accumulated in exosomes that were able to promote angiogenesis in *vitro* and in xenograft models once in contact with recipient cells, thereby evidencing the pro-tumourigenic function of TIMP-1 [47]. In fact, our group previously associated the high plasmatic levels of miR-210 with a worse prognosis in RCC patients, and this evidence could be linked to the higher circulating levels of TIMP-1 EV-derived mRNA but further studies are needed in order to validate this hypothesis [48].

To the best of our knowledge this is the first study to address the potential of TIMP-1 EV-derived mRNA as a prognosis biomarker for ccRCC patients. Future studies in a larger cohort of patients, with an increased follow-up period are needed in order to validate our hypothesis.

Material and Methods

Cell lines

Four renal cell lines were used: HKC-8, RCC-FG2, 786-O and Caki-1. HKC-8 is an immortalized proximal tubular epithelial renal cell line (PTEC), 786-O is a primary ccRCC cell line, and both RCC-FG2 and Caki-1 are metastatic ccRCC cell lines. HKC-8 and

RCC-FG2 were kindly provided by Dr Klaas Kok [19]. And 786-O was provided by Prof Carmen Jerónimo.

Cell culture

The 786-O, Caki-1 and RCC-FG2 cell lines were maintained in RPMI 1640 (1X) medium (*Gibco*®), supplemented with 10% of FBS (Fetal Bovine Serum) (*Gibco*®) and 1% of Pen-Strep (*Gibco*®). HKC-8 was kept in DMEM/F12 medium (*Gibco*®), supplemented with ITS (Insuline-transferrine-selenium) (*Sigma-Aldrich*®), Pen-Strep (*Gibco*®), EGF (Epidermal Growth Factor) (*Sigma-Aldrich*®), Hepes buffer (*Gibco*®) and Hydrocortisone (*Sigma-Aldrich*®). All cell lines were maintained in T75cm^2 flasks with 10 mL of complete medium in a humidified (5% CO_2) incubator at 37ºC. All the cell lines tested negative for mycoplasma contamination. For collection of cells and EVs, the cells were switched at 70% confluence to medium supplemented with exosome-depleted FBS (*Gibco*®) for 72h after which both the cells and the medium containing the EVs were collected. This procedure was repeated five times for each cell line.

Ethics statement

This study was conducted according to the principles of the Helsinky Declaration, having been approved by the ethics committee of the Portuguese Oncology Institute of Porto (project reference: 251/015). All individuals have signed a written informed consent in order to participate in the study.

Study Population

The analysis of the EV-derived TIMP-1, TIMP-2 and MMP-1 mRNA expression was made through a hospital-based study involving a total of 61 ccRCC patients. All individuals were Caucasian from the north of Portugal, with histopathological diagnosis of ccRCC, admitted and treated at the Portuguese Oncology Institute of Porto (IPO-Porto) between November 2015 and June 2019. The patients were divided into two groups: Group A was composed by patients diagnosed with localized disease that were going to undergo surgical intervention; and Group B was composed of patients already presenting metastatic disease. Regarding the 29 patients from group B, 20 developed metastatic disease during the follow-up period after surgery and 7 were already diagnosed with metastatic disease. Clinical characteristics of patients were obtained from their medical records (Table 2). Patients from Group A collected blood twice, before undergoing surgery

and approximately 4 months after surgery and patients from Group B collected blood once. All blood collections were performed during the morning period.

Tumor classification and staging were established according to the tumor-node-metastasis (TNM) classification system of the American Joint Committee on Cancer (AJCC) 8th edition (2018) and the International Society of Urological Pathology (ISUP) Classification of Renal Neoplasia [20].

Table 2- Clinical-pathological characteristics of the study population. Group A is constituted by the patients with localized disease and Group B is constituted by the patients with metastatic disease.

		Group A (N = 32)		Group B (N = 29)	
		N	%	n	%
Gender					
	Male	24	75.0	21	72.4
	Female	8	25.0	8	27.6
Age	Average ± SD	61.9±12.4		62.4±9.9	
Type of surgery					
	Partial nephrectomy	18	56.3	0	0
	Radical nephrectomy	14	43.8	27	93.1
	No surgery	0	0	2	6.9
Tumor size					
	< 7 cm	24	75.0	7	24.1
	≥7 cm	8	25.0	18	62.1
	Undetermined	0	0	4	13.8
T					
	T1	17	53.1	8	27.6
	T2	1	3.1	7	24.1
	T3	13	40.6	10	34.5
	T4	0	0	1	3.4
	Tx	1	3.1	3	10.6
N					
	N0	0	0	15	51.7
	N1-N2	0	0	3	10.3
	Nx	0	0	9	31.0
M					
	M0	32	100	20	68.9
	M1	0	0	7	24.1
	Mx	0	0	2	7.0

Clinical Stage					
	I	17	53.1	7	24.1
	II	1	3.1	6	20.7
	III	13	40.6	8	27.6
	IV	0	0	5	17.2
	Not available	1	3.1	3	10.4
ISUP classification					
	1	2	6.3	1	3.45
	2	18	56.3	12	41.4
	3	11	34.3	7	24,1
	4	1	3.1	5	17.2
	Not available	0	0	4	13.8
Smoking status					
	Smoker	6	18.8	2	6.9
	Ex-smoker	11	34.4	4	13.8
	Non-smoker	14	43.8	23	79.3
	Not available	1	3.1	0	0
Hypertension					
	Yes	20	62.5	14	48.3
	No	11	34.4	15	51.7
	Not available	1	3.1	0	0
Diabetes mellitus					
	DM I	0	0	0	0
	DM II	9	28.1	12	41.4
	No	22	68.8	17	58.6
	Not available	1	3.1	0	0

EVs isolation

EVs were isolated from the cell-culture medium using the Total Exosome Isolation Reagent (from cell culture media) (Invitrogen™) with additional protocol optimizations. Briefly, cell medium was collected and centrifuged 30 minutes at 2000g to remove cells and debris. After centrifugation, the supernatant was recovered and filtered through a 0.22μM filter (GE Healthcare Whatman ™). Then the clarified medium and the exosome isolation reagent were mixed, in a proportion of 2:1, and incubated overnight at 4ºC. Afterwards, the mix was centrifuged 1 hour at 10.000g and the pellet containing the pre-enriched EVs was resuspended in filtered PBS and stored at -80ºC until further analysis.

Regarding the pheripheral blood samples, EVs were isolated from the plasma fraction using the Total Exosome Isolation Kit from Plasma (Invtrogen™) with additional protocol optimizations. Firstly, 8 mL of peripheral blood was collected from the patients in EDTA tubes and centrifuged 5 mins at 1800g to obtain the plasma fraction. The plasma fraction was centrifuged 3 additional times at increasing speeds (300g, 2100g and 10.000g) for a period of 15 mins each in order to obtain platelet-free-plasma (PFP). After centrifugation, the supernatant was recovered and filtered through a 0.22μM filter (GE Healthcare Whatman ™). A 10-minute treatment with proteinase K, the Total Exosome Isolation (TEI) reagent was added to 200μL of PFP and the solution was incubated for 30 min at 4ºC. The precipitated EVs were recovered by a 5 min centrifugation at 10.000g at room temperature. The pellet containing the pre-enriched EVs was resuspended in filtered PBS (0.22 μm membrane filters) and stored at -80ºC until further analysis.

EVs NTA Analysis

All samples were analyzed for particle concentration and size distribution by the NS300 Nanoparticle Tracking Analysis (NTA) system (NanoSight – Malvern Panalytical, UK). Samples were pre-diluted in filtered PBS to achieve a concentration within the range for optimal NTA analysis. Video acquisitions were performed using a camera level of 16 and a threshold between 5 and 7. Five to nine videos of 30 seconds were captured per sample. Analysis of particle concentration per mL and size distribution was performed with NTA software v3.4.

Quantification of vesicular structures by EVs flow cytometry

We employed EVs flow cytometry for quantification of vesicular structures in our EV isolates, as recently described by Maia and colleagues [21]. $2x10^9$ particles of purified

EVs were mixed with 40 µL of PBS containing Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE – Thermo Fisher Scientific - LTI C34554) in a final concentration of 40 µM and incubated for 90 minutes at 37° C. For removal of unbound CFSE, Size Exclusion Chromatography (SEC) columns (iZON - qEV original columns SP1, UK) were used. Samples containing unstained or stained EVs, and appropriate controls, were diluted up to 500 µL of PBS and processed by qEV following manufacturer's instructions. EVs-enriched fractions #7, #8 and #9 were then compiled and retrieved for analysis with the Flow Cytometer Apogee A60-Micro-Plus (Apogee Flow Systems, UK). The A60-Micro-Plus machine is equipped with three spatially separated lasers (488 nm – Position C, 405 nm – Position A and 638 nm – Position B), 7 fluorescence color detectors (525/50, LWP590, 530/30, 574/26, 590/40, 695/40, 676/36) and 3 light scatter detectors (SALS, MALS and LALS). For internal control across assays, before each FC experiment, we used two mixes of beads (Apogee – 1493 and Apogee - 1517). Before being loaded, samples were diluted in filtered PBS (0.22 µm membrane filters) to bring their concentration within the operational range of the equipment (maximum of 3,000 events/second). All samples were run at a flow rate of 1.5 µL/minute using a 405nm – LALS threshold of 70. The 405 nm – LALS PMT noise level was monitored and always maintained below 0.35. For the experiments depicted, the stopping criteria utilized was the number of events acquired, so samples were ran until a minimum of 250,000 events was reached. The acquired data was exported and analyzed with FlowJo software v10.4.2 (FlowJo LLC, US).

Protein extraction and multiplexed MMP array

The pre-enriched EVs and cell pellets were diluted in PBS, 1X RIPA lysis buffer. Halt™ Protease & Phosphatase Inhibitor cocktail (Thermo Scientific®) were added, and the samples were incubated at 4ºC for 15 minutes. Protein was quantified using the BCA protein assay (BioRad®).

Human MMP Antibody Array (Abcam: ab134004) was used to measure MMPs and TIMPs. This ELISA-like multiplex approach offers substantial benefits for examining a defined set of proteins in parallel and detects 7 human MMPs (MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10 and MMP-13) and 3 TIMPs (MMP-13, TIMP-1, TIMP-2 and TIMP-4). Activity was determined in EVs lysates using 100ng of protein input. Quantification was performed by ChemiDoc™ XRS+ System and data analysis was performed using Quantity One® Analysis Software (BioRad®).

RNA extraction and cDNA synthesis

RNA isolation and purification of the cells and cell-derived exosomes was done using the RNeasy Plus Mini Kit (Qiagen®) according to the manufacturer procedure. RNA concentration and purity were measured using the NanoDrop Lite spectrophotometer (Thermo Scientific®) and served as template for cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). The thermal conditions for the cDNA synthesis were as follows: 25ºC for 10minutes followed by 37ºC for 120 minutes and 85ºC for 5 minutes.

Quantitative Real time PCR

MRNA expression levels were analyzed by quantitative real-time PCR. The reactions were carried out in a StepOnePlus™qPCR Real-Time PCR machine, in a volume of 10 ul containing 1X TaqMan™ Fast Advanced Master mix (Applied Biosystems), with 1X TaqMan® mRNA Expression Assays probes (TIMP1: Hs99999139_m1; TIMP2: Hs00234278_m1 and MMP1: Hs00899658_m1 - Applied Biosystems), and cDNA. For mRNA expression normalization two housekeeping controls were used: GAPDH (Hs02758991_g1- Applied Biosystems) and ACTB (Hs01060665_g1-Applied Biosystems). These housekeeping genes were chosen based on the fact that they are reported as typical EVs cargo [22]. The amplification conditions were as follows: holding stage 95ºC for 20 seconds, followed by 45 cycles of 95ºC for 1 second and 60ºC for 20 seconds. Three technical replicates were made for each sample. Data analysis was done using StepOne™ Sofware v2.2 (Applied Biosystems) with the same baseline and threshold set for each plate, in order to generate quantification cycle (Cq) values for all the mRNAs in each sample..

Statistical Analysis

Statistical analyses were done using IBM SPSS Statistics software for Windows (Version 22.0). According the mRNAs levels distribution, the Student t´test or Mann Whitney U test were used in order to evaluate any statistical differences in the normalized expression of the mRNAs. The quality of the housekeeping genes was tested using the BestKeeper software [23]. Both endogenous controls presented a stable behavior, so both were used to normalize the mRNAs levels. The Kaplen Meier method and Log Rank test were used to establish the association of the TIMP-1 derived EV mRNA levels (low *versus* high) to the overall survival. The TIMP-1 derived EV mRNA levels low *versus* high levels were defined using the mean value of the –ΔCq.

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Disclosure of interest

The authors report no conflict of interest.

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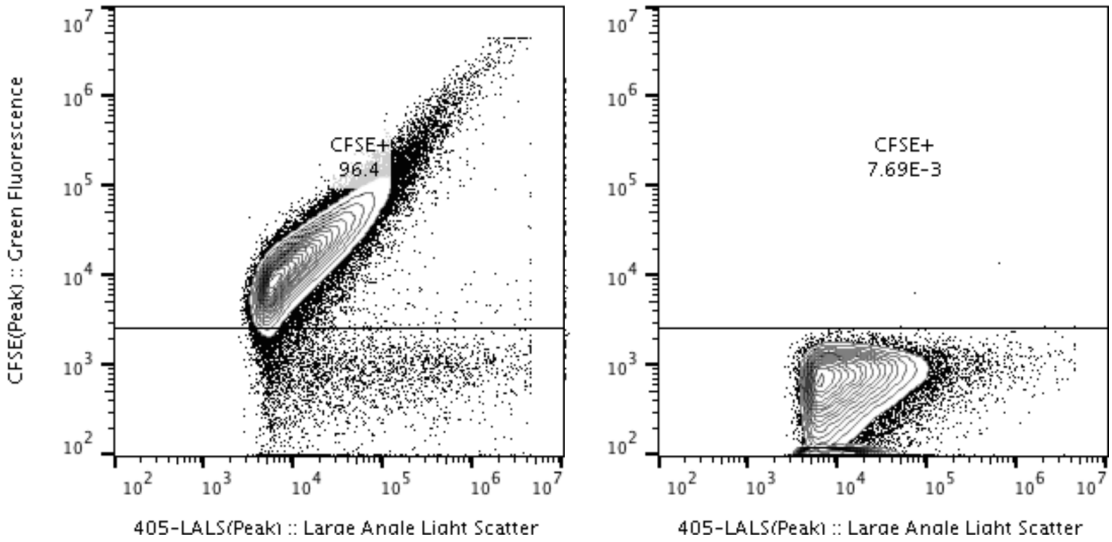
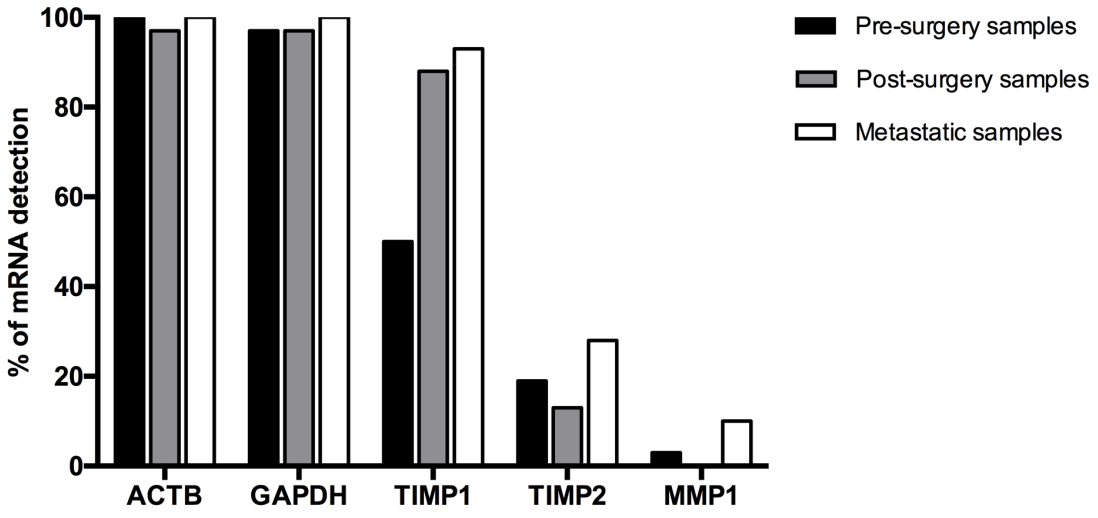
Supplementary material



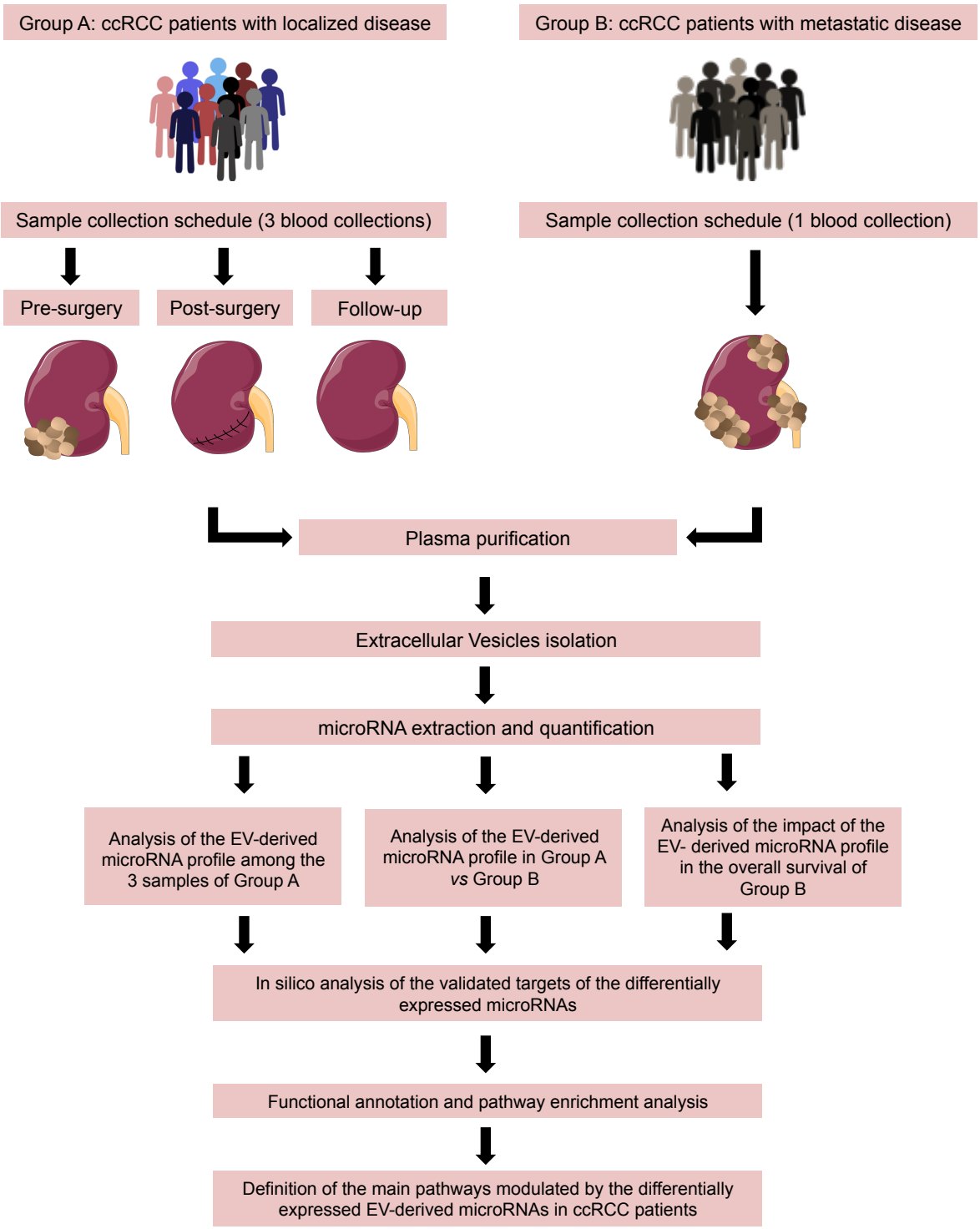
Supplementary Figure 1. Proportion of fluorescent EVs as observed by flow cytometry from a sample EV isolate derived from a patient, either previously stained with CFSE (A) or without previous staining (B).



Supplementary Figure 2. Percentages of ACTB, GAPDH, TIMP-1, TIMP-2 and MMP1 mRNA detection in EVs derived from ccRCC patients.

3. Extracellular vesicles enriched in hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p dynamics in ccRCC patients: potential impact on PI3K/Akt pathway activation

3.1. Study design



Manuscript submitted for publication

Extracellular Vesicles enriched in hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p dynamics in ccRCC patients: potential impact on PI3K/Akt pathway activation

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Abstract

Clear cell Renal Cell Carcinoma (ccRCC) is the most common and aggressive subtype of kidney cancer. One third of ccRCC patients present metastatic disease at the time of diagnosis, and up to 40% of patients submitted to surgery with a curative intent will relapse. Thus, there is an urgent need for the definition of reliable biomarkers that can help stratify patients according to their prognosis. Extracellular Vesicles (EVs) emerged as one of the key players of cancer microenvironment and pre-metastatic niche establishment. The aim of this study is to analyse the impact of a microRNA profile (hsa-miR-25-3p, hsa-miR126-5p, hsa-miR-200c-3p, hsa-miR-210-3p, hsa-miR-301a-3p, hsa-miR-519d-3p, has-miR-1233-5p, hsa-miR-1246 and hsa-miR-1293) in plasma EVs from ccRCC patients with localized disease (before and after surgery) and also in patients with metastatic disease. We observed that the levels of EV-derived hsa-miR-25-3p (P=0.003), hsa-miR-126-5p (P<0.001), hsa-miR-200c-3p (P<0.001) and hsa-miR-301a-3p (P=0.006) decreased after surgery whereas hsa-miR-1293 (P=0.002) EV-levels increased. We also observed that metastatic patients presented higher levels of hsa-miR-301a-3p (P=0.026) and lower levels of miR-1293 (P=0.004) when compared with patients that underwent surgery and are currently with no evidence of disease. When we focused on the miRNAs that decreased after tumor removal we observed that *PTEN,* a tumor suppressor whose major function is the inhibition of the PI3K/AKt pathway, was a common target of hsa-miR-25-3p, hsa-miR-200c-3p and hsa-miR-301a-3p. Furthermore, functional enrichment analysis of the targets of the 4 miRNAs that decreased after surgery resulted in enrichment of terms related to cell cycle, proliferation and metabolism. Taken together, these results suggest that in the presence of the tumor EVs enriched in miRNAs can represent an epigenetic silencing mechanism used by ccRCC to sustain tumor development and growth through *PTEN* inhibition and consequent activation of the PI3K/Akt pathway. Future studies should included larger cohorts and more miRNA involved on the regulation of the PTEN/PI3K/Akt pathway.

Keywords: clear cell Renal Cell Carcinoma, extracellular vesicles, microRNAs, PTEN, PI3K/Akt

Introduction

Renal cell carcinoma (RCC) is the most common solid cancer of the adult kidney and one of the most lethal urologic malignancies [1-3]. The most common histological subtype is clear cell RCC (ccRCC), which arises from the proximal tubular epithelial cells of the nephron and accounts for approximately 80% of all RCC cases [4]. CcRCC development is associated with two key cancer hallmarks: induction of angiogenesis and metabolic reprograming, with several studies implying that ccRCC can be considered a metabolic disease [5-8]. In fact, the majority of known RCC related genes interact with cell metabolism pathways and are involved in energy, nutrient, iron and oxygen sensing [9]. One of the most well characterized pathways involved in the development of ccRCC, and the most studied as well, is the VHL/HIF pathway [10,11]. The loss or inactivation of pVHL function in ccRCC conduct to a state of "pseudohypoxia" were stabilized HIF-1α and HIF-2α induce the transcription of hypoxia responsive genes, resulting in alterations such as increased glucose and ribose metabolism, pH deregulation, cell proliferation and angiogenesis, giving ccRCC a high metastatic potential [1,9,12-14].

Actually one third of ccRCC patients present metastatic disease at the time of diagnosis, and up to 40% of patients submitted to surgery with a curative intent, will relapse within a 5-year period [15]. The therapeutic options for ccRCC patients are limited since the hypoxic microenvironment makes these tumors chemo- and radio- resistant, leaving targeted therapies and immunotherapies as the only options available. However, patients submitted to these therapies tend to develop resistance to therapy within a short period of time [16,17]. Despite that metastatic ccRCC remains incurable, the prognosis for recurrent ccRCC is variable and the detection of early relapse could have an impact on patients' prognosis [18]. Thus, there is an urgent need for the definition of reliable biomarkers that can help stratify patients according to their metastatic risk.

In the recent years, it became clear that one of the key players of tumor microenvironment modulation are the extracellular vesicles (EVs). EVs consist of a mixed population of microvesicles with different sizes, sheded by cells, that enable cell-to-cell communication through the transport of active biomolecules from one cell to another [19,20]. Cancers have been found to highjack EV-mediated communication to facilitate several features of the multi-step metastatic process including cell proliferation, immune suppression, epitelial-to-mesenchymal transition, migration, invasion, angiogenesis and metastasis [5,21,22]. The release of EVs can be induced by several factors including, hypoxia, pH alterations, injury, platelet activation, irradiation and cellular stress, some of them involved in ccRCC progression [23]. One of the most studied classes of

biomolecules carried by EVs are the microRNAs (miRNAs), which consist of small non-coding RNAs that are able to regulate gene expression at a post-transcriptional level, resulting in attenuated translation of target mRNAs [24,25]. Thousands of protein-coding genes are regulated by miRNAs, and miRNAs are master regulators of diverse biological systems and have an impact in the body phisiological responses [26,27]. Several EV-derived miRNAs have been studied and proposed as potential biomarkers in ccRCC [28-38]. However, due to the novelty of the field, most of the studies have been performed in cell lines and only a few used samples from ccRCC patients. Therefore, the aim of this study is to analyse the impact of EV-derived miRNA profile of 9 miRNAs (hsa-miR-25-3p, hsa-miR126-5p, hsa-miR-200c-3p, hsa-miR-210-3p, hsa-miR-301a-3p, hsa-miR-519d-3p, hsa-miR-1233-5p, hsa-miR-1246 and hsa-miR-1293) related to hypoxia and metabolism regulation in plasma EVs from ccRCC patients with localized disease and also in patients with metastatic disease.

Results

EVs characterization

The EVs were characterized according to size, shape and purity. The NTA analysis indicated that the vast majority of isolated EVs presented a size range between 50 and 200 nm, which is consistent with the size of exosomes and small microvesicles (Fig.1A). A Transmission Electron Microscopy (TEM) image (Fig.1B) shows the variability of sizes and morphology present in EVs from purified PFP. We also utilized EVs Flow Cytometry to confirm the purity of our EV isolates [40]. In all cases, more than 80% of particles present in our isolates corresponded to $CFSE^{+}$ vesicular structures (Fig.1C).

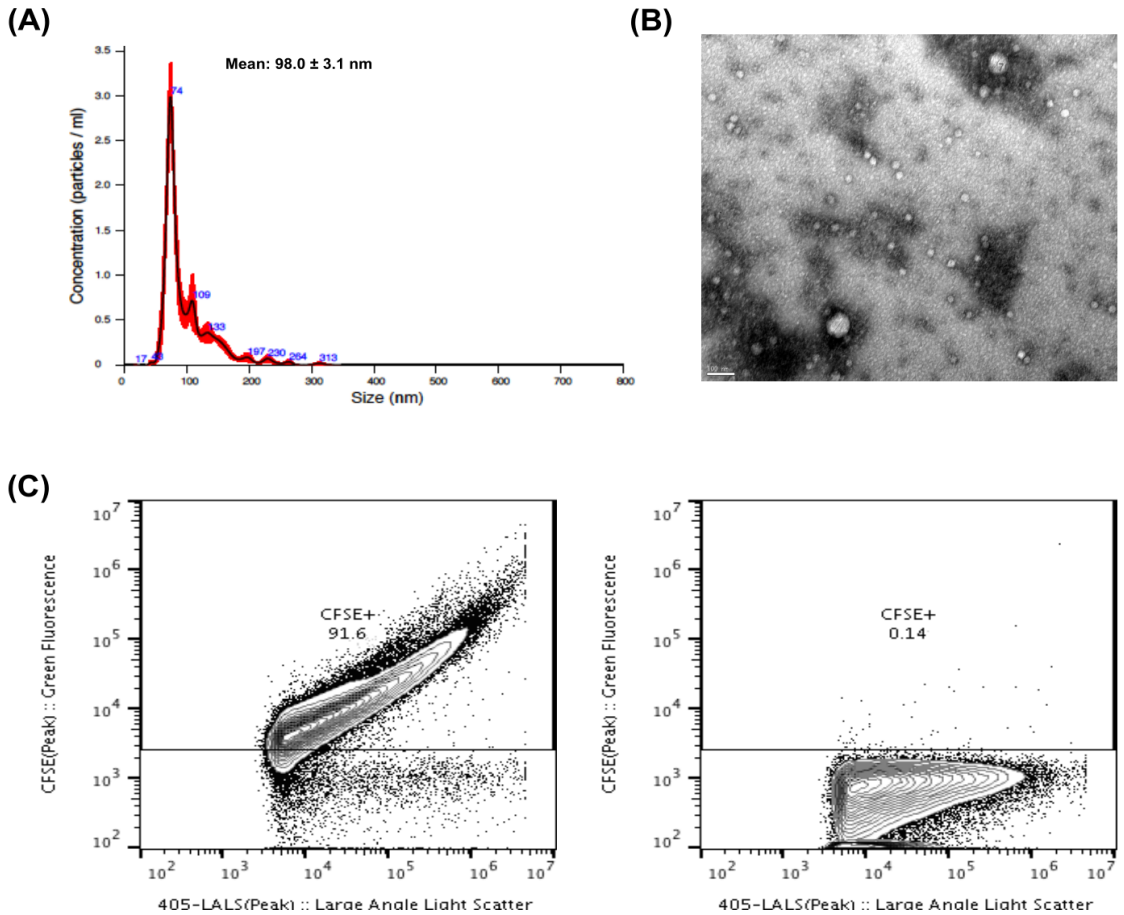

Figure 1. (A) Nanoparticle tracking analysis (NTA) of EVs derived from plasma of ccRCC patients. The red error bars indicate ± 1 standard error of the mean. (B) Transmission electron microscopy (TEM) of EVs from purified platelet-free plasma (PFP). The TEM image was acquired in the Histology and Electron Microscopy platform from I3S Porto using a Transmission Electron Microscope Jeol JEM 1400. (C) Proportion of fluorescent EVs as observed by flow cytometry from a sample EV isolate derived from a patient, either previously stained with CFSE (left panel) or without previous staining (right panel).

EV-derived miRNAs levels in ccRCC patients' plasma samples

Two patients from Group A relapsed during the follow-up and were removed from the analysis. The remaining 30 patients from group A are currently alive with no evidence of disease. In addition to that, two EV-derived miRNAs were exluded from the anlaysis. Hsa-miR-519d-3p was only detected in a small number of samples and hsa-1233-5p was not detected at all, which did not allow a statistical analysis.

The expression levels of the remaining seven miRNAs are represented in Figure 2. Focusing on the patients from Group A, we observed that the levels of EV-derived hsa-miR-25-3p (P=0.003), hsa-miR-126-5p (P<0.001), hsa-miR-200c-3p (P<0.001) and hsa-miR-301a-3p (P=0.006) decreased after surgery (Fig.2A, 2B, 2C and 2E). Hsa-miR-210-3p also decreased after tumor removal but the decrease was slower and only statistical significant when the localized disease samples were compared with the follow-up samples (P=0.010) (Fig.2D). On the other hand, we observed that hsa-miR-1293 (P=0.002) EV-levels increased after tumor removal (Fig.2G). Hsa-miR-1246 EV-levels also increased after tumor removal, but in a slower rate since the levels were only statistical significantly higher when we compared the localized and follow-up samples (P=0.044) (Fig.2F).

When we compare the patients from Group A with the patients from Group B, we observe two scenarios. First, see that some miRNAs have diferences in their expression levels when we compare the samples from localized disease (Group A) with the metastatic disease (Group B). This is the case for hsa-miR-126-5p and hsa-miR-200c-3p that are downregulated in the metastatic patients (P<0.001 and P<0.001, respectively), (Fig.2B and 2C). On the other hand, we can also observe differences in miRNA levels when we compare the follow-up samples with the metastatic samples, with the metastatic samples presenting higher levels of hsa-miR-301a-3p (P=0.026) and lower levels of miR-1293 (P=0.004) (Fig.2E and 2G). In addition to that, hsa-miR-210-3p presented a tendency for being upregulated (P=0.053) and hsa-miR-1246 presented a tendency for being downregulated (P=0.053), both in the metastatic samples, when compared with the follow-up group (Fig.2D and 2F).

Regarding the other clinical pathological characteristics, we observed that patients with localized disease with tumors grater than 7 cm presented higher levels of hsa-miR-126-5p (P=0.013) (Supplementary table 1). Additionally patients who smoke presented higher levels of hsa-miR-1293 (P=0.006) and hsa-miR-210-3p (P=0.034) when compared to non-smokers and ex-smokers, respectively. We did not observe any statistical differences between the EV-derived miRNAs and hypertension and *diabetes mellitus*.

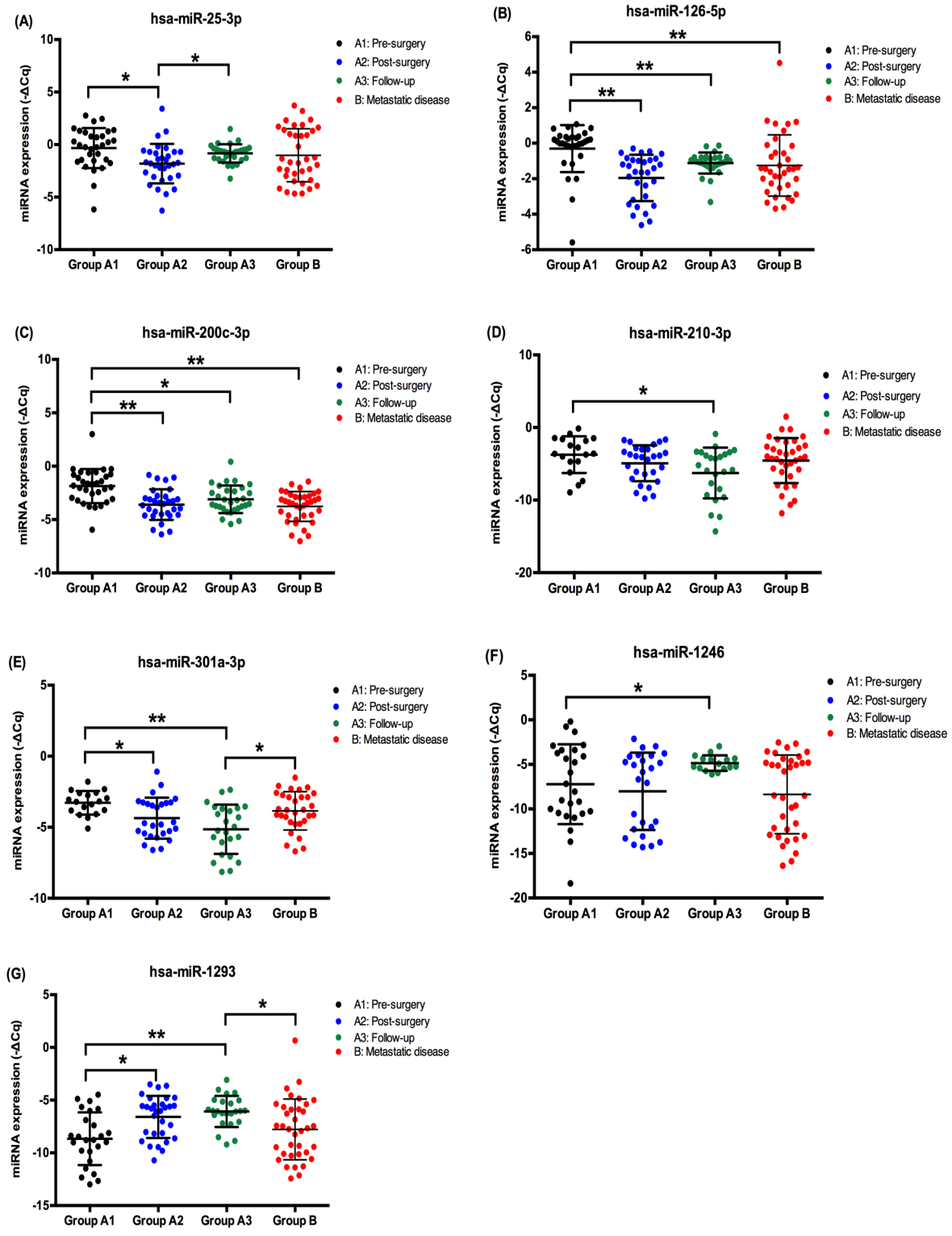

Figure 2. EV-derived miRNA expression (-ΔCq) in pre-surgery, post-surgery, and follow-up samples (Group A) and metastatic disease samples (Group B) of ccRCC patients. *P<0.05, **P<0.001.

EV-derived miRNAs impact on metastatic ccRCC patients overall survival

Since the patients from group A were alive with no evidence of disease on their last observation, we carried out the survival analysis on the patients from group B (Figure 3). The patients were divided in tertiles according the EV-derived miRNA levels using the –ΔCq values of each miRNA (high, intermediate and low levels). Regarding the 7 EV-derived miRNAs analyzed, only the hsa-miR-200c-3p presented statistical significant differences. Patients with a high or low expression level had a lower overall survival compared to patients with intermediate levels of hsa-miR-200c-3p (Log Rank Mantel Cox test, *P*=0.025) (Fig.3C). In addition to that, patients with lower levels of has-miR-25-3p presented a tendency for a lower overall survival (Log Rank Mantel Cox test, *P*=0.054) (Fig.3A).

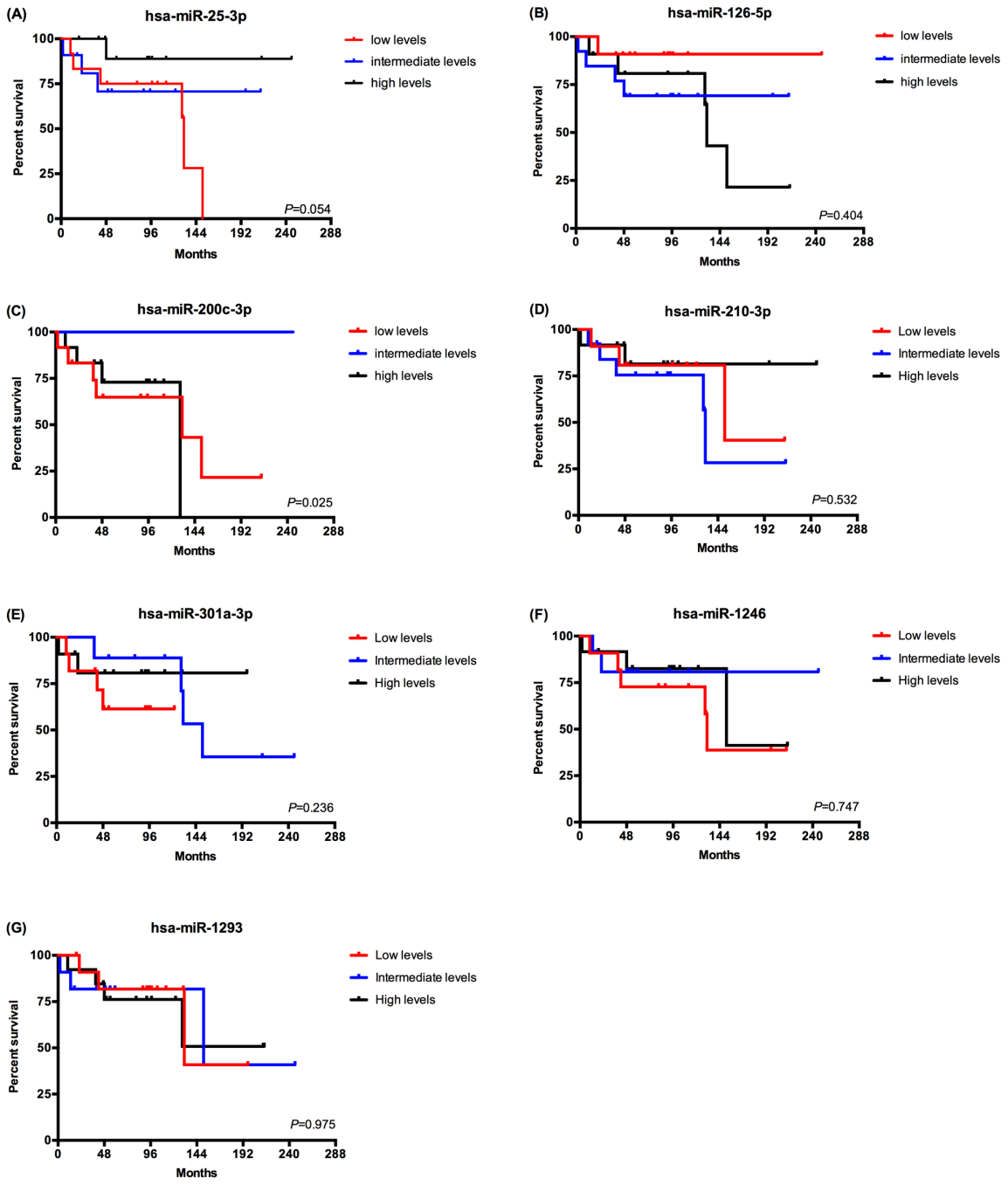

Figure 3. Overall survival analysis of metastic ccRCC patients according to the expression of EV-derived miRNAs. Comparison of survival curves was made with Log-rank (Mantel-Cox) test. (A) hsa-miR-25-3p (low levels – 12 patients; intermediate levels – 11 patients and high levels – 12 patients). (B) hsa-miR-125-5p (low levels – 12 patients; intermediate levels – 13 patients and high levels – 11 patients). (C) hsa-miR-200-3p (low levels – 12 patients; intermediate levels – 12 patients and high levels – 12 patients). (D) hsa-miR-210-3p (low levels – 11 patients; intermediate levels – 13 patients and high levels – 12 patients). (E) hsa-miR-301a-3p (low levels – 11 patients; intermediate levels – 10 patients and high levels – 11 patients). (F) hsa-miR-1246 (low levels – 11 patients; intermediate levels – 11 patients and high levels – 12 patients). (G) hsa-miR-1293 (low levels – 12 patients; intermediate levels – 11 patients and high levels – 12 patients).

Hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p overlaping target genes

Hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p significantly decreased after tumor removal, suggesting that their expression may be related to the presence of the tumor. As so, we used miRTarBase (version 8.0), the largest known online database of validated miRNA:mRNA target interactions, to establish a network of the target mRNAs of this miRNA profile and further evaluate its impact on ccRCC [46].

According to miRTarBase there are a total of 1381 validated target genes for these 4 miRNAs, but we only focused on the the 135 whose miRNA:mRNA interaction was validated according to strong validation methods (such as luciferase reporter assay, western blot and qPCR) (Supplementary table 2). From the 135 validated target genes, *PTEN, VEGFA, TIMP2* and *BCL2L11* were common to more than one of the miRNAs (Figure 3B). *PTEN* was the most common target, being regulated by hsa-miR-25-3p, hsa-miR-200c-3p and hsa-miR-301a-3p (Figure 4).

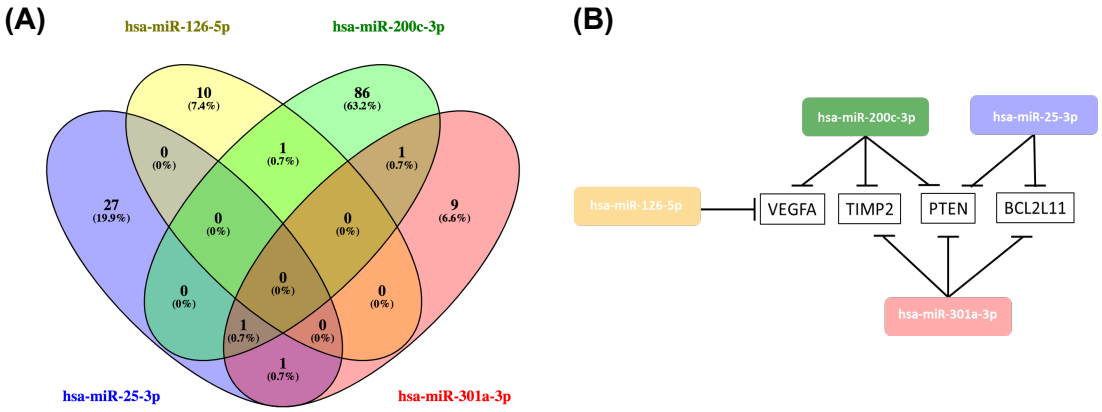

Figure 4. (A) Venn diagram of the validated target genes of hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p obtained using Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/). (B) Representation of the overlaping target genes of hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p.

Functional annotation and pathway enrichment analyses of the hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p targets network

In order to explore the biological impact of the four-miRNA signature in patients with localized disease, we analysed their 135 target genes with the STRINGapp from Cytoscape. A total of 134 of the 135 protein coding genes were filtered into a protein-protein interaction (PPI) network with 134 nodes and 889 edges that presented significant enrichment (P=1.0x10^{-16}). For a deeper understanding of the protein interactions we performed a Markov clustering (MCL), which resulted in the clustering of the proteins into 26 clusters according to their STRING interaction score (Fig.5) (Supplementary table 3).

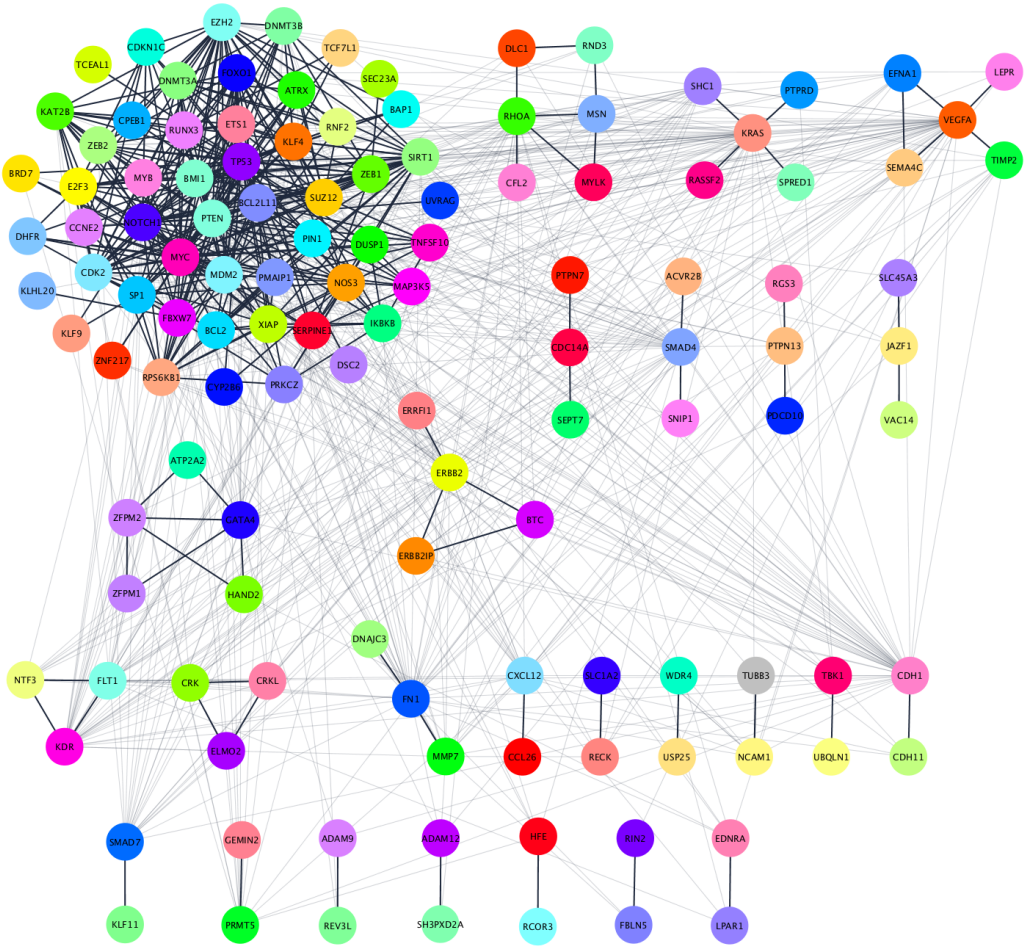

Figure 5. Clustered PPI network of the 134 target genes genes. Clustering was performed using the Markov clustering (MCL) algorithm in the clusterMaker2 Cytoscape app using an inflation value of 4.0. Proteins without any interaction partners within the network (singletons) are omitted from the visualization.

For the functional enrichment analysis we focused on the largest cluster, which reduced the initial network of 134 proteins to 53 proteins. This cluster contained 53 nodes, with PTEN and BCL2L11 among them, and 328 edges and retained the significant PPI enrichment (P=1.0x10^{-16}). The functional enrichment analysis was performed with an FDR threshold of 1%, and the redundant terms were eliminated using a redundancy cutoff of 0.5, which resulted in a total of 549 functinal enriched terms among the Reactome, KEGG and GO categories (Supplementary tables 4, 5,6,7 and 8) The top 20 enriched terms for each category are represented on Figure 6.

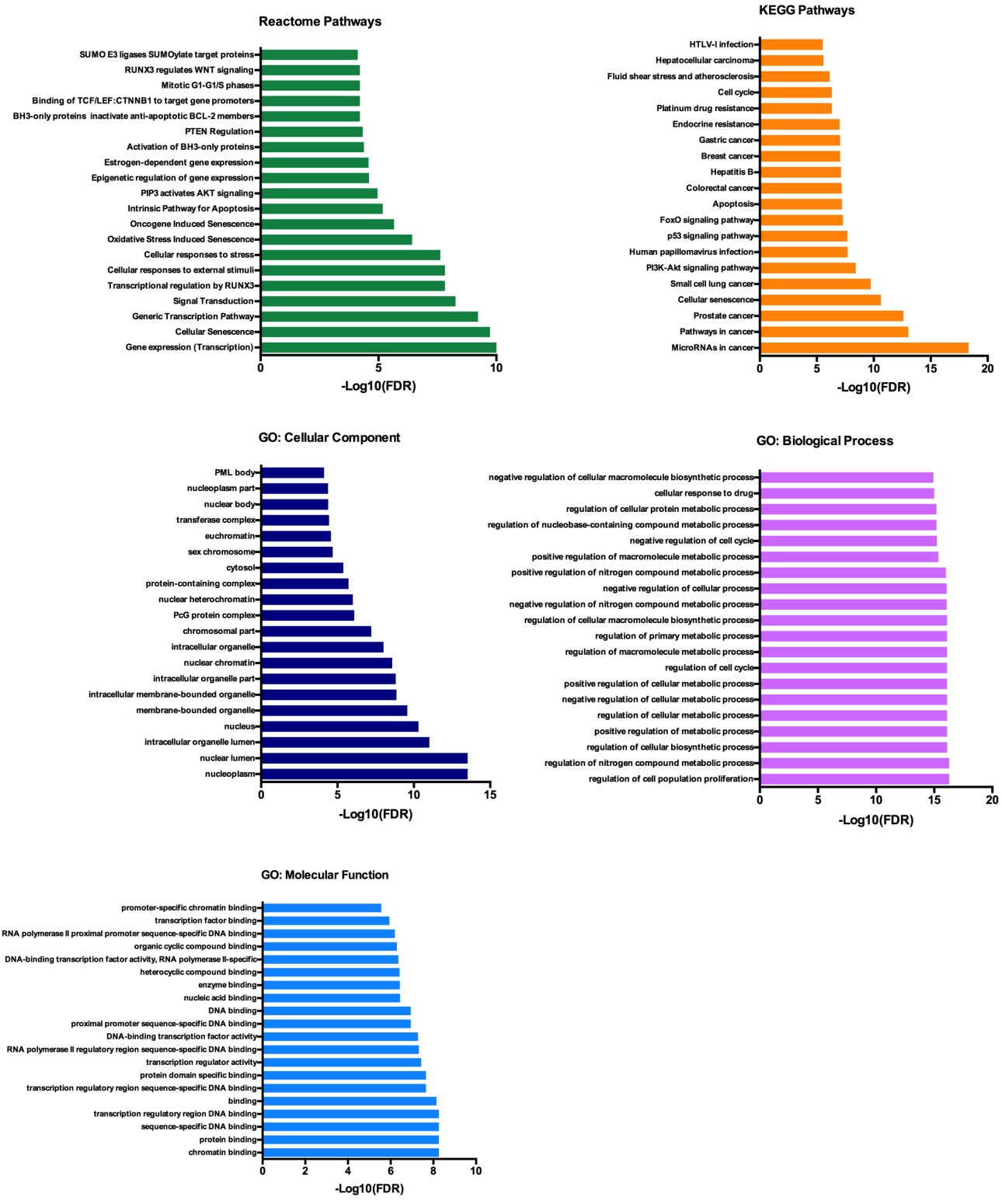

Figure 6. Reactome, KEGG and GO analysis of the 53 selected genes. The functional enrichment anaylis was made with the STRINGapp from Cytoscape.

Among the functionally enriched terms in the Reactome and KEGG pathways we can find AKT, BCL-2, PTEN and p53 signalling. Regarding the GO terms, we can observe that terms related with the Biological Processess were mostly about cellular metabolic processes and also regulation of cell cycle and cell profileration.

Discussion

It is now well stablished that the formation and maintenence of a cancer niche deeply relies in the surrouding microenvironment that is composed by different cell types such as endotelial cells, stem cells, fibroblasts and immune cells [47]. Moreover, EVs play an important role in the complex network of communication that occurs inside the tumor microenvironment and also between the tumor microenvironment and the rest of the human body. This is due to the bioactive molecules they transfer between cells that are able to make a biological impact through the alteration of the recipient cell phenotype.

In our study we observed that patients with localized ccRCC presented an alteration of the pattern of plasmatic EV-derived miRNAs after tumor removal, with a decrease of hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p, and an increase of hsa-miR-1293 approximately 4 months after surgery, suggesting that the presence of the tumor has an key role in the EV network that is established in the patients' body. In fact, hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p have already been reported in EVs, with an impact on cell proliferation, pre-metastatic niche formation, invasion and metastization in other tumor models [48-51]. When we looked into the validated target genes of the four miRNAs that decreased after tumor removal, we observed that four mRNA targets were common to more than one miRNA, which was the case of *PTEN*, *BCL2L11*, *TIMP2* and *VEGFA*. Moreover, *PTEN* was the most common target, being regulated by hsa-miR-25-3p, hsa-miR-200c-3p and miR-301a-3p. Since one miRNA is capable of regulating several targets, and the same mRNA can be regulated by multiple miRNAs, some authors defend that the repressing capability of a single miRNA is quite modest and not capable of inducing significant changes on protein expression [52]. However, the fact that *PTEN* is repressed by three of the studied miRNAs suggests that this gene might be under a stronger repressing environment in the patients with localized disease. *PTEN* is a tumor supressor gene whose major function relies on the inhibition of the PI3K/AKT pathway and consequent regulation pathways related to cell growth and proliferation [53,54]. This tumor supressor gene is frequently mutated in a large number of cancers and ccRCC is no exception [55-57]. Recent data from the The Cancer Genome Atlas (TCGA) showed that *PTEN*

mutations were found in 5% of ccRCCs and were associated with a worse prognosis in survival and disease recurrence [58]. The authors also performed GO and KEGG analysis on the differentially expressed genes of the ccRCC patients harbouring the mutation and concluded that *PTEN* loss of function was associated with a more vigourous cell metabolism and cell growth [58]. In addition that, Lee and colleagues showed that biallelic and monoallelic loss of *PTEN* were more frequently observed in high histologic grade ccRCC tumors, which reinforces the hypothesis that *PTEN* loss of function may be a consequence of the tumor evolution [59]. Moreover, a study performed by Zhang and colleagues demonstrated that astrocyte-derived EVs transferred *PTEN*-targeting miRNAs to metastatic cells, with an impact on mRNA and protein downregulation [60]. This epigenetic silencing of *PTEN* in metastatic cells lead to CCL2 secretion and myeloid cell recruitment, which promoted metastatic cell expansion through reduced apoptosis and enhanced proliferation through PI3K/Akt pathway activation [60].

In our study we performed a functional annotation analysis on the validated target genes of hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p and we observed that the most enriched terms were related to cell growth and proliferation and also with cellular metabolism, with *PTEN, AKT, BCL-2* and *p53* signalling among the most enriched terms. Since these EV-derived miRNAs decrease after tumor removal, our hypothesis is that their EV-enrichment in the presence of the tumor could represent an epigenetic silencing mechanism used by ccRCC to sustain tumor development and growth through *PTEN* inhibition and consequent activation of the PI3K/Akt pathway. In fact, despite the overall mutation rate of PI3K/Akt pathway in ccRCC is relatively low, the overall activation of PI3K/Akt in ccRCC is high compared to other cancers [61-63]. This lead to the conclusion that dyregulation of the PI3K/Akt pathway in ccRCC could be a consequence of epigenetic, post-transcriptional and post-translational mechanisms, which reinforces our hypothesis [62].

Regarding the metastatic patients, EV-derived hsa-miR-301a-3p and hsa-miR-1293 were the most promising miRNAs to differentiate patients in follow-up with no evidence of disease from patients with metastatic disease. Hsa-mir-301a-3p presented a decreasing expression pattern after tumor removal and its levels kept decreasing until follow-up. However, the expression levels were significantly increased in the metastatic group, suggesting that this miRNA could play an important role in the metastization process and might have the potential to be used as prognostic biomarker. To support this hypothesis, a study performed by Wang and colleagues showed that, under a hypoxic microenvironment, pancreatic cancer cells generate EVs enriched in hsa-miR-301a-3p that induce M2 macrophages polarization through the PTEN/PI3K/Akt signalling pathway and promote metastization [49]. Regarding hsa-miR-1293, we observed the opposite

effect since its EV levels started to gradually increase after tumor removal until follow-up, but were significantly decreased in the metastatic group. TIMP-1 is one of the validated targets of has-miR-1293 and its increased expression is associated with a worse ccRCC prognosis [64,65].

Regarding the impact of the EV-derived miRNA profile in the metastatic patients overall survival, only hsa-miR-200c-3p presented significant, but contradictory, results since both its high and low levels were associated with a worse survival. Hsa-miR-200c-3p as been previously described as acting both as an oncomiRNA and tumor suppressor miRNA, which might explain why both its increase and decrease beyond certain expression values could lead to a worse prognosis [50,66]. This result highlights the importance of a balance in EV-derived miRNA expression and how disruptions of that balance can have an impact on the patients' prognosis.

To the best of our knowledge this is the first study monitoring the differences of EV-derived miRNAs during a one-year follow-up period of ccRCC patients with localized disease. This type of study is useful for a better understanding of the disease impact on the host EV dynamics. However, the small samples size in our study may limit the ability to distinguish meaningful differences, being essential the replication of the associations reported in a larger sample size. In addition to that, the patients included in this study should continue to be monitored, and more EV-miRNAs related to the PTEN/PI3K/Akt pathway should be included in the studied profile.

Material and Methods

Ethics statement

This study was conducted according to the principles of the Helsinky Declaration, having been approved by the ethics committee of the Portuguese Oncology Institute of Porto (IPO-Porto) (project reference: 251/015). All individuals have signed a written informed consent in order to participate in the study.

Study Population

The analysis of the EV-derived miRNA profile was conducted through a hospital-based study involving a total of 69 ccRCC patients. All individuals were Caucasian from the north of Portugal, with histopathological diagnosis of ccRCC, and admitted and treated at the IPO-Porto between November 2015 and June 2019. The patients were divided into two groups: Group A was composed by 32 patients diagnosed with localized disease that

underwent surgical intervention; and Group B was composed of 37 patients with metastatic disease. Clinical characteristics of patients were obtained from their medical records (Table 1). Blood from Group A patients was collected three times during the study: before undergoing surgery, approximately 4 months after surgery and approximately 1 year after surgery; from Group B patients blood was collected once. All blood collections were performed during the morning period and stored at 4ºC immediately until sample processing.

Tumor classification and staging was established according to the tumor-node-metastasis (TNM) classification system of the American Joint Committee on Cancer (AJCC) 8th edition (2018) and the International Society of Urological Pathology (ISUP) Classification of Renal Neoplasia [39].

Table 1. Clinical-pathological characteristics of the study population. Group A is constituted by the patients with localized disease and Group B is constituted by the patients with metastatic disease.

		Group A (N = 32)		Group B (N = 37)	
		N	%	n	%
Gender					
	Male	24	75.0	26	70.3
	Female	8	25.0	11	29.7
Age	Average ± SD	61.9±12.4		62.4±9.9	
Type of surgery					
	Partial nephrectomy	18	56.3	0	0.0
	Radical nephrectomy	14	43.8	34	91.9
	No surgery	0	0	3	8.1
Tumor size					
	< 7 cm	24	75.0	9	24.32
	≥7 cm	8	25.0	22	59.46
	Undetermined	0	0	6	16.22
T					
	T1	17	53.1	10	27.03
	T2	1	3.1	6	16.22
	T3	13	40.6	16	43.24
	T4	0	0	3	8.12
	Tx	1	3.1	2	5.40
N					
	N0	0	0	15	40.54
	N1-N2	0	0	5	13.51
	Nx	0	0	17	45.95

M					
	M0	27	100	22	59.46
	M1	0	0	15	40.54
	Mx	5	0	0	0.0
Clinical Stage					
	I	17	53.1	10	27.03
	II	1	3.1	6	16.22
	III	13	40.6	16	43.24
	IV	0	0	3	8.12
	Not available	1	3.1	3	5.40
ISUP classification					
	1	2	6.3	1	2.70
	2	18	56.3	14	37.84
	3	11	34.3	8	21.62
	4	1	3.1	9	24.32
	Not available	0	0	5	13.51
Smoking status					
	Smoker	6	18.8	3	8.11
	Ex-smoker	11	34.4	7	18.92
	Non-smoker	14	43.8	27	72,97
	Not available	1	3.1	0	0.0
Hypertension					
	Yes	20	62.5	15	40.54
	No	11	34.4	22	59.45
	Not available	1	3.1	0	0.0
Diabetes mellitus					
	DM I	0	0.0	0	0.0
	DM II	9	28.1	13	35.14
	No	22	68.8	23	62.16
	Not available	1	3.1	1	2.70

EVs isolation

EVs were isolated from the plasma fraction using the Total Exosome Isolation from Plasma Kit (Invtrogen™) with additional protocol optimizations. Firstly, 8 mL of peripheral blood was collected from the patients in EDTA tubes and centrifuged 5 mins at 1800g to obtain the plasma fraction. The plasma fraction was then centrifuged 3 additional times at increasing speeds (300g, 2100g and 10.000g) for a period of 15 mins each in order to obtain platelet-free-plasma (PFP). After centrifugation, the supernatant was recovered and filtered through a 0.22µM filter (GE Healthcare Whatman ™). After a 10-minute treatment with proteinase K, the Total Exosome Isolation (TEI) reagent was added to 200µL of PFP and the solution was incubated for 30 min at 4ºC. The precipitated EVs were recovered by

5 min centrifugation at 10.000g at room temperature. The pellet containing the pre-enriched EVs was resuspended in filtered PBS (0.22 µm membrane filters) and stored at -80ºC until further analysis.

EVs NTA Analysis

Samples were analyzed for particle concentration and size distribution by the NS300 Nanoparticle Tracking Analysis (NTA) system (NanoSight – Malvern Panalytical, UK). Samples were pre-diluted in filtered PBS to achieve a concentration within the range for optimal NTA analysis. Video acquisitions were performed using a camera level of 16 and a threshold between 5 and 7. Five to nine videos of 30 seconds were captured per sample. Analysis of particle concentration per mL and size distribution was performed with NTA software v3.4.

Quantification of vesicular structures by EVs flow cytometry

We employed EVs flow cytometry procedure for quantification of vesicular structures in our EV isolates, as recently described by Maia and colleagues [40]. Briefly, $2x10^9$ particles of purified EVs were mixed with 40 µL of PBS containing Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE – Thermo Fisher Scientific - LTI C34554) in a final concentration of 40 µM and incubated for 90 minutes at 37° C. For removal of unbound CFSE, Size Exclusion Chromatography (SEC) columns (iZON - qEV original columns SP1, UK) were used. Samples containing unstained or stained EVs, and appropriate controls, were diluted up to 500 µL of PBS and processed by qEV following manufacturer's instructions. EVs-enriched fractions #7, #8 and #9 were then compiled and retrieved for analysis with the Flow Cytometer Apogee A60-Micro-Plus (Apogee Flow Systems, UK). The A60-Micro-Plus machine is equipped with three spatially separated lasers (488 nm – Position C, 405 nm – Position A and 638 nm – Position B), 7 fluorescence color detectors (525/50, LWP590, 530/30, 574/26, 590/40, 695/40, 676/36) and 3 light scatter detectors (SALS, MALS and LALS). For internal control across assays, before each FC experiment, we used two mixes of beads (Apogee – 1493 and Apogee - 1517). Before being loaded, samples were diluted in filtered PBS (0.22 µm membrane filters) to bring their concentration within the operational range of the equipment (maximum of 3,000 events/second). All samples were run at a flow rate of 1.5 µL/minute using a 405nm – LALS threshold of 70. The 405 nm – LALS PMT noise level was monitored and always maintained below 0.35. For the experiments depicted, the stopping criteria utilized was the number of events acquired, so samples were ran until a minimum

of 250,000 events was reached. The acquired data was exported and analyzed with FlowJo software v10.4.2 (FlowJo LLC, US).

miRNA extraction and cDNA synthesis

MiRNA isolation and purification of cell-derived EVs was done using the Plasma/Serum RNA Purifican Mini Kit from NORGEN (Biotek Corporation) according to the manufacturer supplementary protocol for EV RNA purification from EVs already isolated from precipitation methods. RNA concentration and purity were measured using the NanoDrop Lite spectrophotometer (Thermo Scientific®) and served as template for cDNA synthesis using a TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems®) according to the manufacturer protocol.

Quantitative Real time PCR

MiRNA expression levels were analyzed by quantitative real-time PCR. The reactions were carried out in a StepOnePlus™qPCR Real-Time PCR machine, in a volume of 10 µL containing 1X TaqMan™ Fast Advanced Master mix (Applied Biosystems), with 1X TaqMan™ Advanced miRNA Assays probes (hsa-miR-25-3p 477994_mir; hsa-miR-126-5p 477888_mir; hsa-miR-200c-3p 478351_mir; hsa-miR210-3p 477970_mir; hsa-miR-301a-3p 477815_mir; hsa-miR-519d-3p 478986_mir; hsa-miR-1233-5p 479549_mir; hsa-miR-1246 477881_mir and hsa-miR-1293 478692_mir - Applied Biosystems), and 2,5 µL cDNA. For miRNA expression normalization two housekeeping controls were used: hsa-let7a-5p (478575_mir - Applied Biosystems) and hsa-miR-16-5p (477860_mir - Applied Biosystems). These housekeeping miRNAs were chosen based on the fact that they are reported as typical EVs cargo [41]. The amplification conditions were as follows: holding stage 95ºC for 20 seconds, followed by 45 cycles of 95ºC for 1 second and 60ºC for 20 seconds. Three technical replicates were made for each sample. Data analysis was done using StepOne™ Sofware v2.2 (Applied Biosystems) with the same baseline and threshold set for each plate, in order to generate quantification cycle (Cq) values for all the miRNAs in each sample.

Statistical Analysis

Statistical analyses were done using IBM SPSS Statistics software for Windows (Version 22.0). According the mRNAs levels distribution, the Student t´test or Mann Whitney U test were used in order to evaluate any statistical differences in the normalized expression of the EV-derived miRNAs. The quality of the housekeeping miRNAs was

tested using the BestKeeper software [42]. Only has-let-7a-5p presented a stable behavior among all samples and was used to data notmalization. The Kaplen Meier method and Log Rank test were used to establish the association of the EV-derived mRNA levels (low, intermediated and high) to the overall survival.

Protein-Protein Interaction (PPI) Network and cluster analysis

The Search Tool for the Retrieval of Interacting Genes (STRING) database is an online tool that is used to develop protein-protein interaction (PPI) networks [43]. We used the STRINGapp in the Cytoscape software (v3.7.2) to construct the protein interaction relationship of the selected target genes encoding proteins. Those with a combined score of >0.4 were selected as significant. In order to get a better understanding of the resulting PPI network we grouped the proteins according to the strength of their STRING interaction score using the clusterMaker app for Cytoscape to perform Markov clustering (MCL)[44,45].

Functional annotation and pathway enrichment analysis

Gene ontology analysis (GO) is a common useful method for annotating genes and gene products, allowing the identification of characteristic biological attributes for transcriptome data. The functional enrichment analysis of GO, Reactome and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed with the enrichment anaylsis tool of the STRINGapp, with a False Discovery Rate (FDR) of $P<0.01$ as significance threshold. The enrichment results were filtered in order to remove redundant terms. The redundancy filtering takes the list of enriched terms sorted by FDR value and removes the terms that are too similar to any of the previous, better scoring terms that were not themselves removed. The similarity between two terms is measured by the Jaccard índex of the sets of genes annotated by the two terms. A term is added to the filtred list only if it has Jaccard similarity less than the used-specified redundancy cutoff to any other term already in the filtered list.

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Disclosure of interest

The authors report no conflict of interest.

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Supplementary table 1. Association of EV-derived miRNA profile expression with Clinical-pathological characteristics of patients with localized disease (Group A).

miRNA	Hypertension	Diabetes mellitus	Smokers vs Non-smokers	Smokers vs Ex-smokers	Tumor size > 7cm
Hsa-miR-25-3p	*P*=0.528	*P*=0.356	*P*=0.316	*P*=0.422	*P*=0.464
Hsa-miR-126-5p	*P*=0.528	*P*=0.814	*P*=0.108	*P*=0.079	***P*=0.013**
Hsa-miR-200c-3p	*P*=0.555	*P*=0.633	*P*=0.503	*P*=0.617	*P*=0.862
Hsa-miR-210-3p	*P*=0.382	*P*=0.996	*P*=0.211	***P*=0.034**	*P*=0.679
Hsa-miR-301a-3p	*P*=0.820	*P*=0.682	*P*=0.083	*P*=0.120	*P*=0.754
Hsa-miR-1246	*P*=0.958	*P*=0.056	*P*=0.770	*P*=0.558	*P*=0.572
Hsa-miR-1293	*P*=0.837	*P*=0.373	***P*=0.006**	*P*=0.577	*P*=0.194

Supplementary table 2. Hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p mRNA targets validated with strong evidence according miRTarBase V8.0

microRNA	Validated targets
Hsa-miR-25-3p	*WDR4, TP53, TNFSF10, TCEAL1, SMAD7, SEMA4C, RGS3, REV3L, RECK, PTEN, PRMT5, MDM2, MAPK2K4, LATS2, KLF4, KAT2B, HAND2, FBXW7, EZH2, ERBB2, DSC2, DHFR, CYP2B6, CPEB1, CDKN1C, CDH1, CCL26, BCL2L11, ATP2A2*
Hsa-miR-126-5p	*SLC45A3, SPRED1, PTPN7, ADAM9, MMP7, CXCL12, MYC, VEGFA, HOTAIR, CRK, CYLD*
Hsa-miR-200c-3p	*ZNF217, ZFPM2, ZFPM1, ZEB2, ZEB1, XIAP, WDR37, VEGFA, VAC14, USP25, UBQLN1, TUBB3, TIMP2, TCF7L1, TBK1, SUZ12, SP1, SLC1A2, SIRT1, SHC1, SH3PXD2A, SEPT7, SEC23A, RPS6KB1, ROCK2, RNF2, RND3, RIND2, RHOA, RCOR3, RASSF2, PTPRD, PTPN13, PTEN, PRKCZ, PMAIP1, LEPR, KRAS, KLHL20, KLF9, KLF11, KDR, JAZF1, IKBKB, HOXB5, HFE, GEMIN2, GATA4, FOXO1, FN1, FLT1, FBLN5, ETS1, ERRFI1, ERBIN, ELMO2, EFNA1, EDNRA, E2F3, DUSP1, DNMT3B, DNMT3A, DNAJC3, DLC1, CRKL, CFL2, CDK2, CDH11, CCNE2, BTC, BRD7, BMI1, BCL2, BAP1, ATRX, ADAM12, ACVR2B*
Hsa-miR-301a-3p	*MEOX12, NKRF, SERPINE1, SMAD4, RUNX3, BCL2L11, PTEN, UVRAG, SNIP1, MAP3K5, TIMP2, CDC14A*

Supplementary table 3. Protein-protein-interaction (PPI) network clusters according to Marckov Clusterring (MCL) analysis.

Cluster	Clustered proteins
Cluster 1	*ZNF217, KLF9, RPS6KB1, KLF4, NOS3, TCF7L1, SUZ12, BRD7, E2F3, TCEAL1, RNF2, XIAP, SEC23A, ZEB1, ZEB2, KAT2B, SIRT1, ATRX, DNMT3A, DUSP1, DNMT3B, IKBKB, BMI1, PTEN, CDKN1C, EZH2, BAP1, PIN1, MDM2, BCL2, CDK2, SP1, CPEB1, DHFR, KLHL20, UVRAG, PMAIP1, BCL2L11, CYP2B6, FOXO1, PRKCZ, NOTCH1, DSC2, TP53, CCNE2, RUNX3, MAP3K5, TNFSF10, MYB, MYC, ETS1, SERPINE1*
Cluster 2	*DLC1, RHOA, RND3, MSN, CFL2, MYLK*
Cluster 3	*KRAS, SPRED1, PTPRD, SHC1, RASSF2*
Cluster 4	*VEGFA, SEMA4C, TIMP2, EFNA1, LEPR*
Cluster 5	*HAND2, ATP2A2, GATA4, ZFPM1, ZFPM2*
Cluster 6	*ERBB2IP, ERBB2, BTC, ERRFI1*
Cluster 7	*PTPN7, SEPT7, CDC14A*
Cluster 8	*ACVR2B, SMAD4, SNIP1*
Cluster 9	*PTPNI3, PDCD10, RGS3*
Cluster 10	*JAZF1, VAC14, SLC45A3*
Cluster 11	*NTF3, FLT1, KDR*
Cluster 12	*CRK, ELMO2, CRKL*
Cluster 13	*DNAJC3, MMP7, FN1*
Cluster 14	*CCL26, CXCL12*
Cluster 15	*RECK, SLC1A2*
Cluster 16	*USP25, WDR4*
Cluster 17	*NCAM1, TUBB3*
Cluster 18	*UBQLN1, TBK1*
Cluster 19	*CDH11, CDH1*
Cluster 20	*KLF11, SMAD7*
Cluster 21	*PRMT5, GEMIN2*
Cluster 22	*REV3L, ADAM9*
Cluster 23	*SH3PXD2A, ADAM12*
Cluster 24	*RCOR3, HFE*
Cluster 25	*FBLN5, RIN2*
Cluster 26	*LPAR1, EDNRA*

Supplementary table 4. Reactome pathway enrichment analysis for terms with FDR *P* value <0.01.

Number of genes	Term name	Description	FDR value
23	HSA-74160	Gene expression (Transcription)	9,20E-11
11	HSA-2559583	Cellular Senescence	1,85E-10
20	HSA-212436	Generic Transcription Pathway	6,03E-10
27	HSA-162582	Signal Transduction	5,38E-09
8	HSA-8878159	Transcriptional regulation by RUNX3	1,51E-08
13	HSA-8953897	Cellular responses to external stimuli	1,51E-08
12	HSA-2262752	Cellular responses to stress	2,40E-08
7	HSA-2559580	Oxidative Stress Induced Senescence	3,72E-07
5	HSA-2559585	Oncogene Induced Senescence	2,17E-06
5	HSA-109606	Intrinsic Pathway for Apoptosis	6,61E-06
8	HSA-1257604	PIP3 activates AKT signaling	1,09E-05
6	HSA-212165	Epigenetic regulation of gene expression	2,51E-05
6	HSA-9018519	Estrogen-dependent gene expression	2,65E-05
4	HSA-114452	Activation of BH3-only proteins	4,21E-05
6	HSA-6807070	PTEN Regulation	4,68E-05
3	HSA-111453	BH3-only proteins associate with and inactivate anti-apoptotic BCL-2 members	6,23E-05
3	HSA-4411364	Binding of TCF/LEF:CTNNB1 to target gene promoters	6,23E-05
6	HSA-453279	Mitotic G1-G1/S phases	6,23E-05
3	HSA-8951430	RUNX3 regulates WNT signaling	6,23E-05
6	HSA-3108232	SUMO E3 ligases SUMOylate target proteins	7,61E-05
6	HSA-109581	Apoptosis	9.24E-5
8	HSA-3700989	Transcriptional Regulation by TP53	9.24E-5
4	HSA-212300	PRC2 methylates histones and DNA	9.8E-5
5	HSA-6785807	Interleukin-4 and Interleukin-13 signaling	1.6E-4
3	HSA-6804116	TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest	1.6E-4
3	HSA-8941856	RUNX3 regulates NOTCH signaling	1.6E-4
3	HSA-4655427	SUMOylation of DNA methylation proteins	1.7E-4
4	HSA-8943724	Regulation of PTEN gene transcription	2.9E-4
4	HSA-1912408	Pre-NOTCH Transcription and Translation	3.4E-4
4	HSA-69563	p53-Dependent G1 DNA Damage Response	3.4E-4
4	HSA-170834	Signaling by TGF-beta Receptor Complex	5.0E-4
7	HSA-5663202	Diseases of signal transduction	5.4E-4
13	HSA-597592	Post-translational protein modification	5.5E-4
3	HSA-1538133	G0 and Early G1	6.5E-4
5	HSA-5633007	Regulation of TP53 Activity	7.1E-4
3	HSA-6804758	Regulation of TP53 Activity through Acetylation	8.3E-4
2	HSA-69200	Phosphorylation of proteins involved in G1/S transition by active Cyclin E:Cdk2 complexes	9.1E-4
3	HSA-2173796	SMAD2/SMAD3:SMAD4 heterotrimer regulates transcription	9.5E-4
6	HSA-5688426	Deubiquitination	9.5E-4
3	HSA-8953750	Transcriptional Regulation by E2F6	9.5E-4
4	HSA-6804756	Regulation of TP53 Activity through Phosphorylation	0.001

Supplementary table 5. KEGG pathway enrichment analysis. Only ther terms with FDR *P* value <0.01 were considered.

Number of genes	Term name	Description	FDR value
16	hsa05206	MicroRNAs in cancer	4,70E-19
18	hsa05200	Pathways in cancer	9,11E-14
11	hsa05215	Prostate cancer	2,43E-13
11	hsa04218	Cellular senescence	2,37E-11
9	hsa05222	Small cell lung cancer	1,80E-10
12	hsa04151	PI3K-Akt signaling pathway	3,82E-09
11	hsa05165	Human papillomavirus infection	1,95E-08
7	hsa04115	p53 signaling pathway	2,08E-08
8	hsa04068	FoxO signaling pathway	5,12E-08
8	hsa04210	Apoptosis	6,11E-08
7	hsa05210	Colorectal cancer	6,46E-08
8	hsa05161	Hepatitis B	7,43E-08
8	hsa05224	Breast cancer	8,88E-08
8	hsa05226	Gastric cancer	8,88E-08
7	hsa01522	Endocrine resistance	9,79E-08
6	hsa01524	Platinum drug resistance	4,77E-07
7	hsa04110	Cell cycle	4,77E-07
7	hsa05418	Fluid shear stress and atherosclerosis	7,33E-07
7	hsa05225	Hepatocellular carcinoma	2,61E-06
8	hsa05166	HTLV-I infection	2,96E-06
6	hsa04931	Insulin resistance	3.89E-6
6	hsa04919	Thyroid hormone signaling pathway	5.56E-6
6	hsa04071	Sphingolipid signaling pathway	5.58E-6
4	hsa04215	Apoptosis - multiple species	1.35E-5
5	hsa05220	Chronic myeloid leukemia	1.45E-5
4	hsa05219	Bladder cancer	3.48E-5
6	hsa05202	Transcriptional misregulation in cancer	3.86E-5
5	hsa04933	AGE-RAGE signaling pathway in diabetic complications	4.22E-5
6	hsa05203	Viral carcinogenesis	5.58E-5
6	hsa05169	Epstein-Barr virus infection	7.43E-5
4	hsa05213	Endometrial cancer	1.1E-4
4	hsa05221	Acute myeloid leukemia	1.7E-4
4	hsa05214	Glioma	1.8E-4
4	hsa05218	Melanoma	2.2E-4
4	hsa05212	Pancreatic cancer	2.3E-4
5	hsa04934	Cushing's syndrome	2.6E-4
4	hsa01521	EGFR tyrosine kinase inhibitor resistance	2.7E-4
4	hsa04211	Longevity regulating pathway	4.1E-4
4	hsa04066	HIF-1 signaling pathway	5.9E-4
3	hsa05216	Thyroid cancer	6.0E-4
4	hsa04722	Neurotrophin signaling pathway	0.001

Supplementary table 6. Gene Ontology (GO) Cellular Components enrichment analysis. Only the terms with FDR *P* value <0.01 were considered.

Number of genes	Term name	Description	FDR value
37	GO.0005654	nucleoplasm	3,00E-14
39	GO.0031981	nuclear lumen	3,00E-14
40	GO.0070013	intracellular organelle lumen	9,87E-12
44	GO.0005634	nucleus	5,05E-11
52	GO.0043227	membrane-bounded organelle	2,68E-10
50	GO.0043231	intracellular membrane-bounded organelle	1,39E-09
47	GO.0044446	intracellular organelle part	1,55E-09
12	GO.0000790	nuclear chromatin	2,63E-09
52	GO.0043229	intracellular organelle	9,84E-09
15	GO.0044427	chromosomal part	6,21E-08
5	GO.0031519	PcG protein complex	7,95E-07
5	GO.0005720	nuclear heterochromatin	9,89E-07
31	GO.0032991	protein-containing complex	1,91E-06
31	GO.0005829	cytosol	4,14E-06
4	GO.0000803	sex chromosome	2,02E-05
4	GO.0000791	euchromatin	2,68E-05
11	GO.1990234	transferase complex	3,52E-05
11	GO.0016604	nuclear body	4,12E-05
13	GO.0044451	nucleoplasm part	4,17E-05
5	GO.0016605	PML body	7,59E-05
45	GO.0005737	cytoplasm	1.6E-4
7	GO.0005667	transcription factor complex	2.5E-4
2	GO.0097135	cyclin E2-CDK2 complex	3.5E-4
53	GO.0044464	cell part	4.2E-4
4	GO.1902911	protein kinase complex	7.0E-4

Supplementary table 7. Gene Ontology (GO) Biological data enrichment analysis. Only the terms with FDR *P* value <0.01 were considered.

Number of genes	Term name	Description	FDR value
31	GO.0042127	regulation of cell population proliferation	5,21E-17
48	GO.0051171	regulation of nitrogen compound metabolic process	5,21E-17
43	GO.0031326	regulation of cellular biosynthetic process	7,62E-17
39	GO.0009893	positive regulation of metabolic process	7,95E-17
48	GO.0031323	regulation of cellular metabolic process	7,95E-17
35	GO.0031324	negative regulation of cellular metabolic process	7,95E-17
38	GO.0031325	positive regulation of cellular metabolic process	7,95E-17
27	GO.0051726	regulation of cell cycle	7,95E-17
48	GO.0060255	regulation of macromolecule metabolic process	7,95E-17
48	GO.0080090	regulation of primary metabolic process	7,95E-17
42	GO.2000112	regulation of cellular macromolecule biosynthetic process	7,95E-17
34	GO.0051172	negative regulation of nitrogen compound metabolic process	8,53E-17
43	GO.0048523	negative regulation of cellular process	8,56E-17
37	GO.0051173	positive regulation of nitrogen compound metabolic process	1,01E-16
37	GO.0010604	positive regulation of macromolecule metabolic process	4,36E-16
20	GO.0045786	negative regulation of cell cycle	5,86E-16
41	GO.0019219	regulation of nucleobase-containing compound metabolic process	6,42E-16
34	GO.0032268	regulation of cellular protein metabolic process	6,42E-16
17	GO.0035690	cellular response to drug	1,02E-15
27	GO.2000113	negative regulation of cellular macromolecule biosynthetic process	1,17E-15
34	GO.0010605	negative regulation of macromolecule metabolic process	1.19E-15
42	GO.0010468	regulation of gene expression	1.29E-15
27	GO.1901700	response to oxygen-containing compound	3.81E-15
39	GO.0051252	regulation of RNA metabolic process	5.82E-15
38	GO.0006355	regulation of transcription, DNA-templated	7.36E-15
34	GO.0010033	response to organic substance	1.55E-14
33	GO.0006357	regulation of transcription by RNA polymerase II	2.09E-14
23	GO.1901698	response to nitrogen compound	2.09E-14
27	GO.0033554	cellular response to stress	2.23E-14
22	GO.1901701	cellular response to oxygen-containing compound	3.77E-14
22	GO.0042493	response to drug	4.03E-14
21	GO.0000122	negative regulation of transcription by RNA polymerase II	7.01E-14
27	GO.0010941	regulation of cell death	7.5E-14
28	GO.0010628	positive regulation of gene expression	9.96E-14
26	GO.0042981	regulation of apoptotic process	9.98E-14
27	GO.0010629	negative regulation of gene expression	1.13E-13
41	GO.0048522	positive regulation of cellular process	1.41E-13
25	GO.0045934	negative regulation of nucleobase-containing compound metabolic process	3.09E-13
23	GO.0045892	negative regulation of transcription, DNA-templated	5.54E-13
42	GO.0048518	positive regulation of biological process	7.81E-13
14	GO.0010948	negative regulation of cell cycle process	1.25E-12
11	GO.0071236	cellular response to antibiotic	1.28E-12
31	GO.0070887	cellular response to chemical stimulus	1.94E-12

Number of genes	Term name	Description	FDR value
12	GO.0071453	cellular response to oxygen levels	1.94E-12
26	GO.0031399	regulation of protein modification process	2.73E-12
44	GO.0044260	cellular macromolecule metabolic process	3.7E-12
23	GO.0051253	negative regulation of RNA metabolic process	4.45E-12
14	GO.0046677	response to antibiotic	4.57E-12
18	GO.0008285	negative regulation of cell population proliferation	5.03E-12
12	GO.0000302	response to reactive oxygen species	8.51E-12
14	GO.0070482	response to oxygen levels	8.51E-12
23	GO.0009719	response to endogenous stimulus	8.95E-12
16	GO.0010035	response to inorganic substance	8.95E-12
28	GO.0050790	regulation of catalytic activity	1.21E-11
36	GO.0042221	response to chemical	1.83E-11
19	GO.0009725	response to hormone	2.14E-11
19	GO.0043066	negative regulation of apoptotic process	2.33E-11
24	GO.0051247	positive regulation of protein metabolic process	2.4E-11
19	GO.0010243	response to organonitrogen compound	3.14E-11
10	GO.0042542	response to hydrogen peroxide	3.78E-11
13	GO.0001666	response to hypoxia	3.95E-11
12	GO.0034599	cellular response to oxidative stress	4.31E-11
32	GO.0006950	response to stress	4.56E-11
17	GO.0010942	positive regulation of cell death	4.83E-11
14	GO.0006979	response to oxidative stress	4.9E-11
23	GO.0032270	positive regulation of cellular protein metabolic process	5.71E-11
15	GO.0009636	response to toxic substance	5.84E-11
16	GO.1901699	cellular response to nitrogen compound	6.06E-11
17	GO.0010564	regulation of cell cycle process	7.3E-11
10	GO.0034614	cellular response to reactive oxygen species	8.52E-11
34	GO.0048583	regulation of response to stimulus	1.04E-10
27	GO.0051128	regulation of cellular component organization	1.38E-10
16	GO.0043065	positive regulation of apoptotic process	1.4E-10
21	GO.0007049	cell cycle	1.63E-10
51	GO.0050794	regulation of cellular process	1.64E-10
24	GO.0045935	positive regulation of nucleobase-containing compound metabolic process	1.86E-10
23	GO.0051254	positive regulation of RNA metabolic process	1.86E-10
22	GO.0045893	positive regulation of transcription, DNA-templated	1.94E-10
11	GO.0097237	cellular response to toxic substance	2.15E-10
10	GO.0071456	cellular response to hypoxia	2.17E-10
30	GO.0009966	regulation of signal transduction	2.63E-10
27	GO.0050793	regulation of developmental process	3.67E-10
26	GO.0071310	cellular response to organic substance	3.81E-10
31	GO.0010646	regulation of cell communication	4.08E-10
24	GO.0031328	positive regulation of cellular biosynthetic process	4.09E-10
8	GO.0070301	cellular response to hydrogen peroxide	5.0E-10
31	GO.0023051	regulation of signaling	5.14E-10
23	GO.0045595	regulation of cell differentiation	5.42E-10
46	GO.0006807	nitrogen compound metabolic process	9.9E-10

Number of genes	Term name	Description	FDR value
17	GO.0033993	response to lipid	9.9E-10
47	GO.0044237	cellular metabolic process	9.9E-10
23	GO.0010557	positive regulation of macromolecule biosynthetic process	1.07E-9
19	GO.0071495	cellular response to endogenous stimulus	1.08E-9
23	GO.1902531	regulation of intracellular signal transduction	1.13E-9
20	GO.0051094	positive regulation of developmental process	1.6E-9
40	GO.0051716	cellular response to stimulus	2.2E-9
13	GO.1901652	response to peptide	3.21E-9
23	GO.2000026	regulation of multicellular organismal development	3.68E-9
18	GO.0009628	response to abiotic stimulus	4.14E-9
47	GO.0071704	organic substance metabolic process	4.36E-9
46	GO.0044238	primary metabolic process	7.81E-9
27	GO.0051239	regulation of multicellular organismal process	7.93E-9
8	GO.2000134	negative regulation of G1/S transition of mitotic cell cycle	9.01E-9
17	GO.0051338	regulation of transferase activity	9.38E-9
14	GO.0032870	cellular response to hormone stimulus	1.0E-8
13	GO.0071417	cellular response to organonitrogen compound	1.21E-8
29	GO.0065009	regulation of molecular function	1.24E-8
12	GO.2001233	regulation of apoptotic signaling pathway	1.27E-8
20	GO.0042325	regulation of phosphorylation	1.38E-8
14	GO.0007346	regulation of mitotic cell cycle	1.56E-8
21	GO.0019220	regulation of phosphate metabolic process	1.64E-8
28	GO.0006996	organelle organization	1.72E-8
9	GO.1902806	regulation of cell cycle G1/S phase transition	1.74E-8
16	GO.0014070	response to organic cyclic compound	1.84E-8
17	GO.0032269	negative regulation of cellular protein metabolic process	1.84E-8
16	GO.0008284	positive regulation of cell population proliferation	1.99E-8
43	GO.0050896	response to stimulus	2.39E-8
35	GO.0048856	anatomical structure development	2.57E-8
28	GO.0043412	macromolecule modification	2.63E-8
10	GO.0045930	negative regulation of mitotic cell cycle	2.65E-8
19	GO.0001932	regulation of protein phosphorylation	3.0E-8
10	GO.0048511	rhythmic process	3.4E-8
14	GO.0006325	chromatin organization	6.02E-8
17	GO.0045944	positive regulation of transcription by RNA polymerase II	6.02E-8
12	GO.0031667	response to nutrient levels	6.31E-8
17	GO.0044092	negative regulation of molecular function	7.21E-8
33	GO.0007275	multicellular organism development	8.94E-8
18	GO.0080134	regulation of response to stress	9.02E-8
22	GO.0048584	positive regulation of response to stimulus	1.08E-7
16	GO.0051276	chromosome organization	1.1E-7
17	GO.0033043	regulation of organelle organization	1.12E-7
30	GO.0090304	nucleic acid metabolic process	1.17E-7
17	GO.0009968	negative regulation of signal transduction	1.18E-7
9	GO.0043281	regulation of cysteine-type endopeptidase activity involved in apoptotic process	1.2E-7
10	GO.1901653	cellular response to peptide	1.2E-7

Number of genes	Term name	Description	FDR value
9	GO.0032868	response to insulin	1.37E-7
15	GO.0022402	cell cycle process	1.78E-7
19	GO.0051240	positive regulation of multicellular organismal process	1.99E-7
24	GO.0006351	transcription, DNA-templated	2.11E-7
8	GO.0031960	response to corticosteroid	2.11E-7
15	GO.0045597	positive regulation of cell differentiation	2.24E-7
15	GO.0051093	negative regulation of developmental process	2.29E-7
6	GO.2001244	positive regulation of intrinsic apoptotic signaling pathway	2.74E-7
33	GO.1901360	organic cyclic compound metabolic process	2.87E-7
14	GO.0043086	negative regulation of catalytic activity	4.11E-7
19	GO.0010647	positive regulation of cell communication	4.13E-7
8	GO.2001235	positive regulation of apoptotic signaling pathway	4.25E-7
12	GO.0010638	positive regulation of organelle organization	4.26E-7
32	GO.0006725	cellular aromatic compound metabolic process	4.33E-7
19	GO.0023056	positive regulation of signaling	4.33E-7
18	GO.0048585	negative regulation of response to stimulus	5.72E-7
18	GO.0009967	positive regulation of signal transduction	6.3E-7
16	GO.0031401	positive regulation of protein modification process	6.34E-7
33	GO.0016043	cellular component organization	7.5E-7
25	GO.0006464	cellular protein modification process	7.71E-7
10	GO.0043434	response to peptide hormone	7.87E-7
12	GO.0031400	negative regulation of protein modification process	8.52E-7
27	GO.0034645	cellular macromolecule biosynthetic process	8.73E-7
27	GO.0044271	cellular nitrogen compound biosynthetic process	9.23E-7
15	GO.0012501	programmed cell death	1.18E-6
17	GO.0043085	positive regulation of catalytic activity	1.19E-6
7	GO.1901796	regulation of signal transduction by p53 class mediator	1.19E-6
13	GO.0006974	cellular response to DNA damage stimulus	1.24E-6
12	GO.0080135	regulation of cellular response to stress	1.3E-6
10	GO.1901987	regulation of cell cycle phase transition	1.31E-6
25	GO.0018130	heterocycle biosynthetic process	1.36E-6
25	GO.0019438	aromatic compound biosynthetic process	1.39E-6
27	GO.0044267	cellular protein metabolic process	1.39E-6
31	GO.0046483	heterocycle metabolic process	1.46E-6
8	GO.0031669	cellular response to nutrient levels	1.49E-6
10	GO.0052548	regulation of endopeptidase activity	1.54E-6
14	GO.0006915	apoptotic process	1.58E-6
16	GO.0051336	regulation of hydrolase activity	1.58E-6
7	GO.0009267	cellular response to starvation	1.64E-6
7	GO.0051384	response to glucocorticoid	1.71E-6
6	GO.0043502	regulation of muscle adaptation	1.74E-6
21	GO.0051704	multi-organism process	1.76E-6
6	GO.1901216	positive regulation of neuron death	2.25E-6
9	GO.0050678	regulation of epithelial cell proliferation	2.38E-6
32	GO.0034641	cellular nitrogen compound metabolic process	2.42E-6
14	GO.1902533	positive regulation of intracellular signal transduction	2.64E-6
6	GO.0043535	regulation of blood vessel endothelial cell migration	2.66E-6

Number of genes	Term name	Description	FDR value
7	GO.0010212	response to ionizing radiation	2.68E-6
10	GO.0001933	negative regulation of protein phosphorylation	2.71E-6
30	GO.0044249	cellular biosynthetic process	2.74E-6
6	GO.0010822	positive regulation of mitochondrion organization	2.79E-6
15	GO.0051130	positive regulation of cellular component organization	2.81E-6
25	GO.1901362	organic cyclic compound biosynthetic process	2.81E-6
53	GO.0009987	cellular process	2.96E-6
9	GO.0001101	response to acid chemical	3.07E-6
7	GO.0032869	cellular response to insulin stimulus	3.08E-6
9	GO.0048545	response to steroid hormone	3.12E-6
12	GO.0045596	negative regulation of cell differentiation	3.22E-6
7	GO.2001236	regulation of extrinsic apoptotic signaling pathway	3.71E-6
11	GO.0045936	negative regulation of phosphate metabolic process	3.81E-6
7	GO.2001242	regulation of intrinsic apoptotic signaling pathway	3.84E-6
18	GO.0044093	positive regulation of molecular function	3.85E-6
13	GO.0060284	regulation of cell development	4.1E-6
5	GO.0043525	positive regulation of neuron apoptotic process	4.21E-6
13	GO.0043549	regulation of kinase activity	4.23E-6
8	GO.0071375	cellular response to peptide hormone stimulus	4.53E-6
12	GO.0043408	regulation of MAPK cascade	4.75E-6
9	GO.0045862	positive regulation of proteolysis	5.16E-6
28	GO.0048731	system development	5.17E-6
9	GO.1901990	regulation of mitotic cell cycle phase transition	5.59E-6
9	GO.0016569	covalent chromatin modification	6.39E-6
13	GO.0051270	regulation of cellular component movement	6.58E-6
19	GO.0009653	anatomical structure morphogenesis	6.74E-6
4	GO.0051570	regulation of histone H3-K9 methylation	6.79E-6
5	GO.0071385	cellular response to glucocorticoid stimulus	6.8E-6
12	GO.0030162	regulation of proteolysis	6.97E-6
7	GO.1904018	positive regulation of vasculature development	7.65E-6
5	GO.1902041	regulation of extrinsic apoptotic signaling pathway via death domain receptors	7.94E-6
25	GO.0016070	RNA metabolic process	7.97E-6
11	GO.0035239	tube morphogenesis	8.31E-6
10	GO.0071900	regulation of protein serine/threonine kinase activity	8.51E-6
11	GO.0051347	positive regulation of transferase activity	1.04E-5
6	GO.0045814	negative regulation of gene expression, epigenetic	1.1E-5
18	GO.0009605	response to external stimulus	1.13E-5
12	GO.0006366	transcription by RNA polymerase II	1.19E-5
7	GO.0048872	homeostasis of number of cells	1.2E-5
12	GO.0045859	regulation of protein kinase activity	1.25E-5
13	GO.0070647	protein modification by small protein conjugation or removal	1.26E-5
12	GO.0035295	tube development	1.32E-5
8	GO.1901214	regulation of neuron death	1.33E-5
10	GO.0044057	regulation of system process	1.35E-5
6	GO.0043280	positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	1.41E-5

Number of genes	Term name	Description	FDR value
29	GO.1901576	organic substance biosynthetic process	1.43E-5
25	GO.0065008	regulation of biological quality	1.53E-5
12	GO.2000145	regulation of cell motility	1.55E-5
6	GO.0032355	response to estradiol	1.59E-5
6	GO.0048660	regulation of smooth muscle cell proliferation	1.59E-5
6	GO.0072331	signal transduction by p53 class mediator	1.72E-5
9	GO.1903706	regulation of hemopoiesis	1.83E-5
7	GO.0010632	regulation of epithelial cell migration	1.91E-5
16	GO.0035556	intracellular signal transduction	1.93E-5
4	GO.0090200	positive regulation of release of cytochrome c from mitochondria	1.94E-5
6	GO.0031056	regulation of histone modification	2.18E-5
9	GO.0009314	response to radiation	2.3E-5
8	GO.0033044	regulation of chromosome organization	2.3E-5
6	GO.0030856	regulation of epithelial cell differentiation	2.43E-5
4	GO.1903749	positive regulation of establishment of protein localization to mitochondrion	2.43E-5
7	GO.2001234	negative regulation of apoptotic signaling pathway	2.43E-5
6	GO.0009411	response to UV	2.61E-5
9	GO.0051346	negative regulation of hydrolase activity	2.84E-5
7	GO.0090257	regulation of muscle system process	2.84E-5
5	GO.0034644	cellular response to UV	2.98E-5
25	GO.0010467	gene expression	3.48E-5
9	GO.0030335	positive regulation of cell migration	3.6E-5
6	GO.0051147	regulation of muscle cell differentiation	3.6E-5
6	GO.0007050	cell cycle arrest	3.71E-5
15	GO.0048699	generation of neurons	3.79E-5
4	GO.0051443	positive regulation of ubiquitin-protein transferase activity	3.98E-5
34	GO.0032501	multicellular organismal process	4.2E-5
8	GO.0016570	histone modification	4.53E-5
5	GO.0031058	positive regulation of histone modification	4.53E-5
20	GO.0032879	regulation of localization	4.55E-5
5	GO.0097327	response to antineoplastic agent	4.72E-5
11	GO.0030334	regulation of cell migration	4.79E-5
6	GO.0043409	negative regulation of MAPK cascade	4.97E-5
6	GO.0097193	intrinsic apoptotic signaling pathway	4.97E-5
6	GO.2001252	positive regulation of chromosome organization	5.28E-5
5	GO.1901655	cellular response to ketone	5.43E-5
7	GO.0040029	regulation of gene expression, epigenetic	5.46E-5
11	GO.0048609	multicellular organismal reproductive process	5.5E-5
6	GO.0045766	positive regulation of angiogenesis	5.58E-5
4	GO.0070317	negative regulation of G0 to G1 transition	5.58E-5
10	GO.0003006	developmental process involved in reproduction	5.82E-5
7	GO.0007568	aging	5.9E-5
9	GO.0071396	cellular response to lipid	5.9E-5
9	GO.0001944	vasculature development	5.96E-5
3	GO.0014745	negative regulation of muscle adaptation	6.09E-5
3	GO.1902237	positive regulation of endoplasmic reticulum stress-induced	6.09E-5

Number of genes	Term name	Description	FDR value
		intrinsic apoptotic signaling pathway	
10	GO.0000278	mitotic cell cycle	6.17E-5
9	GO.0010720	positive regulation of cell development	6.25E-5
12	GO.0022603	regulation of anatomical structure morphogenesis	7.5E-5
9	GO.0070848	response to growth factor	7.88E-5
8	GO.0051052	regulation of DNA metabolic process	7.99E-5
9	GO.1902532	negative regulation of intracellular signal transduction	8.7E-5
4	GO.0006306	DNA methylation	8.74E-5
7	GO.0070372	regulation of ERK1 and ERK2 cascade	8.75E-5
14	GO.0022414	reproductive process	9.18E-5
7	GO.0045765	regulation of angiogenesis	9.3E-5
18	GO.0007166	cell surface receptor signaling pathway	9.37E-5
5	GO.2001237	negative regulation of extrinsic apoptotic signaling pathway	9.57E-5
18	GO.0007399	nervous system development	9.79E-5
21	GO.0048513	animal organ development	9.81E-5
6	GO.1901654	response to ketone	9.86E-5
11	GO.0048646	anatomical structure formation involved in morphogenesis	1.1E-4
14	GO.0002682	regulation of immune system process	1.2E-4
7	GO.0009416	response to light stimulus	1.2E-4
11	GO.0018193	peptidyl-amino acid modification	1.2E-4
8	GO.0048608	reproductive structure development	1.2E-4
5	GO.0031398	positive regulation of protein ubiquitination	1.4E-4
12	GO.0034097	response to cytokine	1.4E-4
6	GO.0043523	regulation of neuron apoptotic process	1.4E-4
3	GO.0048070	regulation of developmental pigmentation	1.4E-4
6	GO.0008406	gonad development	1.5E-4
23	GO.0048869	cellular developmental process	1.5E-4
15	GO.0009888	tissue development	1.6E-4
4	GO.0010332	response to gamma radiation	1.6E-4
4	GO.0043536	positive regulation of blood vessel endothelial cell migration	1.6E-4
8	GO.0050769	positive regulation of neurogenesis	1.6E-4
29	GO.1901564	organonitrogen compound metabolic process	1.6E-4
12	GO.0045937	positive regulation of phosphate metabolic process	1.7E-4
9	GO.0016032	viral process	1.8E-4
5	GO.0000082	G1/S transition of mitotic cell cycle	1.9E-4
5	GO.0050680	negative regulation of epithelial cell proliferation	1.9E-4
10	GO.0050767	regulation of neurogenesis	1.9E-4
4	GO.0010660	regulation of muscle cell apoptotic process	2.1E-4
4	GO.0014910	regulation of smooth muscle cell migration	2.2E-4
5	GO.0016202	regulation of striated muscle tissue development	2.2E-4
3	GO.1902170	cellular response to reactive nitrogen species	2.2E-4
9	GO.0045664	regulation of neuron differentiation	2.4E-4
12	GO.0051241	negative regulation of multicellular organismal process	2.4E-4
5	GO.0071901	negative regulation of protein serine/threonine kinase activity	2.4E-4
6	GO.0090092	regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	2.4E-4
11	GO.0044703	multi-organism reproductive process	2.5E-4

Number of genes	Term name	Description	FDR value
8	GO.0001568	blood vessel development	2.7E-4
10	GO.0019953	sexual reproduction	2.7E-4
4	GO.0070373	negative regulation of ERK1 and ERK2 cascade	2.7E-4
8	GO.1903827	regulation of cellular protein localization	2.8E-4
4	GO.0001776	leukocyte homeostasis	2.9E-4
11	GO.0001934	positive regulation of protein phosphorylation	2.9E-4
4	GO.0007569	cell aging	2.9E-4
7	GO.0048589	developmental growth	2.9E-4
5	GO.0007623	circadian rhythm	3.0E-4
2	GO.0014740	negative regulation of muscle hyperplasia	3.2E-4
22	GO.0030154	cell differentiation	3.3E-4
9	GO.0030155	regulation of cell adhesion	3.3E-4
4	GO.0045844	positive regulation of striated muscle tissue development	3.3E-4
9	GO.0007276	gamete generation	3.6E-4
4	GO.0008630	intrinsic apoptotic signaling pathway in response to DNA damage	3.6E-4
4	GO.0046902	regulation of mitochondrial membrane permeability	3.6E-4
9	GO.0051129	negative regulation of cellular component organization	3.6E-4
3	GO.0010661	positive regulation of muscle cell apoptotic process	3.8E-4
9	GO.0016567	protein ubiquitination	4.1E-4
10	GO.0072359	circulatory system development	4.1E-4
3	GO.0032461	positive regulation of protein oligomerization	4.2E-4
3	GO.1900740	positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway	4.2E-4
6	GO.0031647	regulation of protein stability	4.6E-4
8	GO.0071407	cellular response to organic cyclic compound	4.6E-4
6	GO.2000146	negative regulation of cell motility	4.8E-4
9	GO.0040008	regulation of growth	4.9E-4
6	GO.0090287	regulation of cellular response to growth factor stimulus	4.9E-4
2	GO.0072717	cellular response to actinomycin D	5.0E-4
2	GO.0090116	C-5 methylation of cytosine	5.0E-4
2	GO.1902263	apoptotic process involved in embryonic digit morphogenesis	5.0E-4
26	GO.0007165	signal transduction	5.4E-4
3	GO.0033032	regulation of myeloid cell apoptotic process	5.4E-4
4	GO.0048661	positive regulation of smooth muscle cell proliferation	5.4E-4
3	GO.0071549	cellular response to dexamethasone stimulus	5.4E-4
6	GO.1902105	regulation of leukocyte differentiation	5.5E-4
6	GO.0009410	response to xenobiotic stimulus	5.6E-4
4	GO.0030330	DNA damage response, signal transduction by p53 class mediator	5.8E-4
3	GO.0048730	epidermis morphogenesis	5.9E-4
4	GO.0097306	cellular response to alcohol	6.0E-4
4	GO.0048145	regulation of fibroblast proliferation	6.3E-4
10	GO.0009887	animal organ morphogenesis	6.7E-4
27	GO.0023052	signaling	6.7E-4
3	GO.0043500	muscle adaptation	7.0E-4
3	GO.0043516	regulation of DNA damage response, signal transduction by p53 class mediator	7.0E-4
3	GO.0045601	regulation of endothelial cell differentiation	7.0E-4

Number of genes	Term name	Description	FDR value
2	GO.0045636	positive regulation of melanocyte differentiation	7.0E-4
2	GO.0051097	negative regulation of helicase activity	7.0E-4
5	GO.2000377	regulation of reactive oxygen species metabolic process	7.0E-4
4	GO.0046620	regulation of organ growth	7.3E-4
7	GO.0051090	regulation of DNA-binding transcription factor activity	7.3E-4
3	GO.0003203	endocardial cushion morphogenesis	7.5E-4
3	GO.1904030	negative regulation of cyclin-dependent protein kinase activity	7.5E-4
5	GO.0018105	peptidyl-serine phosphorylation	7.6E-4
6	GO.0032102	negative regulation of response to external stimulus	7.7E-4
4	GO.0009791	post-embryonic development	7.8E-4
6	GO.0071214	cellular response to abiotic stimulus	7.9E-4
3	GO.0043029	T cell homeostasis	8.0E-4
3	GO.0048873	homeostasis of number of cells within a tissue	8.0E-4
4	GO.1904029	regulation of cyclin-dependent protein kinase activity	8.4E-4
13	GO.0042592	homeostatic process	8.7E-4
4	GO.0060070	canonical Wnt signaling pathway	8.7E-4
27	GO.0007154	cell communication	9.3E-4
3	GO.0034405	response to fluid shear stress	9.3E-4
3	GO.0110111	negative regulation of animal organ morphogenesis	9.3E-4
2	GO.1900378	positive regulation of secondary metabolite biosynthetic process	9.3E-4
6	GO.0030111	regulation of Wnt signaling pathway	9.4E-4
4	GO.0051153	regulation of striated muscle cell differentiation	9.6E-4
3	GO.0010907	positive regulation of glucose metabolic process	9.8E-4
3	GO.0014911	positive regulation of smooth muscle cell migration	9.8E-4
3	GO.0031062	positive regulation of histone methylation	9.8E-4
3	GO.1902042	negative regulation of extrinsic apoptotic signaling pathway via death domain receptors	9.8E-4
6	GO.0001525	angiogenesis	9.9E-4
14	GO.0051049	regulation of transport	9.9E-4
3	GO.0042149	cellular response to glucose starvation	0.001
9	GO.0051345	positive regulation of hydrolase activity	0.001
5	GO.0060485	mesenchyme development	0.001

Supplementary table 8. Gene Ontology (GO) Molecular Function data enrichment analysis. Only the terms with FDR *P* value <0.01 were considered.

Number of genes	Term name	Description	FDR value
14	GO.0003682	chromatin binding	5,52E-09
42	GO.0005515	protein binding	5,52E-09
19	GO.0043565	sequence-specific DNA binding	5,52E-09
17	GO.0044212	transcription regulatory region DNA binding	5,52E-09
52	GO.0005488	binding	7,02E-09
15	GO.0000976	transcription regulatory region sequence-specific DNA binding	2,23E-08
15	GO.0019904	protein domain specific binding	2,23E-08
23	GO.0140110	transcription regulator activity	3,72E-08
14	GO.0000977	RNA polymerase II regulatory region sequence-specific DNA binding	4,83E-08
21	GO.0003700	DNA-binding transcription factor activity	5,19E-08
12	GO.0000987	proximal promoter sequence-specific DNA binding	1,14E-07
24	GO.0003677	DNA binding	1,14E-07
27	GO.0003676	nucleic acid binding	3,60E-07
22	GO.0019899	enzyme binding	3,72E-07
34	GO.1901363	heterocyclic compound binding	3,81E-07
19	GO.0000981	DNA-binding transcription factor activity, RNA polymerase II-specific	4,35E-07
34	GO.0097159	organic cyclic compound binding	5,09E-07
11	GO.0000978	RNA polymerase II proximal promoter sequence-specific DNA binding	6,26E-07
12	GO.0008134	transcription factor binding	1,15E-06
5	GO.1990841	promoter-specific chromatin binding	2,73E-06
11	GO.0019901	protein kinase binding	7.86E-6
9	GO.0001228	DNA-binding transcription activator activity, RNA polymerase II-specific	2.13E-5
4	GO.0051721	protein phosphatase 2A binding	2.3E-5
26	GO.0046872	metal ion binding	5.93E-5
18	GO.0140096	catalytic activity, acting on a protein	1.0E-4
5	GO.0042826	histone deacetylase binding	1.6E-4
5	GO.0019903	protein phosphatase binding	2.5E-4
4	GO.0002039	p53 binding	5.6E-4
2	GO.0003886	DNA (cytosine-5-)-methyltransferase activity	7.0E-4
3	GO.0035035	histone acetyltransferase binding	7.0E-4
2	GO.0050816	phosphothreonine residue binding	7.0E-4
4	GO.0008013	beta-catenin binding	7.7E-4
3	GO.0016538	cyclin-dependent protein serine/threonine kinase regulator activity	9.0E-4
30	GO.0043167	ion binding	9.0E-4
2	GO.0071535	RING-like zinc finger domain binding	0.0012
14	GO.0042802	identical protein binding	0.0014
16	GO.0016740	transferase activity	0.0016
10	GO.0044877	protein-containing complex binding	0.0019
2	GO.0001134	transcription regulator recruiting activity	0.0034

Number of genes	Term name	Description	FDR value
10	GO.0046914	transition metal ion binding	0.0034
4	GO.0002020	protease binding	0.0038
3	GO.0097110	scaffold protein binding	0.0039
2	GO.0004861	cyclin-dependent protein serine/threonine kinase inhibitor activity	0.0043
2	GO.0000900	translation repressor activity, mRNA regulatory element binding	0.0049
6	GO.0004674	protein serine/threonine kinase activity	0.0085
8	GO.0008270	zinc ion binding	0.0088
9	GO.0030234	enzyme regulator activity	0.0098
5	GO.0031625	ubiquitin protein ligase binding	0.0099

GENERAL DISCUSSION

EVs applicability in ccRCC patients' management

CcRCC is characterized by difficulty in early diagnosis and a high metastatic potential. Despite the constant improvement of imaging techniques, about 20 to 30% of all patients present metastatic disease at the time of diagnosis and up to 40% of the patients with localized disease submited to nephrectomy will relapse in distant sites [1,2]. When patients are diagnosed with metastatic disease, they usually present a 5-year survival rate lower than 10% [3]. Metastatic ccRCC treatment focus on the control of its angiogenic and proliferation potential using TKIs and mTOR inhibitors, but patients develop resistance within a short period of time, leaving the clinicians with limited options. In the last few years, immune checkpoint inhibitors entered the treatment options as a new hope with good clinical trial results, but even with a good initial response, the patients ultimately develop resistance and there is also a subgroup of patients that does not seem to benifit from this type of therapy [4,5].

Despite metastatic ccRCC remains incurable, the prognosis of recurrent ccRCC is variable and the detection of early relapse could allow patients' stratification according to metastatic risk and contribute to a more personalized medicine approach in patient management [6]. The TCGA consortium has made considerable efforts to molecularly characterize the different subtypes of RCC in order to get a deeper knowledge of their molecular biology and improve personalized medice aproaches on these patients [7]. In addition to the well stablished deregulated VHL/HIF pathway, and consequent increase in the expression of a vasculature development gene signature, the TCGA reported increased metabolic, immune and mitotic cell cycle gene signatures in ccRCC [8]. However, this information did not seem enough for the improvement of ccRCC patients' care so far. Additional molecular biomarkers, aside from the classical genetic alterations markers that are commonly addressed by large genomic studies, could be the missing puzzle piece that could allow a better understanding of this complex and multivariable disease.

With the evolution of the oncology field, cancer is now seen as a entity with ecosystem features due to its clonal heterogeneity and microenvironment of its own [9]. It is now known that an important part of the complex communication netwotks established between the tumor, its microenvironment and the host are mediated by EVs [10-12]. In fact, the intratumor heterogeinity characteristic of ccRCC is considered to be one of the major contributers to treatment failure and tumor recurrence, with some authors defending that the EVs released by different populations of renal cancer stem cells (CSCs) are related to its aggressiveness [13-17]. Other studies reported the ccRCC derived EVs also have immunomodulation capacity, namely in terms of CD8+ T cell suppression and

inhibition of NK cells cytotoxicity [18,19]. However, one must keep in mind that the majority of these studies have been performed *in vitro* or with animal models and further validation is needed. So far, the studies using patients remain a few, but were able to study EVs in tissue-exudate, serum and urine from ccRCC patients [20-26]. These studies mainly focused on the EV-cargo aplicability as diagnosis and prognosis biomarkers, with special interest on miRNAs.

One of the main goals of our project was to follow the dynamic changes on miRNA and MMP/TIMP EV cargo in ccRCC patients with localized disease before and after surgery, and also during their follow-up period, and compare it with patients with metastatic disease. We observed that patients with localized ccRCC tumors had EVs enriched in hsa-miR-25-3p, miR-126-5p, miR-200c-3p and miR-301a-p, and that enrichment decreased after tumor removal. On the other hand, patients with metastatic disease had EVs enriched in hsa-miR-301a-3p and TIMP-1 mRNA, being the later associaded with a lower overall survival. Both the miRNAs and TIMP-1 are able to activate the PI3K/Akt pathway. Hsa-miR-25-3p, hsa-miR-200c-3p and hsa-miR-301a-3p modulate PI3K/AKT pathway by inhibition of PTEN expression, and TIMP-1 is able to activate the same pathway through receptor mediated signalling [27-30]. The PI3K/Akt pathway is a signal transduction cascade that is responsible for many physiological functions, including cell cycle, cell survival, protein synthesis and growth, metabolism, motility and angiogenesis [31]. The VHL/HIF and PI3K/AKT pathways contribute to ccRCC development through an extensive crosstalk that gives rise to a large signaling network. It begins with *VHL* loss or innactivation that leads to HIF upregulation and consequent expression of a variety of growth factors, such as VEGF, PDGF and TGF, which in turn activate the PI3K/Akt pathway through tyrosine kinase receptors [32,33]. PI3K/Akt activation leads to and increase of mTORC1 and mTORC2 that will promote HIF-1α and HIF-2α expression, therefore forming a positive feedback loop that will result in a constitutive activation of this signalling network in the ccRCC landscape [31]. Although the overall mutation rate of the PI3K/Akt pathway in ccRCC is relatively low compared with other cancer types, the overall activation of PI3K/Akt in ccRCC is high, which means that are other mechanisms behind its activation are present [34,35].

Our results hint to the hypothesis of an EV-mediated Akt activation through the transfer of hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p, hsa-miR-301a-3p and TIMP-1 mRNA but additional studies are needed to validate this hypothesis. Moreover, depending on the disease phase (localized or metastatic) there were diferences in the miRNA and mRNA enriched cargo of EVs, suggesting that Akt activation maybe sustained by different molecular mechanisms in different stages of the disease. In addition to its relation with disease evolution, a deeper understanding of the Akt activation through EV-

mediated cargo would allow a better understanding of the mechanisms behind therapy resistance in ccRCC since Akt is part of the molecular cascades that both TKIs and mTOR inhibitors are used to inhibit.

In conclusion, we observed that it is possible to relate the EVs content with ccRCC development, and establish potencial molecular mechanisms behind it. This study highlights the potential use of EV-cargo as potentital biomakers for ccRCC, using a minimally invasive approach.

EV-free miRNAs or EV-derived miRNAs?

The is no doubt that miRNAs revolutionized the biomarker discovery field in the past years. Due to their impact on gene expression, small size and stability, they were widely studied in several types of biological samples of cancer patients to test their applicability as biomarkers. In fact, it is now possible to distinguish between different RCC subtypes and even different prognosis subgrups within the same histological RCC subtype according to different miRNA profiles [36-39]. However, these studies are performed in tissue samples, which maintain the need for conventinal biopsy. There are also studies in urine, serum and plasma which require non-invasive or minimally invasive sample collection techniques, such as a blood colection. Circulating miRNA studies focus more in tumor detection and prognostic applicability rather than histological subtype differentiation and several miRNAs demonstrated promising results [40-46]. Nevertheless, none have made to the clinical setting so far due to lack of validation. Another issue that must be addressed regarding EV-free miRNAs is wether or not they have a biological impact. Besides EVs, miRNAs are also able to circulate bounded to proteins, being AGO2 the most common carrier [47]. So far there are is no evidence of active release of protein-miRNA complexes from cells or their uptake by recipient cells in mammals [48]. Moreover, some authors defend that circulating protein-bounded miRNAs are apparently non-specific remnants resulting from cellular activity or cell death [49,50]. The hypothesis that protein-bound miRNAs are released in a non-selective manner after cell death is in line with the fact several authors reported an increase of tissue–specific miRNAs in the bloodstream after certain tissues suffer from damage or toxicity [51-55].

According to our results, hsa-miR-210-3p, hsa-miR-221 and hsa-miR-1233 plasma levels could differentiate ccRCC patients from healthy individuals and their expression was also related to clinicalpathological features such as higher tumor size and metastasis. In addition to that, higher plasmatic levels of these miRNAs were associated with a lower cancer-specific survival and improved the capacity to predict death by ccRCC in 8.4% when compared to the current variables used by clinicians. These results support the

applicability of circulating miRNAs as prognostic biomarkers for RCC. However, when we studied hsa-miR-210-3p and hsa-miR-1233-5p in EVs, the scenario was a bit different. We weren´t able to dectect hsa-miR-1233-5p in ccRCC patients EVs, which suggests that this miRNA doesn't circulate in EVs and rather uses other carriers. On the other hand, hsa-miR-210-3p was detected in ccRCC plasma EVs, with its levels decreasing between surgery and follow-up and also a tendency to increase in the metastatic group. Based on these results, we can hypothesize that hsa-miR-1233-5p may be a remnant of cellular activity and hsa-miR-210-3p may exert a biological effect. Since both of these miRNAs are increased by hypoxia, a well known cellular stress inducer and microenvironment modulator, one plausible theory could be that hypoxia modulates their expression through different mechanisms and with different intents. Hsa-miR-1233-5p can be released in a protein carrier as consequence of the stress that hypoxia induces in the cell, while hsa-miR-210-3p is loaded and released in EVs by hypoxic cells and can be used as a microenvironment modulator. In fact, there are studies resporting that EV-derived hsa-miR-210-3p is involved in angiogenesis induction and metastasis formation [56-58].

In conclusion, both EV-free and EV-derived miRNAs have great potential as cancer biomarkers and can be used in a complementary way. EV-free miRNAs have the advantage that their isolation protocols are well established and less time consuming. On the other hand, due to the fact that EVs are agents of cell-to-cell communication, the study of EV-miRNAs will deepen the knowledge regarding how the tumor evolves and impacts its host.

Concluding remarks

The current state of the art of EV research focuses on the identification of EV-cargo that can be used as diagnostic or prognostic biomarker in cancer has its main goal. In the light of the current knowledge, there is no doubt that EVs play a crucial role in tumor development and pre-metastatic niche establishment. However, most of the evidence available to date was obtained from *in vitro* studies or animal models, both of which presenting several limitations associated. The studies involving human patients are starting to increase but there is still a need for stantardization in terms of biofluid selection and processing, and respective EV isolation methods, in order to allow the correct comparison between studies. In addition to that, future studies should use larger cohorts of patients with larger follow-up periods for a better understanding of the EVs network dynamics in the host.

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