PROGNOSTIC SIGNIFICANCE OF PROTEOMICS AND MULTI-OMICS STUDIES IN RENAL CARCINOMA.

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ABSTRACT

Introduction Renal carcinoma and in particular its most common variant, the clear cell subtype, is often diagnosed incidentally through abdominal imaging. Rather frequently, the tumour is discovered at an early stage. However, 20% to 40% of patients undergoing nephrectomy for clinically localized renal cancer, even after accurate histological and clinical classification, will develop metastasis or recurrence, justifying the associated mortality rate. Therefore, even if renal carcinoma is not among the most frequent nor deadly cancers, a better prognostication is needed.

Areas covered Recently proteomics or other -omics combinations have been applied to both cancer tissues, on the neoplasia itself and surrounding microenvironment, cultured cells and biological fluids (so-called liquid biopsy) generating a list of prognostic molecular tools that will be reviewed in the present paper.

Expert opinion. Although promising, none of the approaches listed above has been yet translated in clinics. This is likely due to the peculiar genetic and phenotypic heterogeneity of this cancer, which makes nearly each tumour different from all the others. Attempts to overcome this issue will be also revised. In particular we will discuss how the application of -omics integrated approaches could provide the determinants of response to the different targeted drugs.

Keywords: cancer heterogeneity, liquid biopsy, multi-omics, proteomics, renal cell carcinoma

Article highlights

- The prognostic requirements for renal cell carcinoma (RCC) are even more critical than for other common cancers

- The issue of genomic and phenotypic cancer-associated heterogeneity strongly affects the research for prognostic markers in RCC

- Application of proteomics to tissues, cultured cells, and biological fluids has provided some advancement into RCC prognostic significance assessment

- The integration of different -omic strategies and system biology can contribute in identifying sound prognostic panels

- None of the newly proposed prognostic factors, either single or in combination has reached enough acceptance to be applied in the patients' follow up

1. INTRODUCTION

Renal cell carcinoma is among the top ten most commonly diagnosed cancers worldwide, representing 2-3% of all cancers, with the highest incidence rates in developed countries [1]. Histologically, kidney tumours

comprise a wide variety of different subtypes with the clear cell variant being the most common, accounting for 75-90% of all renal cancers [2].

In this review, we will focus on clear cell renal cell carcinoma, from now on named simply RCC, revising the most recent papers in the field (last five years).

In general, RCC are sporadic diseases, but they can also arise in familial forms. The most frequent genetic mutation causing RCC is a deletion on chromosome 3(LOH 3p) in three possible positions: 3p14 (Fragile Histidine Triad Diadenosine Triphosphatase, FHIT gene), 3p21.3 e 3p25 (Von Hippel Lindau, VHL gene) [3]. Subsequent genomic alterations involving Protein polybromo-1 (PBRM1) [4], SET Domain Containing 2, Histone Lysine Methyltransferase, SETD2, [5], or BRCA1 associated protein 1, BAP1 [6], all present on chromosome 3p21-3p25 in the VHL region, are required for disease progression and are associated with aggressive phenotypes [7-9].

In the past years, the typical symptomatology of RCC was flank pain, haematuria and a palpable abdominal renal mass, but nowadays only a minority of patients with RCC displays this classic triad [10]. At present, a renal mass is often discovered following abdominal imaging investigation for different causes, and the most common therapeutic strategy is partial or total nephrectomy [11]. Moreover, it is frequent that RCC identification and treatment are applied rather early in the natural history of disease, in a preclinical phase. Therefore, there is not a stringent need of diagnostic markers. The surveillance protocols for follow-up of RCC patients after radical nephrectomy are based on the American Joint Committee on Cancers (AJCC) pathological tumour-node-metastasis (TNM) classification system [12]. Other comprehensive staging modalities have emerged and have been implemented by combining different pathological and clinical variables, including Fuhrman nuclear grade and Leibovich score [13].

However, even early stage tumours remain at risk of metastatic progression after surgical resection and about 30% of patients undergoing nephrectomy for clinically localized RCC will develop a recurrence. Identifying this high-risk group of RCC patients remains a challenge. Hence, novel molecular prognostic biomarkers are urgently needed to better predict clinical outcomes.

An intensive search for predictive and prognostic markers has been ongoing for the past few years, leading to the discovery of three markers which have been validated in RCC. These are VHL, vascular endothelial growth factor (VEGF) and carbonic anhydrase 9 (CA9) [14]. Nonetheless, the use of these markers is still debated and none of them has yet been implemented in clinical routine. One of the main issues concerning marker efficacy is the peculiar RCC heterogeneity, which entails considerable molecular diversity both between and within tumours. Dissecting such issue is crucial in order to clarify the diverse but converging mechanisms of RCC progression. It is likely that neoplastic cells adopt common pathways of aggressiveness, shared with other carcinomas, such as angiogenesis stimulation, apoptosis inhibition and PDL1 expression [15], but it is also intriguing to hypothesize that deepening RCC molecular pathophysiology concurs to discover new and more specific routes of malignancy development, possibly actionable by future drugs.

2. RCC HETEROGENEITY

Tumour heterogeneity may be defined as the molecular, morphological and behavioural variability that cancer cells display within a single tumour. It depends on genetic, epigenetic, and microenvironmental factors and presents a challenge for cancer diagnosis and therapy [16]. However, it may well be responsible for the failure to find suitable candidate markers able to help in assigning a meaningful prognosis to each patient. This heterogeneity is characteristic of virtually any solid tumour, but many observations concur in showing that it is particularly marked in the case of RCC. This may explain why currently used methodologies in diagnostic pathology often fail in indicating the correct prognosis, lacking the capability to discern deeper genotypic and subtler phenotypic differences among individual patients.

In fact, phenotypically, RCC shows a wide range of disease courses. For example, metastatic disease may develop in patients either as synchronous (*de novo*) or metachronous (after surgical intervention) disease, showing highly variable temporal and spatial patterns of progression [17]. This can vary from indolent, sequential seeding of solitary or oligometastatic sites, typically involving the lungs, pancreas, or thyroid, with long periods of latency between events, to early multiorgan dissemination within months following primary nephrectomy [18]. In the same patient, tumours with rapid and slow growth often co-exist next to each other. Differences in growth kinetics are suggestive of a state of transient tumour dormancy [19].

From a genetic point of view, extensive DNA sequencing has shed light on the diversity of individual cancer genomes (multigenic nature) and their evolution among RCC patients and has led to different classification proposals, recently reviewed in [20]. Based on marked genetic intra-tumour and inter-metastases heterogeneity of RCC, different molecular tumour subtypes were characterized, defining a "braided cancer river model" [21]. An intriguing interpretation of this peculiar behaviour is offered by evolutionary theory, based on the trajectories of the variations in chromosomal complexity during RCC course. This approach showed the correlation between the paths of driver mutations and the clinical phenotypes of RCC, including the metastatic potential and therapeutic response [22].

A further confirmation of the peculiar characteristic of RCC was offered by a very recent bioinformatics analysis on proteomic and phospho-proteomic data sets of six different cancer types, (breast, colon, ovarian, lung, endometrial cancer and RCC) [23]. Results showed that all types form one cluster, except for RCC, displaying decreased phosphorylation status -as compared to the other five cancers - thus placing it on a distinct branch. For this reason, RCC was excluded from the phosphorylation signature typical of all the other cancers [23].

Hence, while the heterogeneity of RCC tumours represents a challenge, it also provides important insights for clinical proteomic research, with the aim of increasing the effectiveness of intervention on metastatic tumours with targeted therapies [21]. In particular, distinct molecular subtypes of RCC according to the mutation status of PBRM1, BAP1 and KDM5C could have potential biomarker values for patients with metastatic RCC treated with targeted agents [24]

Moreover, we believe that what dictates the different aggressiveness of otherwise clinically indistinguishable tumours may reside in phenotypic characteristics that could be effectively detected and monitored through proteomic or multi-omic approaches. A signature or a panel of biomarkers could be a more practical and

plausible route to the management of this cancer, and could assist in evaluating tumour progression after surgical treatment.

3. SEARCH OF PROGNOSTIC MARKERS FOR RCC THROUGH PROTEOMICS

3.1 TISSUE

Tissue represents a very common starting sample for RCC proteomic approach; in fact, after the detection of a renal mass by conventional or innovative imaging techniques, the most frequent therapeutic option is nephrectomy. Therefore, this can provide material for proteomic assay in different formats. Fresh frozen tissues are utilised most commonly but formalin-fixed paraffin-embedded samples (FFPE) can also be used. This latter option allows the exploitation of archival specimens, potentially available in all world biobanks and therefore its adoption for proteomic studies looks promising. However, efficacy and reproducibility of fixed versus fresh tissues needs further validation, this was recently reviewed in [25].

Three kinds of strategies are usually applied to RCC tissues. Firstly, one can simply compare the global protein profile of RCC to that of the adjacent normal kidney (ANK). However, this approach has little chance of giving prognostic meaning to the differential proteome. A second strategy, hypothetically more informative, consists in grouping patients based on the staging/grading pathological classification. In addition, a third possibility is to correlate differences with patient clinical outcomes. This has the highest chance to provide prognostic significance. However, this type of investigation requires close collaboration not only between lab investigators and clinicians, but also between different clinical settings. In fact, the management of the patient in the surgical phase is assigned to urologists, while the follow-up, especially in cases of recurrence and/or metastasis, is in the hands of medical oncologists. Unfortunately, in this phase, there is an increasing risk of losing contact with the patient and hence miss precious information.

A few papers were recently published exploring these strategies and adopting different MS-based protocols (Table 1).

Song at el worked on 14 pairs of RCC and ANK frozen tissues, to identify dysregulated proteins [26]. They performed a quantitative proteomic approach based on data-independent acquisition (DIA), an unbiased and high-throughput MS approach. Among more than 4000 identified proteins, 436 of them were differentially expressed in RCC tissues, showing the dysregulation of multiple pathways such as that of oxidative phosphorylation. Moreover, the over-expression of 4 differential proteins (L-lactate dehydrogenase A chain, annexin A4, nicotinamide N-methyltransferase, and perilipin-2) was confirmed by RT-qPCR, western blot, and immunohistochemistry, validating the results of this approach. Although no attempt was made in order to find a prognostic significance to these results, it may be interesting to consider that a majority of the dysregulated proteins are related to exosomes, nanovesicles identified as cell-cell communication effectors. This finding allows us to associate exosomes with ccRCC progression [26], opening up new research paths (see below).

Another study was conducted in 2017 on a small cohort of patients (4), comparing RCC to ANK frozen tissues [27]. Label-free quantitative analysis using LC-MS/MS allowed the identification of 210 dysregulated

proteins, many of them resulted again involved in oxidative phosphorylation. In order to interpret their results and to investigate the potential prognostic significance of the dysregulated proteins, authors looked for correlations among the expression levels of the mRNA of the identified differential proteins. They used mRNA microarray data from 47 paired RCC and ANK tissues and the survival data available online. Finally, they proposed a few protein species as potential prognostic factors for RCC, both negative and positive [27].

Very recently, a study was published on 18 pairs of RCC and ANK frozen tissues, from patients grouped according to stage [28]. Using iTRAQ-based proteomics analysis, authors identified 130 differentially expressed proteins, but none of them allowed discriminating among different tumour stages, apart from enoyl-CoA hydratase, short chain 1 (ECHS1), a key enzyme in fatty acid metabolism, whose downregulation in RCC tissues discriminated stage I from ANK tissues (AUROC> 0.7). Although ECHS1 did not give any prognostic information, it was identified as a tumour suppressor, as its overexpression in RCC cultured cells inhibited proliferation and migration through inhibiting mTOR pathway activation [28].

Zhang et al. [29] used a targeted approach studying sodium/potassium-transporting ATPase subunit alpha-1 (ATP1A1) expression level in RCC tissue compared to ANK (80 pairs), by SILAC technique. They reported a 3.7-fold decrease of ATP1A1, also confirmed by differential immunoreactivity. In particular they showed that ATP1A1 downregulation correlates with RCC malignant grade and patients' poor survival. Furthermore, the exogenous expression of ATP1A1 inhibits RCC cell proliferation and cell migration possibly by increasing ROS production, and induces cell apoptosis. The data indicates ATP1A1 as a novel potential suppressor protein and a role for ATP1A1 inhibition in RCC progression both in vitro and in vivo [29].

Particular mention should be paid to MALDI imaging mass spectrometry, as it combines molecular and spatial information directly on tissue samples. The approach of histology-guided MALDI-MSI was applied by Stella et al. [30] to FFPE samples from RCC patients in order to highlight the proteomic alterations associated to the different RCC grades. They identified vimentin and three histones that were able to discriminate among RCC grades. Although Vimentin is a protein that can be detected after all kind of kidney injuries, and it cannot be recommended as specific biomarker, differences in the levels of some of its fragments could also be due to the presence of advanced tumour grade-specific exoproteases. Furthermore, the authors found a good correlation between the molecular profiles generated for each grade and the different cancer-specific survival rate at 10 years post-surgery. Such findings could represent a valuable starting point for further clarification of the molecular events that occur during RCC development [30].

3.2 CULTURED CELLS

It is quite common, as mentioned above, following a comparison between tumour tissue and ANK, to transfer the information obtained to in vitro models, such as cell cultures. In fact, the use of cultured cells allows the application of different approaches, potentially very useful for the deepening of prognostic significance of single molecules or pathways. These strategies consist in the comparison of cell proteomic profiles, and consequent behaviour modifications, with and without drug treatments, or silencing/interference of any key element. In particular, variable native or acquired resistance both to traditional chemotherapy and

to new targeted drugs is particularly relevant for RCC prognostic definition, since unpredictable response to treatment represents an open challenge. Therefore, a valuable tool to study the determinants of RCC progression may be to investigate the drivers of its characteristic drug resistance [31]. All these approaches have been adopted in RCC research (Table 1).

In particular, Giuliano et al. [31] performed transcriptomic and proteomic analyses on sunitinib sensitive and resistant RCC cultured cells with the aim to identify specific molecular signatures of acquired resistance to sunitinib, highlighting the role of CXCL5-mediated lysosomotropic drug resistance. In a prospective clinical trial, CXCL5 was demonstrated to be a marker of advanced disease and relapse [31]. The role of CXCL5 was supported by the results obtained using an in vivo mouse model and a human clinical sample survey [32]. They also confirmed the role of Androgen receptor (AR) signalling in promoting RCC progression by altering the pathway of HIF-2α/VEGF and AKT /NF-κB/CXCL5, which reflects the enhancement and the recruitment of endothelial cells. This finding may drive the development of new therapies to slow RCC progression [32].

Sunitinib resistance was also investigated through Tandem Mass Tag labelling of peptides by studying sunitinib-conditioned, resistant and wild-type Caki-1 cell lines [33]. The results confirmed the role of Y-box binding protein 1 (YB-1) and ATP-binding cassette sub-family B member 1 (ABCB-1) in acquired sunitinib-resistance development, proposing a potential new target to overcome this effect.

Another important process likely involved in RCC progression is metastasis-associated epithelial-to-mesenchymal transition (EMT). This process allows the outgrowth of metastatic cells from the primary tumour mass and increases therapy resistance, while the reverse mesenchymal-to-epithelial transition (MET) leads to colonization of new tissues by metastatic cells [34]. Some papers agree in awarding an important role to histone deacetylases (HDAC) in these processes, even if it is not yet clear whether HDAC promote or suppress EMT. However, recent evidence shows that class I HDACs are frequently overexpressed in RCC [35]. Kiweler et al. [36] evaluated the expression levels of over 5,000 proteins by proteomics of whole cell lysates of primary human RCC cells before and after treatment with HDAC inhibitors (HDACi). Their results show that HDACi do not trigger EMT, but induce a more complex dysregulation of EMT- and cell adhesion-associated proteins, involving the expression and function of E-cadherin and β-catenin, markers of epithelial and mesenchymal state, respectively. These effects were then mimicked by genetic targeting of HDAC1 and HDAC2 with RNA interference. Authors conclude that HDACs contribute to EMT, which leads to outgrowth of metastatic cells from the primary tumour mass and increases therapy resistance [36]. The same group recently confirmed HDACs involvement in metastatic events both in RCC and in other cancer types by proteomics, quantitative PCR, immunoblot, single cell DNA damage assays, and flow cytometry. The results showed that HDACi not only suppress the EMT but also compromise DNA repair processes of cancer cells [37]. The involvement of EMT was confirmed in another recent paper [38], where they observed that the knockdown of AT-rich interactive domain 1A (ARID1A), a novel tumour suppressor gene, by siRNA both in non-malignant MDCK and malignant 786-O RCC cells, triggers EMT. The down-regulation of ARID1A protein expression was confirmed also in human RCC tissues.

Some studies have addressed the role of apoptosis resistance as a driver of RCC progression [39,40]. The X-linked inhibitor of apoptosis protein (XIAP) is a potent inhibitor of the caspase pathway, thereby promoting cell survival during tumour progression. Two consecutive studies employed iTRAQ-MS approach comparing Caki-1 cell lines with high or low XIAP expression established using RNA interference technology and stably transfected XIAP-knockdown Caki-1 cells, in order to investigate the regulatory mechanism of XIAP in RCC. The identified differentially expressed proteins were involved in numerous biological processes connected to apoptosis. Given these functions, XIAP may play a key role in determining the resistance to apoptosis in RCC in response to chemo- and radio-therapy agents. This suggests that the overexpression of XIAP in RCC may serve as a molecular prognostic marker in RCC and improve the staging of RCC [41,42].

3.3 LIQUID BIOPSY

Liquid biopsy is a non-invasive alternative to tissue surgical biopsy. Indeed, it represents a surrogate of the tissue from which it originates, reflecting its correspondent physiological and pathophysiological status. One could define a liquid biopsy as any element that provides an easily accessible window of the parental cell or tissue, such as circulating tumour cells (CTC), cell-free DNA and RNA, extracellular vesicles and their cargo, proteins, peptides and metabolites, all isolated from various body fluids (e.g. blood, urine, saliva, faeces, ascites, pleural effusion, cerebral spinal fluid) [43-45]. Moreover, liquid biopsy can be readily obtained at different disease stages, allowing longitudinal studies for the active surveillance and/or for the monitoring of the response to drug treatment. Nowadays, a solid foundation has been laid for the development in the field of the routine analysis of 'liquid biopsy' for some common cancers, but not yet for RCC.

Liquid biopsy based on circulating tumour cells (CTCs) and circulating tumour DNA (CtDNA) has been very recently reviewed by [46]. Authors conclude that although the specificity of CTC/CtDNA is usually very high, the concordance with tumour-based biopsy is generally low. According to authors, this depends mainly on RCC heterogeneity, as mentioned, besides few technical issues in genotyping. Another paper also reported a low concordance (8.6%) of genomic alterations assessed by next-generation sequencing (NGS) between tumour tissue DNA and CtDNA in metastatic RCC, suggesting that ctDNA NGS may be more reflective of dynamic tumour genomic heterogeneity [47]. It is intriguing to observe that the concentration of ctDNA increases with stage and decreases when RCC is responding to therapy suggesting one could use simply CtDNA level as biomarker to monitor therapy response. Presence of detectable ctDNA has been proposed as a fourth parameter in a modified staging system. However, even if ctDNA is more stable than cells and easier to isolate, information at single-cell level, functional assays along with proteomics, transcriptomics and metabolomics studies can be performed only in CTCs [47]. However, to this day no one has yet started proteomic analysis of CTCs.

Blood serum and plasma proteomics remains challenging because of their complexity and the presence of highly abundant protein species. A promising approach is represented by the DIA-based MS workflow, able

to detect numerous peptides and proteins with better reproducibility starting from a low sample amount [49]. This approach showed the high potential of serum peptidome study in RCC: the comparison of 31 RCC patients and 31 healthy subjects led to discover 833 differential peptides, most of which are related to extracellular matrix degradation [50]. However, alterations in serum peptidome can also derive by non-specific action of proteases during (and after) clotting, whose risk increases with higher cancer stage [51]. Although these papers did not address the study from a prognostic point of view, it does provide solid basis for future development in this respect.

For RCC we also have the possibility to search prognostic markers in a biological fluid, such as urine, easily accessible and available over time, allowing to verify the reproducibility of the proposed signature. In fact, although other tumours can release detectable markers in urine and/or determine some kind of modification of urine composition, a prompt variation of any urinary parameter, potentially corresponding to that encountered in the tissue, is expected in the presence of RCC. Moreover, urines are proposed for biomarker research as alternatives to plasma not only because of the proximity of urine to the site of cancers but also because the wide dynamic range of protein concentrations in plasma makes it a particularly challenging matrix (Table 1) [52].

Di Meo et al. approached this issue, investigating the urinary peptidome and proteome of small renal masses in two sequential papers [52,53]. They employed a quantitative label-free liquid LC-MS/MS method and targeted parallel-reaction monitoring in urine samples of 56 RCC cases, about half progressive and half non-progressive, and 26 healthy controls. They identified a two-protein signature able to distinguish progressive from non-progressive RCC. These results open the possibility to use urinary protein profile obtained before surgery to classify patients for different RCC progression and potentially help choosing appropriate follow up strategies.

An original approach was recently applied in the field of liquid biopsy by comparing proteome profile of serum and urine of the same RCC patients [54]. Authors investigated how blood and urine "proteomically" reflect the changes occurring during RCC infiltration into renal vein, which is a well-known clinical hallmark of progressive RCC. A panel of 26 urinary proteins was found directly correlated with infiltration: their increased levels paralleled the extension of RCC into renal vein. From a functional point of view, these proteins are involved mainly in immune-system and defense processes. Moreover, this work highlighted the complementarity of blood and urine and the relevance of an integrative approach in the study of a dynamic system such as RCC.

We hope that liquid biopsy will provide a novel inventory of disease biomarkers, acting as a potential complementary method to the tissue analysis in diagnosis, prognosis, treatment response and resistance prediction in the era of personalized medicine.

3.4 MICROENVIRONMENT AND EXTRACELLULAR VESCICLES

As a final element to be taken into consideration for the assessment of prognostic significance, but also related to liquid biopsy, we would like to draw attention on tumour microenvironment (TME). In fact TME

represents the target of some innovative therapeutic approaches for RCC, aimed at inhibiting angiogenesis, stimulating a more effective response by the immune system, and inhibiting immune checkpoints. However, TME is a dynamic and heterogeneous system, able to adapt to therapies by establishing resistance processes. An in-depth exploration of the cellular components of RCC TME, such as fibroblasts, endothelial cells and immune cells and of their contribution to disease progression is out of the scope of the present review, therefore reference can be made to a recently published report [55]. However, in this contest, it is useful to consider also extracellular vesicles as component of TME: in fact, these nanometer-sized vesicles released by most cell types play a recognized role in cell to cell communication. The term "extracellular vesicles" (EV), as stated by the recently updated guidelines of the International Society for Extracellular Vesicles (ISEV), stands for "particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate" [56]. EV are a heterogeneous family of vesicles, differing in their biogenesis, size and molecular composition. Among them, the most studied are exosomes and microvesicles. The secretion of exosomes by different cells is reported to contribute in the establishment of a favourable microenvironment to promote tumour progression and metastatization [57]. As such, they can be considered both as part of the TME and as components of liquid biopsy, once they exit the tumour area and reach biological fluids. Giving these premises, EVs released by tumour cells could be promising sources of prognostic factors [58]. An example is the finding that exosomes expressing CA9 in hypoxic RCC cell lines may promote angiogenesis in TME, leading to cancer progression [59]. It confirms our previous observation about CA9 over-expression in urinary exosomes of RCC patients, by proteomic profiling [60]. However, it has to be said that the majority of exosomal biomarker studies in RCC has so far focused on miRNAs. This matter was deeply investigated in a recent review focused on the contribution of EV released by RCC cells and stem cells in RCC progression [58]. Thus, tumour-derived EV act on the microenvironment favouring tumour aggressiveness, may contribute to angiogenesis through both direct and indirect mechanisms and are involved in tumour immune escape [58].

4. OTHER -OMICS PROGNOSTIC SIGNIFICANCE IN RCC

Proteomic-based approaches allow the analyses of proteins not only at the translational levels, but also at complex post-translational levels, particularly they allow identification of protein modifications like phosphorylation and glycosylation, which are not detected by gene analysis. Moreover, the -omics approaches have shed light on tissue-specificity metabolome remodelling. Only a few studies have tackled the issue of other -omics applications in the search for prognostic biomarkers for RCC. However, these papers suggest clues for future research.

Regarding phosphoproteomics, the already cited investigation performed on six different cancer types highlighted the peculiar phosphorylation pattern of RCC, compared to the other malignancies [23]. A few other papers analysed specific pathways through different approaches. A proteomics SELDI-based analysis of 93 urinary samples of patients affected by RCC, compared with healthy subjects and other urological conditions, provided signatures specific for each condition. In particular, urinary excretion of Raf Kinase

Inhibitor Protein - a key regulator of cell signalling - and its phosphorylated form was shown to be predictive for cancer-specific survival and progression-free survival in RCC [61]. van der Mijn et al [62] evaluated global tyrosine phosphorylation by LC-MS/MS after immunoprecipitation with an antiphosphotyrosine antibody in RCC 786-O cells treated or not with sunitinib. 180 phospho-peptides resulted differentially regulated, among which AXL, a cell surface receptor tyrosine kinase, exhibited a significant upregulation. Its inhibition was shown to improve the antitumor activity of sunitinib [62]. A quantitative phosphoproteomic approach was applied to identify pyruvate kinase M2 (PKM2) substrates in RCC cells: the results underlined the importance of two phosphorylation sites on mTORC1 inhibitor AKT1 substrate 1, whose activation leads to accelerated oncogenic growth and autophagy inhibition in cancer cells. The deepening of PKM2 phosphorylome revealed a constitutive mTORC1 activating mechanism in RCC cells [63]. In a comparative study on RCC tissue and ANK, the authors investigated the role of Golgi phosphoprotein 3 (GOLPH3), a proto-oncogene product. Its expression was found to promote the proliferative and invasive capacity of RCC cells via phosphorylation of FAK/Raf1/MEK and further activation of Wnt/β-catenin signaling pathways, using a high-throughput phospho-proteome array verified by immunoblotting [64].

Glycosylation is one of the most important post-translational modifications and N-glycosylation patterns are reported to be involved in the progression and spreading of tumours. However, only recently this issue was investigated for RCC by Santorelli et al. [65]. They proposed an innovative strategy based on glycoproteomic study of urine of RCC patients at early and advanced stages, and healthy subjects. In this pilot study, they observed increased glycosylation events at non-consensus sequence sites indicating alterations of glycosylation processes and showing the presence of a specific tumour stage glycosignature. Moreover, thanks to the enrichment of glycopeptides, it was possible to detect proteins that had never been identified before in urine, such as phospholipid transfer protein and complement factor H.

In the field of metabolomics, the last ten years have brought some advancement. Mainly two techniques - mass spectrometry and nuclear magnetic resonance spectroscopy- have been employed. Although these were not focused on prognosis, two recent reviews analyse literature regarding metabolomics, centred on diagnostic markers identification and pathophysiological mechanism, respectively in [66] and in [67]. Metabolomics-based research in RCC is reported as still in its infancy stage [68].

Nevertheless, an attractive approach was recently adopted in a metabolomic study on preoperative fasting urine and serum samples from patients with clinical renal masses. Even if results are as expected, since differential metabolites are mainly involved in glycolytic and tricarboxylic acid pathways, they allowed to distinguish benign from cancerous cases and different stages of RCC. Moreover, the results showed that urine samples appear to be better predictors of RCC stages than serum samples [69]. In another metabolomic investigation, mainly aimed at finding specific lipidomic signature of different type of RCC (clear cell, papillary, chromophobe…), a part was devoted to analyse RCC metastasis. Interestingly, authors showed that metastasis display similar metabolite levels as primary tumours, irrespective of the organ where the metastasis was harboured [70]. Another recent pilot study performed urine metabolomics untargeted

metabolomic analysis of RCC. Interestingly, the analysis of RCC urine samples one year post-nephrectomy reveals isobutyryl-l-carnitine and l-proline betaine as potential prognostic markers [68].

Finally, an exome-wide approach (WES) was applied for the first time to matched tumour and normal sample pairs from RCC patients, to assess the association between somatic mutation burden in metastatic RCC primary tumours, and patient survival [71]. All patients were at stage IV and similarly treated. Results provide evidence for two candidate genes associated with RCC prognosis.

5. MULTI-OMICS

The multi-omic approach is in principle much more likely to achieve real progress in the management of solid cancers and RCC in particular. An analysis of past, but also recent literature clearly shows that the use of single -omic strategies, such as investigating the genome for cancer-specific mutations and identifying cancer-associated alteration in epigenetic-processes or by exploring the differential expression of mRNA and protein through transcriptomics and proteomics techniques, respectively, is failing to provide effective biomarkers (Figure 1). In particular, since these approaches lack the resolving-power to link molecular signatures to the phenotypic manifestation, it is unlike that any single -omics tool will obtain enough prognostic significance to be used in routine clinical application [72].

On the contrary, the multidimensional -omics approaches have the potential to dissect both the intricate molecular mechanisms underlying different phenotypic manifestations of cancer progression, such as metastasis and angiogenesis, and the drivers of RCC typical drug resistance, in order to discover molecular candidates with prognostic value.

The integration of -omics methodologies requires a very strong advancement both in bioinformatics and in technical procedures [73]. Moreover, it obviously requires a tight multidisciplinary network among specialist with very different competences. Finally, we cannot disregard the improvement brought on by the application of artificial intelligence and machine learning to this field. Such an example is the "Group lass regularized Deep learning for cancer Prognosis", a computational tool for survival prediction using both clinical and multi-omics data from different cancers, including RCC [74]. A recent system biology approach exploits advances in computational technology and methods to integrate diverse sets of data in the generic area of kidney diseases. It has the potential to unravel the interplay of multiple genes, proteins, and molecular mechanisms that drive key functions in kidney health and disease. The development of large, comprehensive, multilevel biologic and clinical data from national and international databases, cohort studies, and trials now provides the framework needed for significant application of systems biology approaches in nephrology [75].

Regarding the technical aspect, it has to be considered that the -omics separation methods are often mutually exclusive and the destructive nature of the procedures may be an obstacle when sample is limited. In a recent paper, the Banks' group [76] describes a simple and effective detergent-free method that facilitates direct measurement of proteome and metabolome in the same sample extract. This "single-pot" multi-omics processing was applied in a proof-of-principle integrated study of RCC tissue biopsies, resulting well suited for this kind of samples, characterized by limited amounts and high heterogeneity.

An overview of papers that show the potential of the integration of -omics techniques is provided below.

By integrating genomic and quantitative proteomic analysis, Li et al. [77] elucidated the mechanisms through which deficiency of SETD2, often lost in RCC, contributes to tumour development. Multiple SETD2-regulated cellular pathways were identified that suppress cancer development [77].

Combined transcriptomic and metabolomic analysis were performed on 35 RCC tumours and matched controls [78]. Metabolite profiling by GC-MS and transcriptomic analysis by qPCR-arrays pointed out 93 metabolic genes, involved in metabolism of succinate, beta-alanine, purines, glucose and myo-inositol. As the cohort of 468 RCC patients was monitored for more than three years, the authors were able to correlate the alteration of those pathways with poor survival of RCC patients.

A similar approach was used in another paper [79], where a large-scale metabolomic profile integrated with transcriptomic data was performed on RCC tissue specimens and also on primary tumour cell cultures. In this investigation as well, although based on innovative and high throughput procedures, an increased glucose uptake and an imbalance of the glycolysis reactions were confirmed, according to the well-known Warburg effect. Moreover, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2 (NDUFA4L2) resulted as the most highly expressed gene in RCC cells and functional analysis in cultured cells highlighted its role in sustaining angiogenesis, chemoresistance, and mitochondrial dysfunction [79].

Koch et al. [80] underlined the potential of a "cross-omics" pathway analysis approach, based on proteomics and mRNA sequencing data obtained from RCC and ANK FFPE tissues of 11 patients, and on published miRNA sequencing data from an overlapping patient cohort. This combinatorial strategy allowed the individuation of RCC specific pathways: in particular, it pointed out an alteration of the antigen presentation pathway, identifying CD74 as a candidate targets for the treatment of RCC.

In the large-scale comprehensive proteogenomic study performed by the Clinical Proteomics Tumor Analysis Consortium (CPTAC), 110 treatment-naïve RCC patient tissue and whole blood were analysed in order to show the effects of genomic and epigenomic events on the transcriptome, proteome, and phosphoproteome at the functional level [81]. Moreover, it once again confirmed the great heterogeneity of RCC tumours and assigned immune signatures able to stratify RCC patients, aiming to develop better personalized therapies.

Human RCC xenograft models of sunitinib-treated resistant tumours were analysed by an integrative systems biology approach based on proteomics and transcriptomics, which identified cathepsin B, a cysteine proteinase of the papain family, as a key protein involved in the resistance to tyrosine kinase inhibitors [82]. Furthermore, its overexpression was associated with low survival rate by a survival analysis across The Cancer Genome Atlas (TCGA) pan-cancer dataset.

The strategy to combine and integrate several different "big data" repositories, and then applying specific platform for the search of their correlation and statistical significance was adopted in several studies. We here report a few observations about the papers that have been published in this area. TCGA expression data were evaluated by multi-omic integration in two papers published shortly after each other [83,84]. Zhao et al. [83] combined interaction network, expression, somatic mutation, copy number variant and DNA

methylation data and found five core clusters related to the activation of the immune and inflammation systems involved in RCC progression. Moreover, they created a risk-score formula associating the five core clusters with survival rate. Hu et al. [84] focused on DNA methylation and gene expression data analysis, revealing 863 methylated differentially expressed genes. Seven of them were integrated into a prognostic risk score model, allowing to stratify RCC patients into high- and low-risk groups and to predict overall survival.

Starting from *in silico* microarray bioinformatics and complex correlation and functional analysis, Fan et al. [85] identified the nuclear antigen-associated factor KIAA0101 as the driver of the side effects of Erythropoietin (EPO) in anaemic RCC patients. Comparative proteomics also confirmed the up-regulation of KIAA0101 in response to EPO stimulus. Moreover, consultation of Oncomine database (https://www.oncomine.org) showed KIAA0101 and EPO negative correlation with 5-year survival in patients.

A recent paper [86] proposed an interesting modelling for integrating H&E-stained histopathology images and proteomics data through machine learning, including deep neural networks, based on the CPTAC from RCC patient datasets. An imaging-based classification model generated a set of predictions pointing on proteins significantly implicated in immune responses, extracellular matrix reorganization, and metabolism; such findings offer new research opportunities in this and other cancer types [86].

EXPERT COMMENTARY

I reread the expert commentary of the paper [87] we wrote 4 years ago on a similar subject and I regret to confirm nearly the same considerations that we report in that paper.

The main issue that surgeons pose to basic scientist is to have a way to predict whether the tumour that they have treated, usually by RCC resection, will relapse or metastasize. To this day, this request remains unmet. Even the most accurate post-surgery pathological evaluation can fail to indicate a correct prognosis, since early stage RCC show metastatic progression or recurrence in 20-40% of cases.

Moreover, when RCC is metastatic, innovative treatment options are now available, relying on up to 12 drugs with 3 newly approved ones [88], i.e. immune checkpoint-blockade PD-1 antibody Nivolumab, VEGF/cMET inhibitor Cabozantinib, and VEGF/FGF inhibitor Lenvatinib. For this reason, the support of basic research is needed to help clinicians to assign the patient to the best, personalized and state-of-art therapy.

As a first concluding observation, the issue of correctly stratifying patients conflicts with the extreme heterogeneity of RCC, which have confirmed by very recent and deep investigations [89]. Although cancer heterogeneity is often regarded as a practical obstacle in the search for biomarkers for many tumours, it seems particularly challenging for RCC.

In fact, in two above cited publications [23, 81] it has been underlined that RCC behaves differently from other carcinomas. Moreover, the observation that several cellular pathways displayed opposite regulation at the transcriptomic and proteomic levels is actually puzzling, and looks specific of RCC [81].

Moreover, it is disappointing that advanced investigations, relying on both integrated multilevel approaches and applying sophisticated computerized/AI-based data elaboration, mostly end up identifying genes, transcripts, proteins or metabolites that are linked to a the well-known phenomenon, first discovered in early thirties, called the "Warburg effect". It consists essentially in activation of glycolytic pathway and/or down regulation of mitochondrial oxidative phosphorylation [26, 27, 67, 78, 90]. This is surely a constant feature of RCC, but it likely will not bring any substantial advancement.

The two above summarized observation, which we have intentionally kept as a thread throughout the entire discussion, may found a common explanation. The heterogeneity of RCC could derive from the fact that this cancer not only is formed by different neoplastic cells, likely deriving from clones at different phases of evolution, but is also influenced by the heterogeneity of TME, to which the tumour displays a tight cross-talk and strong dependence and whose composition is itself variable. Spatial and temporal heterogeneity may permit the tumour as a whole to adapt to a fluctuating tumour microenvironment [91].

Regarding the issue of neoplastic cells, in a recent study, authors developed different cell models as "mini-tumours in a dish", starting from RCC patient-derived surgical specimens, and showed that these models mirror in vitro inter- and intratumour heterogeneity of RCC [92]. Moreover, after serial passaging of cells in vitro, clonal dynamics were evident, and also affected drug responsiveness [93].

As far as TME is concerned, not only cells and extracellular matrix should be considered, but we suggest that a fundamental role could be played by extracellular vesicles as vectors of cell to cell communication.

Perhaps what is still missing is the technical chance to apply proteomics and more likely integrated multi-omics approaches to these small entities, whether they are single tumour-derived cells, such as CTCs, or clones in culture or extracellular vesicles.

We expect that new initiatives that integrate proteomics into multi-omics studies will open the way for rapid translation of laboratory discoveries to bedside and ultimately have an impact in improving clinical management and outcomes of RCC patients in the next few years, by providing novel molecular prognostic biomarkers.

Table 1. Proteomic studies with prognostic significance applied to RCC tissues, cultured cells and biofluids

Authors	Cohort/Samples	Method	Involved pathway	Proteins of interest	Prognostic value
Tissues					
Song et al. 2017 [26]	14 pairs RCC and ANK frozen tissue	DIA.based LC-MS	Oxidative phosphorylation	↑ LDHA, ANXA4, NNMT, PLIN2	na
Sun et al. 2016 [27]	4 pairs RCC and ANK frozen tissue	nano-LC-MS/MS	Oxidative phosphorylation	↑ RPN1, DARS, RPL27A; ↓ CYP4F2, GSTM3	↓ OS
Wang et al. 2020 [28]	18 pairs RCC and ANK frozen tissue	iTRAQ-UPLC-MS/MS	Fatty acid metabolism	↓ ECHS1	Not significant
Zhang et al. 2019 [29]	80 pairs RCC and ANK frozen tissue	SILAC-LC-MS/MS	ROS production	↓ ATP1A1	↓ OS
Stella et al. 2019 [30]	13 FFPE RCC tissues	MALDI imaging MS combined with nLC-ESI-MS/MS	Gene accessibility, EMT	↑ H2A, H3, H4, VIME	↑ Grade
Cultured cells					
Giuliano et al. 2019 [31]	sunitinib sensitive and resistant RCC cells	Transcriptomics and immunoblotting	ROS production, cytokine-dependent signalling	↑ CXCL5 in resistant RCC cells	↑ sunitinib restistance ↓ OS
D'Costa et al 2020 [33]	sunitinib-conditioned resistant and wild-type Caki-1 cell lines	TMT- LC-MS/MS/MS	mTOR pathways	↑ YB-1, ABCB-1	↑ sunitinib restistance
Kiweler et al. 2018 [37]	primary human RCC cell treated with HDACi	Gel-based LC-MS/MS	EMT, DNA repair processes	↓ CDH ↓ ITGB1 ↓ ACK1	HDACi potential drug
Liu et al. 2019 [41] Chen et al. 2018 [42]	XIAP silenced Caki-1 cell line XIAP-knockdown Caki-1 cell line	iTRAQ-LC-MS/MS	apoptosis	XIAP	resistance to apoptosis induced by chemo- and radio-therapy
Biological fluids					
Lin et al. 2018 [49] Lin et al. 2020 [50]	Serum from 31 RCC and 31 healthy subjects	DIA.based LC-MS	EMC degradation, adhesion/junction associated pathways and actin-cytoskeleton regulation, complement and coagulation	↑ SERPINA5 ↓ H3, TAGLN2, S100A9 ↑ Number of peptides	na
Di Meo et al. 2020 [52] Di Meo et al. 2019 [53]	Urine from 56 RCC and 26 healthy subjects	LC-MS/MS and PRM	na	↑ Two protein signature: EPS8L2 and CCT6A	↓ OS
Chinello et al. 2019 [54]	Urine and plasma from 9 RCC patients	label-free LC MS/MS	immune-system process and defense	↑ HPT, TTHY, ITIH2, KAIN, TETN, PGRP2, FINC, APOE, IGJ, IGHA1,	correlate with the extension of RCC into renal vein

	CO9, IGHG4, HV320, IGHM, K2C1, LV302, A2GL, A1AG1, CRP, IGHG3, IGHG2, AACT, APOA, CERU, CFAB, CFAI

Legend: A1AG1, alpha-1-acid glycoprotein 1; A2GL, leucine-rich alpha -2-glycoprotein; AACT, alpha-1-antichymotrypsin; ABCB-1, ATP-binding cassette sub-family B member 1; ACK1, Activated CDC42 kinase 1; ANXA4, annexin A4; APOA, apolipoprotein(a); APOE, apolipoprotein E; ATP1A1, sodium/potassium-transporting ATPase subunit alpha-1; CCT6A, T-complex protein 1 subunit zeta; CDH, E-cadherin; CERU, ceruloplasmin; CFAB, complement factor B; CFAI, complement factor I; CO9, complement component C9; CRP, C-reactive protein; CXCL5, C-X-C motif chemokine 5; CYP4F2, Cytochrome P450 4F2; DARS, Aspartate-tRNA ligase; ECHS1, enoyl-CoA hydratase, short chain 1; EPS8L2, Epidermal growth factor receptor kinase substrate 8-like protein 2; FINC, fibronectin; GSTM3, Glutathione S-transferase Mu 3; H2A, histone 2A; H3, histone 3; H4, histone 4; HDACi, histone deacetylases inhibitors; HPT, Haptoglobin; HV320, immunoglobulin heavy chain V-III region GAL; IGHA1, immunoglobulin alpha-1 chain C region; IGHG2, immunoglobulin gamma-2 chain C region; IGHG3, immunoglobulin gamma-3 chain C region; IGHG4, immunoglobulin gamma-4 chain C region; IGHM, immunoglobulin mu chain C region; IGJ, immunoglobulin J chain; ITGB1, integrin-beta 1; ITIH2, Inter-alpha-trypsin inhibitor heavy chain H2; K2C1, keratin type II cytoskeletal 1; KAIN, kallistatin; LDHA, L-lactate dehydrogenase A chain; LV302, immunoglobulin lambda chain V-III region LOI; na, not available; NNMT, nicotinamide N-methyltransferase; OS, overall survival; PGRP2, N-acetylmuramoyl-L-alanine amidase; PLIN2, perilipin-2; PRM, parallel-reaction monitoring; RKIP, raf kinase inhibitor protein; RPL27A, 60S ribosomal protein L27a; RPN1, Ribophorin 1; S100A9, Protein S100-A9, SERPINA5, plasma serine protease inhibitor; TAGLN2, Transgelin-2; TETN, tetranectin; TMT, Tandem Mass Tag labelling of peptides; TTHY, Transthyretin; VIME, vimentin; XIAP, X-linked inhibitor of apoptosis protein; YB-1, Y-box binding protein 1.
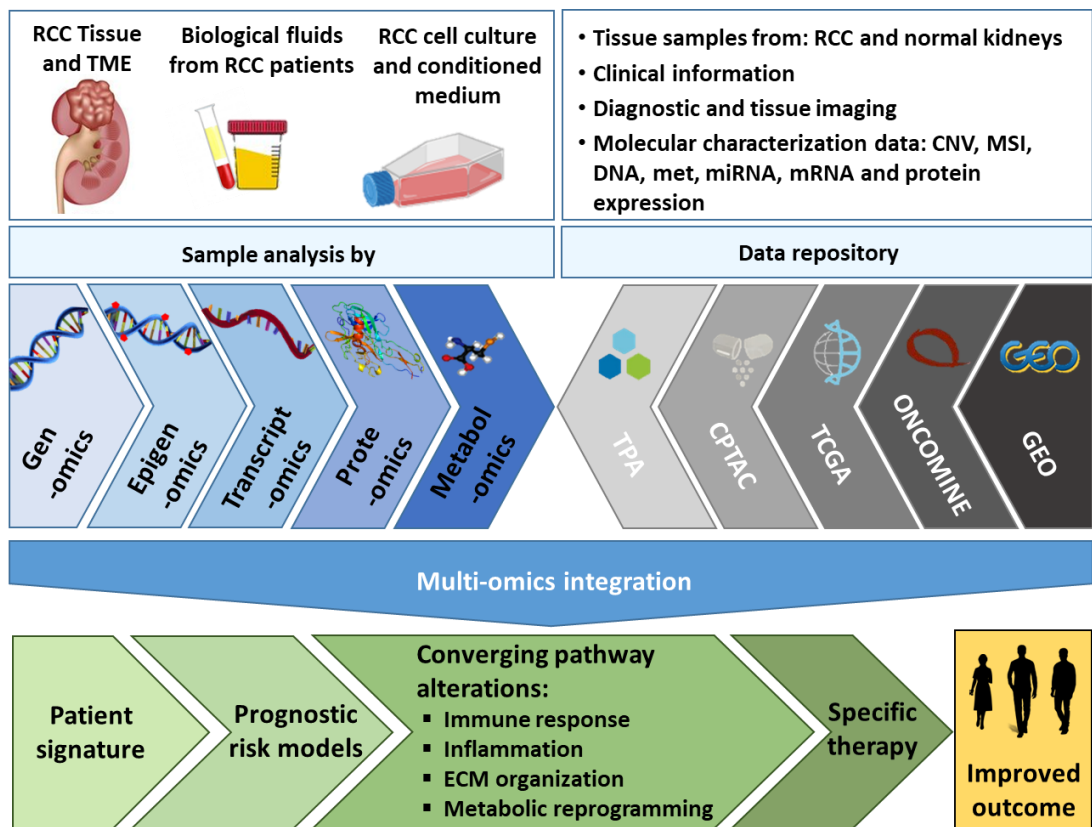
Figure 1. One of the fundamental aims of proteomic and multi-omic applications to RCC is to improve prognosis definition. It can be reached by exploiting single markers or panels of proteins, genes or metabolites to generate prognostic risk models. These models could then be used to stratify patients according to their cancer progression risk and to assign them to the best state-of-art personalised therapeutic regimen in the hope of improving their outcome. To reach this goal, extensive integration among results of single studies, either relying of samples of RCC tissues, plasma, serum, urine or other biological sources, as well as international open source data repositories is needed.

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