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Linkage of microRNA and Proteome-Based Profiling Data Sets: A Perspective for the Priorization of Candidate Biomarkers in Renal Cell Carcinoma?

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Abstract

Despite recent advances in the understanding of the biology of renal cell carcinoma (RCC) and the implementation of novel targeted therapies, the overall 5 years' survival rate for RCC patients remains disappointing. Late presentation, tumor heterogeneity and in particular the lack of molecular biomarkers for early detection and classification represent major obstacles. Global, untargeted comparative analysis of RCC vs tumor adjacent renal epithelium (NN) samples by high throughput analyses both at the transcriptome and proteome level have identified signatures, which might further clarify the molecular differences of RCC subtypes and might allow the identification of suitable therapeutic targets and diagnostic/prognostic biomarkers, but none thereof has yet been implemented in routine clinical use. The increasing knowledge regarding the functional role of noncoding microRNA (miR) in physiological, developmental, and pathophysiological processes by shaping the protein expression profile might provide an important link to improve the definition of disease-relevant regulatory networks. Taking into account that miR profiling of RCC and NN provides robust signatures discriminating between malignant and normal tissues, the concept of evaluating and scoring miR/protein pairs might represent a strategy for the selection and prioritization of potential biomarkers and their translation into practical use.

Keywords

RCC; miR; proteomics; biomarker

Introduction

Over the last 20 years, the incidence of renal cell carcinoma (RCC) has increased in the Western world, thereby currently accounting for approximately 3% of cancers and 2% of all cancer-related deaths.[1] RCC represents a highly aggressive tumor entity. At the time of diagnosis approximately 30% of the patients have developed metastases.[2] Clear cell RCC (ccRCC) accounting for almost 70% of all kidney cancers is the most common histological subtype of this disease and is characterized by the loss of the von Hippel Lindau (VHL) tumor suppressor gene expression either due to deletions, mutations, loss of heterozygosity

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and/or hypermethylation.[3] In addition, ccRCC exhibits a wide range of natural histories and varying response rates to targeted therapies.[4] In particular, early stage tumors have a significantly better disease-free survival after resection than tumors of higher stage and grade.[5–7] In addition, to structural abnormalities or epigenetic silencing of VHL other molecular factors directly associated with the initiation and progression of this disease are largely unknown. Therefore, the molecular basis for the diversity regarding histological grade, clinical behavior and response to targeted therapy has to be better characterized to gain further insights into the heterogeneity of RCC. The identification of deregulated molecules and their interactions is required to elucidate the pathophysiology of RCC and to improve the development of new treatment strategies.

Despite major efforts within the past decade to establish and implement genomic and proteomic profiling strategies into clinical routine so far only the MammaPrint has been approved by the Food and Drug Administration (FDA, FDA Clears Breast Cancer Specific Molecular Prognostic Test, 2007).[8] Although (c)DNA microarrays represent the most advanced and robust high throughput technology, the assessment of expression profilings still face a number of challenges such as laboratory to laboratory and platform to platform reproducibility.[9] Moreover, most expression patterns were established with rather small sizes of patients' cohorts and/or numbers of samples analyzed. Nevertheless, gene expression profilings have been performed with samples from RCC patient-matched specimens and cell lines using different cDNA microarray platforms, which resulted in the development of preliminary expression signatures and array-comparative genomic hybridization patterns, but only in rare cases the potential targets were validated.[10,11] Thus, in the clinical setting tumor stage and grade are hitherto still used as predictors for the outcome of RCC patients.

Since mRNA expression patterns can not account for frequently occurring posttranslational modifications at the protein level, which are often associated with key regulatory or functional consequences profilings restricted to the transcriptomic level allow only limited prediction of the disease. Therefore, side by side evaluations of the genomic/transcriptomic and proteomic status are critical to enhance the knowledge of disease pathogenesis and the underlying molecular mechanisms of the differential mRNA and protein expression levels. An important link within the regulatory network between the available genomic information and its outcome at the proteomic level is represented by a class of small noncoding RNAs, termed miRs. miRs have been shown to play a fundamental role in different physiological and pathophysiologcal processes including cell proliferation, cell motility, differentiation, apoptosis and carcinogenesis. At the functional level they regulate from few to up to thousands of genes in regard to their individual mRNA transcript and/or protein expression levels. With the development of miR arrays, systematic expression profilings could be performed, which may contribute (i) to define the effects of miRs on the regulation of mRNA transcripts and proteins under defined conditions and (ii) to elucidate their biological role by gaining further insights into relevant regulatory networks.[12]

Important Features of miRs

miRs are a class of small noncoding RNAs of approximately 22 nucleotides in length frequently conserved across species. They bind to complementary sequences in the 3′ untranslated region (UTR) of their target genes and thus regulate mRNA transcription levels by cleavage of the target mRNA or by repression of protein synthesis in a tissue-specific manner.[13,14] However, it is noteworthy that translation of some proteins can also be up-regulated by miRs under some circumstances.[15–17] This might be explained by oscillation of miRs between repression and activation in coordination with the cell cycle or by

transcription of miRs as a polycistron, which preferentially coregulates proteins in close proximity.[16]

It is estimated that approximately 30% of the human genes are regulated by miRs.[18] However, miRs not only serve as direct regulators of gene expression but are likely regulated themselves by multiple factors. Most miRs are located in genomic regions distant from the annotated target genes and also encoded in distinct transcription units.[19] Approximately 25% of miR were processed from introns, whereas others are clustered in the genome and seem to exhibit a functional relationship. Although more than 900 miRs that have been identified so far in mammals, their biological relevance and functional targets still remain largely unknown. However, there is evidence that miRs have been shown to be involved in modulating key cellular processes which might also be influenced by oncogenes as well as tumor suppressor genes.[20] In addition, miRs have also been investigated in several solid and hematologic malignancies revealing deregulations in many tumors, including renal cell carcinoma.[21–26] This functional correlation is further strengthened by the finding that miR encoding genes are frequently located at fragile sites or chromosomal regions undergoing point mutations, amplifications, deletions or translocations in tumors.[27]

Analytical Issues Linked to the Profiling of miRs

High Throughput Analysis for miR Identification

Despite the existence of several high throughput approaches to identify and/or quantify miRs in tissue samples there exists no gold standard. cDNA oligonucleotide micro- and polymerase chain reaction (PCR)-based arrays are currently applied as global scale techniques for miR profiling.[28] Recently, bead-based flow cytometric miR expression profiling has been developed as a new emerging technology, which uses xMAP beads with locked nucleic acid capture probes to detect target specific miRs.[29] Furthermore, cloning of so far known miRs allows the identification of the all expressed miRs. However, these technologies have limitations regarding their specificity and/or ability to detect novel so far unknown RNAs. With the establishment of the next generation small RNA (smRNA) deep sequencing[30,31] all smRNAs present in the samples analyzed including novel and under-expressed miRs, small nucleolar RNAs (snoRNAs), small cytoplasmic RNAs and small nuclear RNAs can be detected.[32–34] Profiling of miRs by miR arrays and deep sequencing is nowadays also applicable for formalin-fixed paraffin-embedded tissues,[35] allowing the retrospective analysis of large cohorts of clinical samples including material from clinical trial-based studies. This technology provides an extremely powerful tool in the identification of clinical relevant markers as well as therapeutic targets in cancer research.[36] Although different strategies have been applied to identify specific miR profiles and functions, the question, how many of these changes are critical for the function of an individual miR, still remains elusive.

miRNA Target Prediction

So far, computational algorithms have been the major driving force in predicting miR targets, which are primarily based on sequence complementarities between the 5′ end of most miRs and the 3′ UTR of target genes and free energy predictions. Due to the ubiquitous nature of miR/mRNA targeting, independent prediction databases using 11 different algorithms have been developed. The miRWalk database (http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/), is covering information based on eight established miR target prediction algorithms, such as e. g. RNA22,[37] miRanda,[38] miRDB,[39] TargetScan,[40] RNAhybrid,[41] PITA,[42] PICTAR[43] and Diana-microT.[44]

miRs can target from few to more than thousand mRNAs causing significant expression changes of the targeted transcripts and/or proteins.[40,45] However, *in silico* approaches might

not necessarily be reliable predictors for miR target interactions due to the high rates of false positive and false negative miR targets.

miR Experimental Target Validation

The lack of validation data for predicted miR target genes currently represents the bottleneck in the functional characterization of miR-dependent regulatory networks. However, this information is essential to gain further insights into the molecular mechanisms of miR-controlled pathogenic processes such as tumorigenesis, invasiveness of tumors and metastasis formation.[46] The regulatory role of miRs makes them per se strong candidate oncogenes and tumor suppressor genes.[47,48] Examples of both oncogenic miRs and those that suppress tumors have been reported.[49–51] In addition, mutations and single nucleotide polymorphisms (SNPs) within the seed sequence of miRs or alterations in their epigenetic control might affect miR processing leading to reduced miR expression.[52–55]

Expression and Function of miRs in RCC

There exists a growing body of literature focusing on the analysis and the role of miRs in RCC, in particular in clear cell RCC. Studies investigating global changes in the miR pattern of RCC lesions compared to normal kidney epithelium focusing on the effects of individual miR on the given RCC subtype.[21–23,25,54,56] Hierarchical cluster analysis of established miR expression profiles allowed the classification of matched tissue pairs into RCC lesions and nontumoral kidney parenchyma and in some cases even according to histological distinct RCC subtypes as well as tumor staging and grading.[21–23,25,35,57,58] Thus, there exist unique miR signatures for RCC lesions, each renal tumor subtype, autologous kidney parenchyma, primary and metastatic lesions.[25] Furthermore, epigenetic changes might also modulate the miR expression and function as demonstrated for the miR-9 family exhibiting an altered methylation status in primary RCC lesions.[54] Moreover, genetic polymorphisms of the miR processing machinery might contribute to the risk of RCC as demonstrated by Horikawa and co-workers (2008).[55] These data suggest that miRs are involved in the initiation as well as progression of RCC and might serve as diagnostic, prognostic and therapeutic tools. Until now 184 miRs have been identified to be differentially expressed between RCC and NN.[26,35,56–60] The numbers of tissue samples analyzed ranged from 3 to >72, but not for all of these studies pairs of RCC and normal corresponding kidney epithelium were available. A similar miR regulation pattern was also defined in various other tumor types, such as ovarian, breast, pancreas, lung and stomach cancer. In order to determine differentially expressed miRs the independently identified miRs were subjected to strict selection criteria such as the availability of at least 3 independent identifications along with a consistent regulation pattern leading to a subset of 12 down-regulated and 8 up-regulated miRs (Table 1). The group of down-regulated miRs in RCC lesions comprised the members miR-30a, –133b, –138, –141, –200a, –200b, –200c, –204, –429, –510, and –514 (Table 1), whereas the panel of up-regulated miRs is represented by miR 34a, –34b, 106a, 106b, –155, –185, and –224 (Table 1). Although predominantly classified as up-regulated (Table 1)[56] miR-21 is also included in a set of down-regulated miRs.

miR-141 (6 identifications) and miR-200c (7 identifications) were not only the most frequently down-regulated miRs when compared to normal renal epithelium but also mapped to the same locus. Moreover, the down-regulation of both miRs seems to be involved in the induction of epithelial-to-mesenchymal transition (EMT) by up-regulating the expression of zinc finger E box-binding homeobox 2 (ZFHXIB, ZEB2), a transcriptional repressor of CDH1/E-cadherin.[61] This is associated with an increased aggressiveness of the tumor, which is in line with the report of Park and coauthors demonstrating that the miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2.[62]

The most frequently up-regulated miRs in RCC were miR-155 and miR-224, which are mapped to distinct chromosomal loci. Some of the up-regulated miRs (miR-21, miR-34) have so far been associated with cancer-related processes such as proliferation, invasion or metastases formation,[63,64] others like miR-106b have been linked to disease progression, in particular with prediction of metastatic recurrence after nephrectomy and to poor patients survival.[60]

Matching patterns between chromosomal alterations in RCC lesions and miR deregulation was found for 84% of the selected miRs (refs 26, 35, 56–60 and Table 1). The panel of selected miRs is distributed over 11 chromosomes. Four of the differentially expressed miRs were located on chromosome 1 (miR-200a, - 200b, -429 and -34a) or X (miR-510, -514, -106b and -224), whereas miR-30a and -133b were mapped to chromosome 6 and the EMT-associated miR-141 and -200c to chromosome 12, respectively. The remaining miRs were mapped to chromosome 3 (miR-138), 7 (miR-106b), 9 (miR-204), 11 (miR-34b), 17 (miR-21), 21 (miR-155) and 22 (miR-185). Their regulation pattern was mostly in line with genetic alterations previously defined in RCC by comparative genomic hybridization[65,66] with the exception of chromosome 12, for which rather gains in a frequency of 20% for both 12p and 12q was described and of chromosome 21 for which no frequent genetic alterations yet exist in RCC. In terms of the miRs mapped to chromosome X a 10% frequency of Xp loss as well as Xp and/or Xq gain was reported.[65]

Concerning validation and functional studies, the distinct miR expression pattern detected in RCC vs NN was only confirmed in a limited number of samples analyzed by quantitative real-time PCR,[56,59,67] even less information is available in terms of the functional characterization of the differentially expressed miRs.

Proteomic Profiling of RCC

Protein profiling is a complementary approach to the mRNA-based analysis, which has several advantages despite its limited high throughput capacity.[68] Studies at the proteome level not only allow to bridge potential gaps in respect to correlations between mRNA and protein expression levels, but also allow the detection and subsequent identification of posttranslational modifications (PTM), such as e. g. glycosylation and phosphorylation in combination with mass spectrometric analyses. This is of great interest because in most cases PTMs have an enormous impact on the protein function and/or its biological activity.

Various proteome-based strategies have been employed for the proteomic profiling of RCC, mostly in the context of identification of candidate biomarkers in RCC,[69–86] which despite certain advantages and limitations complement for the most part each other.[68,87] Next to gel-based technologies using conventional two-dimensional polyacrylamide gel electrophoresis (2-DE), a number of gel-free approaches, in particular liquid chromatography coupled with matrix-assisted laser desortion ionization or electro-spray ionization MS, have been employed to determine changes in the protein expression profiles between RCC and normal renal tissues and/or respective primary or established RCC cell cultures. The latter approach complements 2D-PAGE-based profilings by covering not only low abundant or membrane proteins but also proteins excluded from gel-based approaches due to limitations such as size or extreme isoelectric points. In analogy to miR studies, the number of RCC lesions and corresponding epithelium analyzed significantly varied from 5 to >50 paired samples. Although the comparative analysis of RCC lesions vs patient-matched NN or representative RCC cell lines generated a pool of 336 differentially expressed proteins, only a limited number of proteins have been validated by RT-PCR, Western blot and/ or immunohistochemistry and demonstrated a general RCC or RCC subtype-specific

expression.[68,86–88] Furthermore, the functional relevance of candidate biomarkers has only been addressed for a limited number of candidate targets.[87]

Can the Correlation of miR and Proteomic Profiling Data Lead to a Further Improvement in the Search for Biomarkers?

Using distinct high throughput platforms, a number of studies have identified panels of transcripts, miRs, and proteins that are differentially expressed between normal renal tissue and RCC lesions or between lesions of distinct RCC subtypes.[26,68,77,81,89–93] The simultaneous profiling of the miR and mRNA expression pattern was recently performed and demonstrated an inverse relationship between the miR expression profile and that of a number of putative targets.[94–96] The linkage between miR and mRNA expression levels led to the identification of deregulated miR/mRNA pairs involved in the initiation or maintenance of disease processes.[26,97] However, this approach has its limitations since it cannot identify targets that are regulated at the posttranscriptional level. For such targets the changes at the mRNA levels remain unaffected, whereas the corresponding protein levels will be frequently reduced.[98]

In contrast, it could be postulated that proteome-based approaches can detect and quantify changes at the protein expression level induced by specific miRs. Since there exists increasing evidence that translational repression instead of mRNA degradation is the dominant miR regulatory mechanism the analysis of the correlation between miR and protein expression levels is gaining more importance.[97,99] It is suggested that broad changes in protein synthesis are induced by miRs.[45,100] Therefore, the concept to perform parallel miR and proteomic profiling represents a novel experimental strategy to define the protein output[45,100] and might be further applied to define potential biomarkers in any disease, including RCC. On the basis of this assumption, the determination of the inverse correlation between differentially expressed miRs and proteins in tumor lesions and corresponding normal tissues might lead to the optimization of the selection process for candidate biomarkers.

Despite the majority of miRs exhibit an impact on the general protein expression profile,[45,100] a comparative global proteomic and miR profiling in malignancies has not yet been performed. It is postulated that the comparison of proteome-based data to available miR profiling data sets will demonstrate a significant inverse regulation between differentially expressed miRs and their potential protein targets (Table 1). However, the disadvantage of this selection strategy is the elimination of indirect regulations since only those miR/protein pairs with strictly inverse correlated expression levels will be retained. This approach will reduce the number of putative, pair-matched-based protein targets extracted from databases and might provide highly robust subsets of relevant functional targets. However, the complex biology and tissue specificity of miRs make it still difficult to elucidate their precise role in the initiation and progression of disease and their link to deregulated genes and/or protein expression.

Identification of Correlative Signatures between miR and Protein Pairs in RCC and their Association to Pathways

To determine a link between miR and protein expression the subset of frequently and consistently deregulated miRs was subjected to a miRWalk search to collect their predicted targets. Only miR targets (Table 1), which were found in at least four of the eight prediction algorithms, were subsequently stored in an in-house database. These were then aligned to the experimentally defined differentially expressed proteins in RCC samples. With the exception of the up-regulated miR-21, counter-regulated miR/protein pairs could be defined

(Table 1) for the subset of miRs fulfilling the selection criteria. In addition, the chromosomal location of the miRs are in line with chromosomal alterations in RCC.[65,66,101–104]

In the next step, the identified pairs of counter-regulated miRs and proteins were dedicated to their respective pathway according to the KEGG database (Table 2 and Figure 1). Interestingly several metabolic pathways known to be involved in cancer seemed to be altered by different miRs such as the nucleotide, energy as well as amino acid metabolism. Regarding the purine metabolism, the guanine deaminase protein was down-regulated in RCC,[85] which was accompanied by an up-regulation of miRs-106a and –185. Since this enzyme is responsible for the guanine degradation the diminished expression might be a prerequisite for tumor cells to reach an increased proliferation rate as large amounts of DNA are required for cell division. In contrast, the up-regulation of the nucleoside diphosphate kinase might lead to an increased RCC proliferation since the required nucleoside triphosphates required for DNA synthesis can be provided. Concerning the energy metabolism miR-21 down-regulation influencing the expression of phosphoglucomutase was detected.[56,69] This enzyme is up-regulated at the proteome level, suggesting that miR-21 has potential regulatory effects for the glycolytic flux and might therefore contribute to the metabolic transformed phenotype. In this context it is noteworthy that miR-106b targeting phosphofructokinase (PFKP), the rate-determining enzyme of glycolysis, is up-regulated in RCC,[59] but there also exist controversial results regarding the expression of this enzyme[82,84] in RCC lesions.[79] Furthermore the observed up-regulation of glutaminase in RCC is accompagnied by a decreased expression of miR-141, which is in line with the enhanced glutaminolysis frequently observed during metabolic transformation of tumor cells.[26,35,56,58–60,77] The decreased expression of miR-200b, –200c, –429 as well as miR-204, which targets moesin and ezrin, respectively, might be the underlying molecular mechanism for the increased cell migration of RCC caused by an up-regulation of the moesin/ezrin/radixin family frequently detected in proteome studies of RCC. Taken together miRs are potential key regulators for the expression of different metabolic enzymes that are required for the altered metabolic demands of RCC.

Conclusions

Until now, high throughput RNA, DNA, and protein analyses of tumors have not given comprehensive information about the initiation and progression of malignancies. In addition, there exist only a limited number of reports analyzing the miR expression pattern in RCC lesions.[26,35,56–60,69–86] The link of the miR profile to respective protein expression patterns of RCC lesions and normal kidney parenchyma might lead to an increased understanding of the molecular mechanisms involved in the pathogenesis of this disease.

In the future, the correlative expression profile of miRs and proteins have to be extended to a larger sample number to use the coordinate by regulated miR and protein pairs as diagnostic and prognostic tools not only for RCC but also for other cancers. Nevertheless, the number of tumor samples analyzed so far is too low to draw general conclusions or to refer to a common RCC-specific miR/protein profile. In addition, there exists only limited information about (i) the clinical significance of miRs by correlating miR expression levels with the outcome of RCC patients and (ii) about the relevance of miR in the alteration of fundamental processes in RCC.

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Abbreviations

ccRCC	clear cell RCC
HIF1-α	hypoxia inducible factor
miR	microRNA
NN	tumor adjacent renal epithelium
PFKP	phosphofructokinase
RCC	renal cell carcinoma
UTR	untranslated region
VHL	von Hippel Lindau

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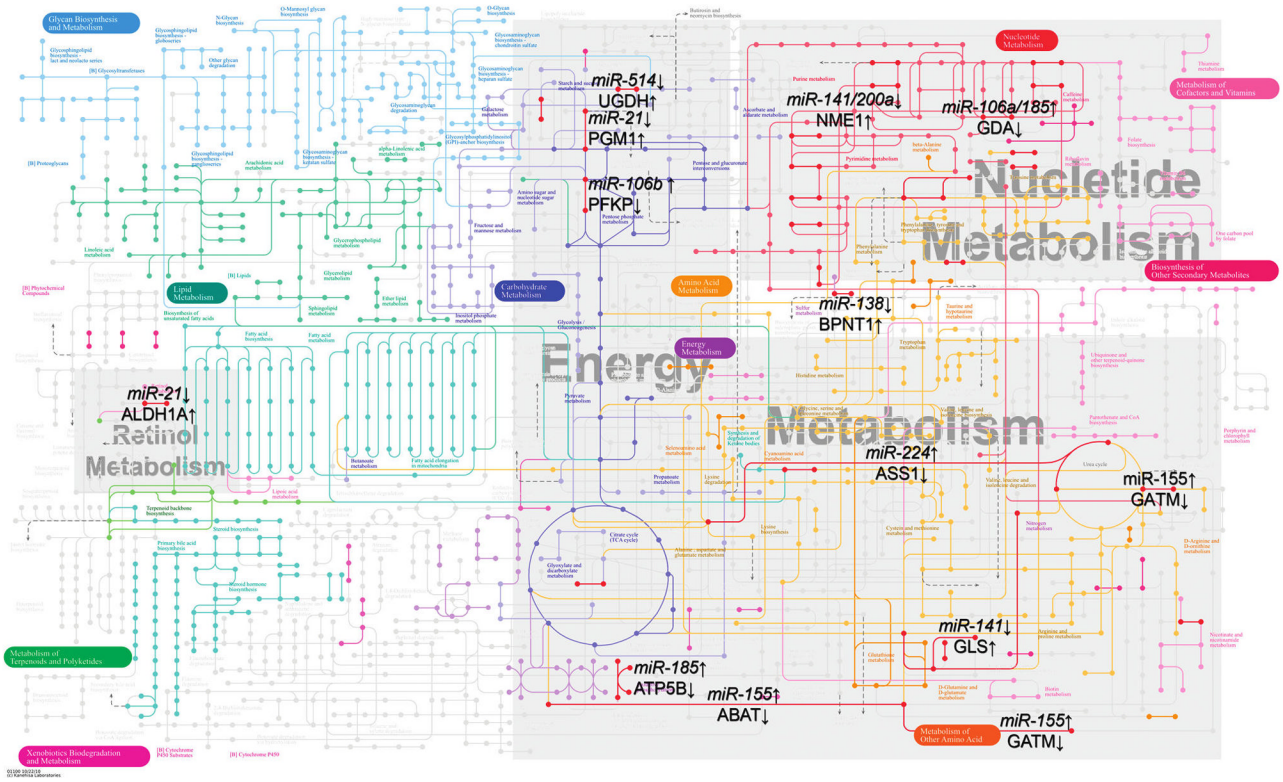

Figure 1.

Overview of influenced metabolic pathways. The scheme assigns the inverse correlated miRs and proteins to their respective metabolic pathways according to the KEGG database. The gray boxes represent metabolic parts. ↑, up-regulated; ↓, down-regulated.

Table 1

Comparison of Differentially Regulated miRs with the Counter Regulated Proteins Identified

miRs				target proteins	
name	**reference**	**DNA locus**	**RCC related region**	**name**	**reference**
			Down-regulated miRNA		
miR-21[a]	56	17q23	17q21 loss of het.[101]	Retinal dehydrogenase 1	78, 79, 82, 84
				Phosphoglucomutase-1	69
miR-30a	26, 58, 59	6q13	loss 6q[66,103]	Dihydropyrimidinase-related protein 2	77, 84
miR-133b	58, 59	6p12	loss 6p[65]	Ferritin light chain	86
				N-myc downstream-regulated gene 1 protein	84
miR-138	35, 59	3p21	loss of 3p,[65,103] loss of 3p21-pter[66]	3′(2′),5′-bisphosphate nucleotidase 1	83
miR-141	26, 35, 56, 58–60	12p13		Glutaminase kidney isoform	77
				Nucleoside diphosphate kinase A	86
miR-200a	56, 58, 59	1p36	loss 1p[102]	Aspartyl-tRNA synthetase	84
				Nucleoside diphosphate kinase A	86
miR-200b	26, 57–60	1p36	loss 1p[102]	Chloride intracellular channel protein 4	69
				Fibronectin	82
				Stress-70 protein	79
				Moesin	77
miR-200c	26, 35, 56–60	12p13		Chloride intracellular channel protein 4	69
				Stress-70 protein	79
				Moesin	77
miR-204	26, 56, 58, 59	9q21	loss 9[66]	Ezrin	82
				Lamin-A	82
miR-429	26, 56, 58, 59	1p36	loss 1p[102]	Chloride intracellular channel protein 4	69
				Four and a half LIM domains protein 1	82
				Fibronectin	82, 84
				Moesin	77
miR-510	26, 58, 59	Xq27	loss Xq[65,66,102]	Peptidyl-prolyl cis-trans isomerase A	78
miR-514	26, 35, 58, 59	Xq27	loss Xq[65,66,102]	UDP-glucose 6-dehydrogenase	83

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miRs				target proteins	
name	**reference**	**DNA locus**	**RCC related region**	**name**	**reference**
			Up-regulated miRNA		
miR-21	26, 57–59	17q23	gain 17q[65]		
miR-34a	56, 58, 59	1p36	add(1)(p34)[104]	Calbindin	81, 85
miR-34b	56, 58, 59	11q23		Calbindin	81, 85
miR-106a	57, 59, 60	Xq26	gain Xq[65]	Guanosine-diphosphatase	85
				Sorting nexin-9	69
miR-106b	57–60	7q22	trisomy 7,[103] gain 7[65,66]	6-phosphofructokinase type C	78
				Sorting nexin-9	69
miR-155	26, 35, 58–60	21q21	del(21)(q22)[104]	4-aminobutyrate aminotransferase	75, 82, 84
				Glycine amidinotransferase	81, 82, 84, 85
miR-185	26, 58, 59	22q11		ATP synthase subunit beta	83, 84
				Guanosine-diphosphatase	85
				14-3-3 protein gamma	85
miR-224	26, 35, 58, 59	Xq28	gain Xq[65]	Argininosuccinate synthase	75, 81, 84
				Fibrinogen beta chain	82

[a] miR-21: 6 times detected, only one time a down-regulation; het.: heterozygosity.

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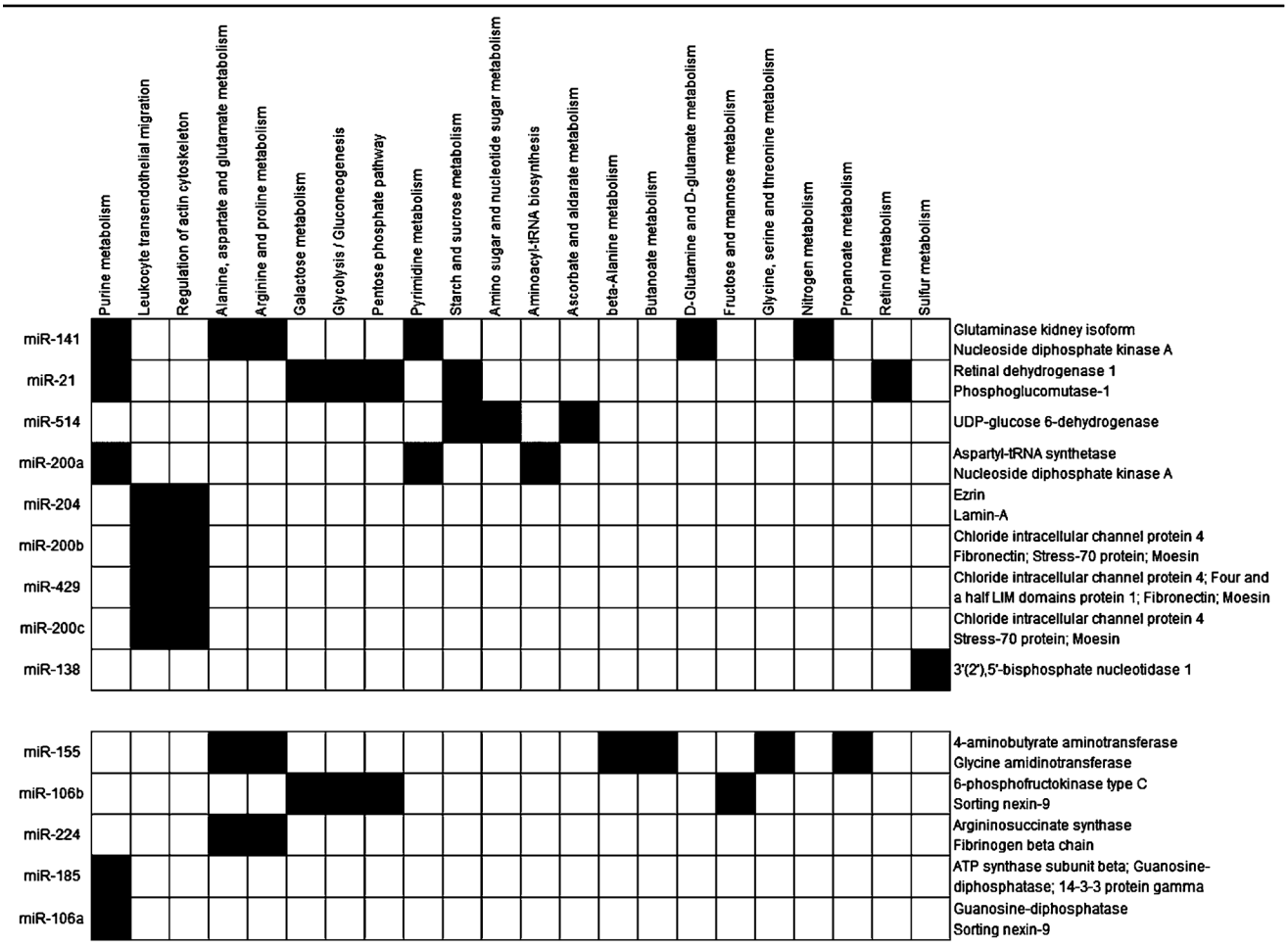
Table 2

Differentially Regulated miR/Protein Pairs Identified in RCC Associated to Their Metabolic Pathways[a]



[a]Upper part, down- regulated miRs; lower part, up-regulated miRs.