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RESEARCH ARTICLE

Exploring the role of miRNAs in renal cell carcinoma progression and metastasis through bioinformatic and experimental analyses

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Abstract Metastasis results in most of the cancer deaths in clear cell renal cell carcinoma (ccRCC). MicroRNAs (miRNAs) regulate many important cell functions and play important roles in tumor development, metastasis and progression. In our previous study, we identified a miRNA signature for metastatic RCC. In this study, we validated the top differentially expressed miRNAs on matched primary and metastatic ccRCC pairs by quantitative polymerase chain reaction. We performed bioinformatics analyses including target prediction and combinatorial analysis of previously reported miRNAs involved in tumour progression and metastasis. We also examined the co-expression of the miRNAs clusters and compared expression of intronic miRNAs and their host genes. We observed significant dysregulation between primary and metastatic tumours from the same patient. This indicates that, at least in part, the metastatic signature develops gradually during tumour progression. We identified metastasis-dysregulated miRNAs that can target a number of genes previously found to be involved in metastasis of kidney cancer as well as other malignancies. In addition, we found a negative correlation of expression of miR-126 and its target vascular endothelial growth factor (VEGF)-A. Cluster analysis showed that members of the same miRNA cluster follow the same expression pattern, suggesting the presence of a locus control regulation. We also observed a positive correlation of expression between intronic miRNAs and their host genes, thus revealing another potential control mechanism

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for miRNAs. Many of the significantly dysregulated miRNAs in metastatic ccRCC are highly conserved among species. Our analysis suggests that miRNAs are involved in ccRCC metastasis and may represent potential biomarkers.

Keywords Renal cell carcinoma · VEGF-A · MicroRNA · Tumour markers · Metastasis · Prognosis

Abbreviations

ccRCC	Clear cell renal cell carcinoma
EMT	Epithelial to mesenchymal
miRNA	microRNA
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RCC	Renal cell carcinoma
VEGF	Vascular endothelial growth factor
RPLPO	Acidic ribosomal phosphoprotein
EGFL7	Epidermal growth factor like 7
FAK	Focal adhesion kinase
IGF1	Targeting insulin-like growth factor 1
MMP2	Matrix metalopeptidase 2
HIF1A	Hypoxia inducible factor 1 alpha subunit
PDGFB	Platelet-derived growth factor B
PDGFC	Platelet-derived growth factor C
MDM2	Murine double minute 2
TYMS	Thymidylate synthase
VHL	Von Hippel Lindau

Introduction

Renal cell carcinoma (RCC) is the most common neoplasm of the adult kidney accounting for about 90% of adult kidney cancers with clear cell renal cell carcinoma (ccRCC) representing the most common subtype [1]. RCC is one of the top prevalent cancers in North America [2]. In USA, RCC incidence and mortality rates increased over the past decades [1, 3].

Patients diagnosed at the metastatic stage have relatively poor prognosis with only a 9% 5-year-survival rate, while a favourable prognosis is associated with the early diagnosis and treatment of ccRCC [4]. Therefore, there is a crucial need for more understanding of the molecular pathways that underlie ccRCC metastasis to identify new biomarkers and targeted therapies for this aggressive malignancy.

Metastasis is responsible for about 90% of cancer associated mortality [5]. The process of metastatic formation was considered for long time as the final stage of the multistep process of primary tumour progression [6, 7]. However, recent evidences suggested that malignant cells' dissemination can occur early in tumourigenesis [7–9]. Hunter et al. reported that the metastatic process may start early in about 60% to 70% of patients [10]. Hanahan and Weinberg have shown that the malignant cells in the metastatic colonies may not only continue to disseminate to other new sites in the body but may also return back to their tumours of origin [7]. Kim et al. demonstrated that this self-seeding process can further increase the tumour growth through many mediators [11]. The mechanisms triggering and controlling metastasis are not yet fully elucidated. miRNAs represent an interesting new players in this field [12].

MicroRNAs (miRNAs) are the small non-coding RNAs that regulate the expression of protein-coding genes by binding to the 3′ untranslated region of the RNA transcript leading to transcriptional repression or mRNA cleavage and degradation [13]. miRNAs regulate many important cell functions including proliferation, differentiation and apoptosis and play an important role in tumour development, progression and metastasis [14]. Several studies have reported miRNA metastatic signature in many cancers and revealed that many of these miRNAs target genes involved in regulation of cell to cell adhesion, cell motility and cell–matrix interactions [13]. Accumulating reports show that miRNAs are dysregulated in kidney cancer [15–19] and that they are involved in RCC pathogenesis [20–23]. Potential mechanisms through which miRNAs can contribute to RCC pathogenesis have been recently outlined [24, 25].

We and others identified miRNAs that are altered in metastatic ccRCC compared to the primary tumours [26, 27]. In order to further explore the role of these miRNAs in tumour progression, we compared the expression of these miRNAs in pairs of primary and metastatic tumours from the same patient. We also performed bioinformatics analyses including target prediction as well as combinatorial analysis of miRNAs previously reported to be involved in metastasis. In addition, we examined the negative correlation between miR-126 and its target vascular endothelial growth factor (VEGF)-A. Moreover, we examined the co-expression of the dysregulated miRNA clusters as well as the expression profiles of intronic miRNAs and their host genes. Furthermore, we examined conservation among species for the most dysregulated miRNAs in metastatic ccRCC.

Materials and methods

Specimen collection

We compared the expression of miR-10b, miR-126, miR-196a, miR-204, miR-215, miR-192 and miR-194 by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in 20 pairs of formalin-fixed paraffin-embedded tissues of matched primary and metastatic ccRCC from the

same patient. Specimens were collected from St. Michael's Hospital, Toronto General Hospital, Toronto and London Health Sciences Center, London, Canada. Areas of pure tumour tissues were identified by a pathologist. All the procedures were done following the Research Ethics Board at St. Michael's Hospital.

Total RNA extraction

Nucleic acid isolation was done using six cores of formalin-fixed paraffin-embedded tissues of primary and metastatic ccRCC tissues. Total RNA was extracted using miRNeasy (Qiagen, Mississauga, Canada) according to the manufacture's protocol. Total RNA concentrations were determined spectrophotometrically. Samples suitable for analysis were stored at −80°C.

qRT-PCR

MiRNA specific reverse transcription was performed with 5 ng total RNA using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer for miR-10b, miR-126, miR-196a, miR-204, miR-215, miR-192 and miR-194. qRT-PCR was performed using the TaqMan microRNA Assay® Kit on the Step One™ Plus Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were according to the manufacture's fast protocol, and all reactions were performed in triplicate. Relative expression was determined using the $\Delta\Delta C_T$ method and expression values were normalized to small nuclear RNA, *RNU44* (Applied Biosystems).

The primer sequences for VEGF-A were as follows: forward-5′-CTTGCCTTGCTGCTCTACCT-3′ and reverse-5′-GTGATGATTCTGCCCTCCTC-3′. Reverse transcription was preformed with High capacity RNA-to-cDNA kit (Applied Biosystems) as per the manufacturer's instructions. qRT-PCR was performed using the Fast Syber Green Master Mix (Applied Biosystems). Acidic ribosomal phosphoprotein (RPLP0) was used as endogenous control and its primers were as follows: forward-5′-GGCGACCTGGAAGTCCAACT-3′ and reverse-5′-CCATCAGCACCACAGCCTTC-3′.

Bioinformatics analysis

MiRNA target prediction analysis

Bioinformatics analysis was performed on the top differentially expressed miRNAs in metastasis that was identified in our previous study [27] (Supplementary Table 1). We performed target prediction analysis using two programs. TargetScan version 5.1 (http://www.targetscan.org/) and TragetCombo (http://www.diana.pcbi.upenn.edu/cgi-bin/TargetCombo.cgi) [28]. We selected the option "Predicted Targets: Union" for the TargetCombo analysis. This option identifies all targets predicted by any of the four prediction programs included in TargetCombo which are DIANA-microT, PicTar, TargetScanS and miRanda.

MiRNA family and cluster analyses

Chromosomal locations and distances between miRNAs detected in our miRNA microarray (875 miRNAs) were identified by miRBase (Release 14). MiRNAs with an inter-miRNA distance of <50,000 nucleotides were included as part of a miRNA cluster.

Intronic miRNA analysis

Intronic miRNAs are those miRNAs located within the introns of their host genes. We identified the intronic miRNAs among the differentially expressed miRNAs in metastatic ccRCC and their host genes. We compared the expression pattern of these miRNAs and their host genes in metastasis of different malignancies. MiRBase (Release 14) was used to identify intronic miRNAs while Ensemble Genome Browser, UniProt Knowledgebase and literature search were used to identify host gene expression in metastasis of different types of malignancies.

Phylogenetic analysis

The University of California Santa Cruz Genome Browser was used for sequence comparison of the most differentially expressed miRNAs in metastatic ccRCC. Conservation among species of these miRNAs was examined with sequence alignment in the genomes of 28 vertebrate species, including 17 mammalian species.

Results

MiRNA microarray experimental validation

In order to validate our metastatic miRNA signature, we experimentally verified the expression level of seven of the top significantly differentially expressed miRNAs (based on our miRNA microarray results), miR-10b, miR-126, miR-196a, miR-204, miR-215, miR-192 and miR-194 in an independent set of 20 matched pairs of metastatic ccRCC and their matched primary tumours from the same patient with the "gold standard" qRT-PCR using miRNA-specific probes. Figure 1 shows the expression of these seven miRNAs in metastatic ccRCC cases compared to their primary matched pairs. qRT-PCR results indicated that miRNA-10b, miR-126, miR-196a, miR-204, miR-215, miR-192 and miR-194 showed decreased expression in

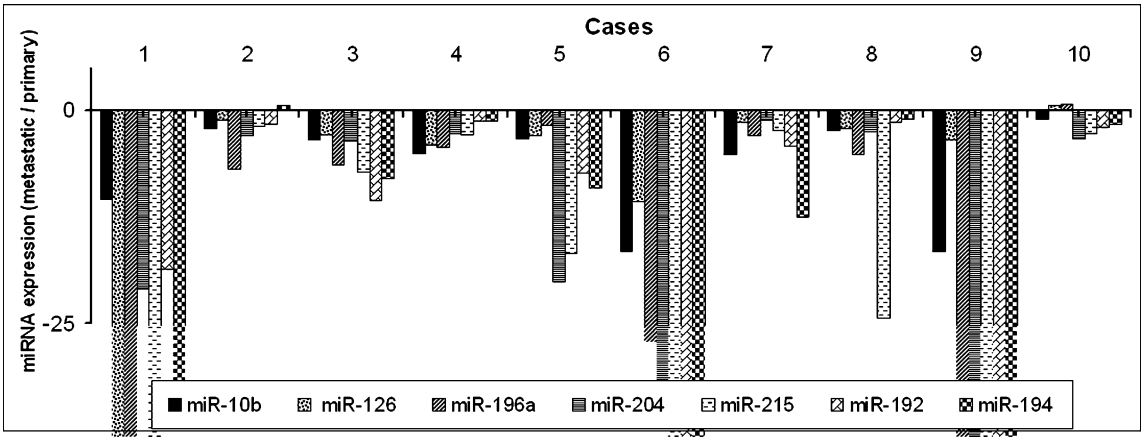

Fig. 1 A representative bar graph showing miRNA expression in 10 pairs of matched metastatic and primary ccRCC. The expression levels of seven miRNAs were assessed by qRT-PCR in matched pairs of primary and metastatic tumours from the same patient. miRNA expressions are shown as the ratio of metastatic to primary after normalization with a house keeping internal control. All seven miRNAs showed a trend towards downregulation in metastasis compared to primary tumours, in agreement with miRNA microarray data. In few cases, the levels of expression were comparable in both the primary and metastatic tumours

15/20, 13/20, 16/20, 14/20, 14/20, 16/20 and 14/20 of cases, respectively, when the metastatic ccRCC was compared to their matched primary tumours. These results are consistent with microarray data. This trend of down-regulation in metastasis indicates that some of these changes are gradually developed during the process of tumour progression. In other cases, the expression was comparable between primary and metastasis indicating that metastatic signature was manifested in the primary tumour.

The role of miRNAs in ccRCC progression and metastasis

In order to explore the role of miRNAs in ccRCC metastasis, we performed target prediction analysis of the miRNAs that are differentially expressed in metastatic ccRCC. We then cross-matched those targets with genes that are reported to be involved in metastatic ccRCC. Our target prediction analysis showed that miRNAs that are differentially expressed in metastasis can play a role in metastatic ccRCC pathogenesis by targeting key molecules such as VEGF, hypoxia inducible factor 1 alpha subunit (HIF1A), platelet-derived growth factor B (PDGFB), platelet-derived growth factor C (PDGFC), MMP2 and murine double minute 2 (MDM2), as summarized in Table 1.

We validated the negative correlation between miR-126 and one of its predicted targets, VEGF-A by qRT-PCR on matched pairs of primary metastatic kidney tissues from the same patient. As shown in Fig. 2, we observed a negative correlation between the miRNA and its target, with lower levels of miR-126 associated with higher VEGF-A and vice versa. This provides indirect evidence that VEGF-A is a target of miR-126 in vivo.

Moreover, literature search identified many of the genes that are experimentally proven to be involved in the process of tumour progression and metastasis in many cancers to be predicted targets of miRNAs that are differentially expressed in metastatic ccRCC as shown in Table 2. For example, miR-361-5p, miR-29c, miR-29b, miR-29a and miR-126 are predicted to target VEGFA which is involved in regulation of cell cycle, cell growth and proliferation. miR-1275, miR-1207-5p, miR-361-5p, miR-30a, miR-27b, miR-424, miR-26b, miR-1 and miR-27a can target IGF1, a positive regulator of cell proliferation.

Furthermore, we found that many of the genes that are reported to be differentially expressed in metastasis of different cancers are predicted targets of the significantly dysregulated miRNAs in metastatic ccRCC as partial list is shown in Supplementary Table 2. For example, matrix metalloproteinases are involved in metastasis of many cancers. let-7f, let-7e, let-7g and miR-98 can target matrix metalopeptidase 11 (MMP11) which is reported to be dysregulated in colon cancer metastasis. Matrix metalopeptidase 16 (MMP16) which is reported to be differentially expressed in metastasis of breast cancer is a predicted target for miR-182, miR-24, miR-27b, miR-106b, miR-181b, miR-15a, miR-195, miR-30a, miR-27a, miR-194, miR-200a. Matrix metalopeptidase 13 (MMP13) and matrix metalopeptidase 2 (MMP2) are dysregulated in the metastasis of esophageal squamous cell carcinoma, hepatocellular carcinoma, colorectal cancer and breast cancer. MMP13 is a predicted target miR-27b and miR-27a while MMP2 is a predicted target for miR-106b, miR-29c, miR-29b and miR-17.

Mechanisms that control miRNA expression

Next, we explored two mechanisms that can contribute to coordinated regulation of miRNAs during tumour progression, miRNA clusters and intronic miRNAs.

Table 1 Genes involved in metastatic ccRCC are predicted to be targeted by miRNAs that are differentially expressed in RCC metastasis

Genes involved in metastatic ccRCC		Targeting miRNA
Gene symbol	Reference	
GALNTL2	[43]	let-7d, let-7f, let-7g, let-7e, miR-98
NFASC	[43]	miR-1207-5p, 182, 521, 10a
PPM1H	[43]	miR-548m, 331-3p
PTPN12	[43]	miR-374a, 10a, 107, 103, 200b, 145, 30a, 194, 126*
TSPAN7	[43]	miR-130a, 27a, 27b
TUBB2B	[43]	miR-106b, 29c, 29b, 106a
VEGF	[44]	miR-361-5p, 29c, 29b, 29a, 126, 1640, 27a
HIF1A	[44]	miR-106b, 93, 20a, 20b, 106a
PDGFB	[44]	let-7d, let-7f, let-7g, let-7e, miR-126*, 98, 29c, 29b, 29a, 1
PDGFC	[44]	miR-186, 130a, 29c, 29b, 29a
MMP2	[36]	miR-106b, 29c, 29b, 17
MDM2	[37, 45]	miR-192, 194, 215
TYMS	[38]	miR-192, 215

Coordinated regulation of miRNA dysregulation in ccRCC: MiRNA clusters and families

Using an inter-miRNAs cut-off distance of 50,000 nucleotides as suggested by literature [29, 30], 57% (64 miRNAs) of the differentially expressed miRNAs in metastatic ccRCC were found to be organized within 31 clusters. Supplementary Table 3 and Fig. 3 display the clusters of miRNAs dysregulated in metastasis of ccRCC. The largest numbers of clusters were found on chromosome 1 and X (five clusters in each).

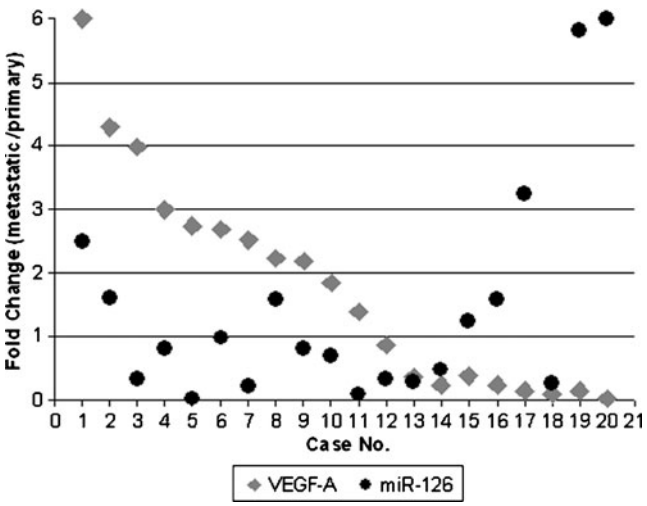

Fig. 2 Representative blot showing the negative correlation between miR-126 and its predicted target, VEGF-A. Expression levels of both molecules are shown as a fold change (metastasis over primary) after normalization with an internal control of a house keeping small RNA molecule. Our results show the presence of a negative correlation between the miRNA and its target with lower expression of miR-126 correlating with higher expression of VEGF-A and vice versa. This provides indirect evidence that VEGF-A is a direct target of miR-126. Patient cases are shown on the *X*-axes and the fold changes are represented along the *Y*-axes

Most of these clusters showed the same pattern of expression for all miRNA members of the same cluster. For example, in the miR-215 and miR-194-1 cluster located on chromosome 1 (cluster E in Fig. 3), both miRNAs are downregulated. Members of another cluster, miR-182, miR-96 and miR-183 (cluster B on chromosome 7), are all upregulated. This not only suggests that members of the same cluster may have coordinated functions to regulate the same biological processes but also suggests that they may have common control mechanisms. Interestingly, many of these clusters are found in chromosomal locations related to metastasis of different types of cancer such as 3p21.1[31] and 11q13.1[32] where the let-7g/miR-135a-1 and the miR-192/miR-194-2 clusters are located. For other clusters, as the miR-17-92a cluster which is located on chromosome 13, two miRNAs (miR-18a and miR-92a-1) are upregulated while the remaining miRNAs are downregulated.

Intronic miRNAs and their host gene expression in metastatic tumours

We identified 58 (51.8%) of the 112 miRNAs differentially expressed in metastasis to be intronic miRNAs (i.e. located within an intron of their host genes) (Supplementary Table 4). As shown in Table 3, literature search demonstrates that many of the intronic miRNAs and their host genes have the same pattern of expression in metastatic cancers e.g. miR-126 and its host gene epidermal growth factor like 7 (EGFL7) and also miR-151-5p and its host gene focal adhesion kinase (FAK). This suggests that, at least in part, intronic miRNAs and their host genes can be under the same transcriptional control. On the other hand,

Table 2 Differentially expressed miRNAs in ccRCC metastasis are predicted to target key molecules involved in tumour progression and metastasis in different cancers

Gene	Function	Targeting miRNAs
APC	Cell to cell adhesion	miR-374a, 107, 187, 103, 27b, 181b, 181a, 26b, 15a, 30a, 27a
CD44	Cell to cell adhesion, transmembrane receptor	miR-27b, 15a, 27a
PNN	Cell to cell adhesion	miR-107, 103, 106b, 186, 30b, 30c, 30d, 1, 106a, 30a*
VEGF	Cell to cell adhesion	miR-361-5p, 29c, 29b, 29a, 126, 1640, 27a
FN1	Cell adhesion	miR-200b, 1, 27b, 27a
MTSS1	Cell adhesion	miR-182, 1384, 1385, 107, 103, 186, 29c, 200b, 181b, 26b, 15a, 130a, 1, 195, 194, 200a
MMP11	Matrix metalloproteinase	let-7f, let-7e, let-7g, miR-98
MMP13	Matrix metalloproteinase	miR-27b, 27a
MMP2	Matrix metalloproteinase	miR-106b, 29c, 29b, 17
TIMP2	MMP inhibitor	miR-421, 17, 106b, 30b, 30c, 30d
TIMP3	MMP inhibitor, induction of apoptosis	miR-107, 103, 106b, 30b, 20a, 181b, 181a, 30c, 30d, 20b, 143, 15a, 1, 181d, 195, 101, 200a, 106a, 30a, 30a*
COL4A2	Extracellular matrix proteins	let-7d, let-7f, 29c, 29b, let-7g, let-7e, 98
KRAS	Regulation of the cell cycle	miR-421, 27b, 30b, 181b, 181a, 30c, 30d, 143, 27a
TGFB1	Regulation of the cell cycle, cell growth and proliferation, anti-apoptosis	miR-663, 744
VEGFA	Regulation of the cell cycle, cell growth and proliferation	miR-361-5p, 29c, 29b, 29a, 126
NF2	Regulation of cell cycle	miR-107, 103, 15a, 195, 2604
PTEN	Regulation of cell cycle	miR-548, 498, 1491, 374b, 1492, 10a, 106b, 93, 29c, 20a, 29b, 29a, 26b
IGF1	Positive regulation of cell proliferation	miR-1275, 1207-5p, 361-5p, 30a, 27b, 424, 26b, 1, 27a
SMAD4	Transcription factor	miR-498, 664, 28-5p, 204, 26b
TCF20	Transcription factor	miR-186, 30c, 204
CHD4	Transcription regulator	let-7d, let-7f, let-7g, let-7e, 194, 98
SMAD2	Transcription regulator	miR-1786,455-3p, 30a, 1787

miR-149 and its host gene glypican-1 (GPC-1) have different expression patterns.

Differentially expressed miRNAs in metastatic ccRCC are conserved among species

Our sequence analysis of the 112 significantly dysregulated miRNAs in metastatic ccRCC showed that 99 (88%) of them are highly conserved among species. Ten (52.6%) out of the 19 upregulated miRNAs and 89 (95.7%) out of the 93 downregulated miRNAs were highly conserved among species. Figure 4 shows the high conservation of miR-204 among 28 species. Conservation among species indicates that these miRNAs have critical functions that might be shared among different organisms.

Discussion

In our previous work, we identified the miRNA signature of metastatic ccRCC using miRNA microarray and validated our miRNA microarray results by qRT-PCR. In the current study, we further confirmed the presence of differentially regulated miRNAs in metastatic compared to primary renal cell carcinomas on an independent set of matched primary and metastatic tissues from the same patient. Our data show that in a number of cases, the metastatic signature develops gradually during tumour progression, as demonstrated by comparing primary and metastatic tumours from the same patient. In other cases, however, the "metastatic signature" was present in the primary tumour with no much difference of expression levels between primary and metastatic tumours.

A recent review explored the role of miRNAs in the process of metastasis including their effects on migration, invasion, proliferation, angiogenesis and apoptosis [12]. Bueno and Malumbres discussed the cell cycle regulation by miRNAs and its contribution to tumour development and progression [33].

We identified some key molecules involved in metastasis of ccRCC to be predicted targets of the significantly dysregulated miRNAs in metastatic ccRCC such as VEGF, HIF1A, PDGFB, PDGFC, MMP2, MDM2 and thymidylate synthase (TYMS). We also demonstrated

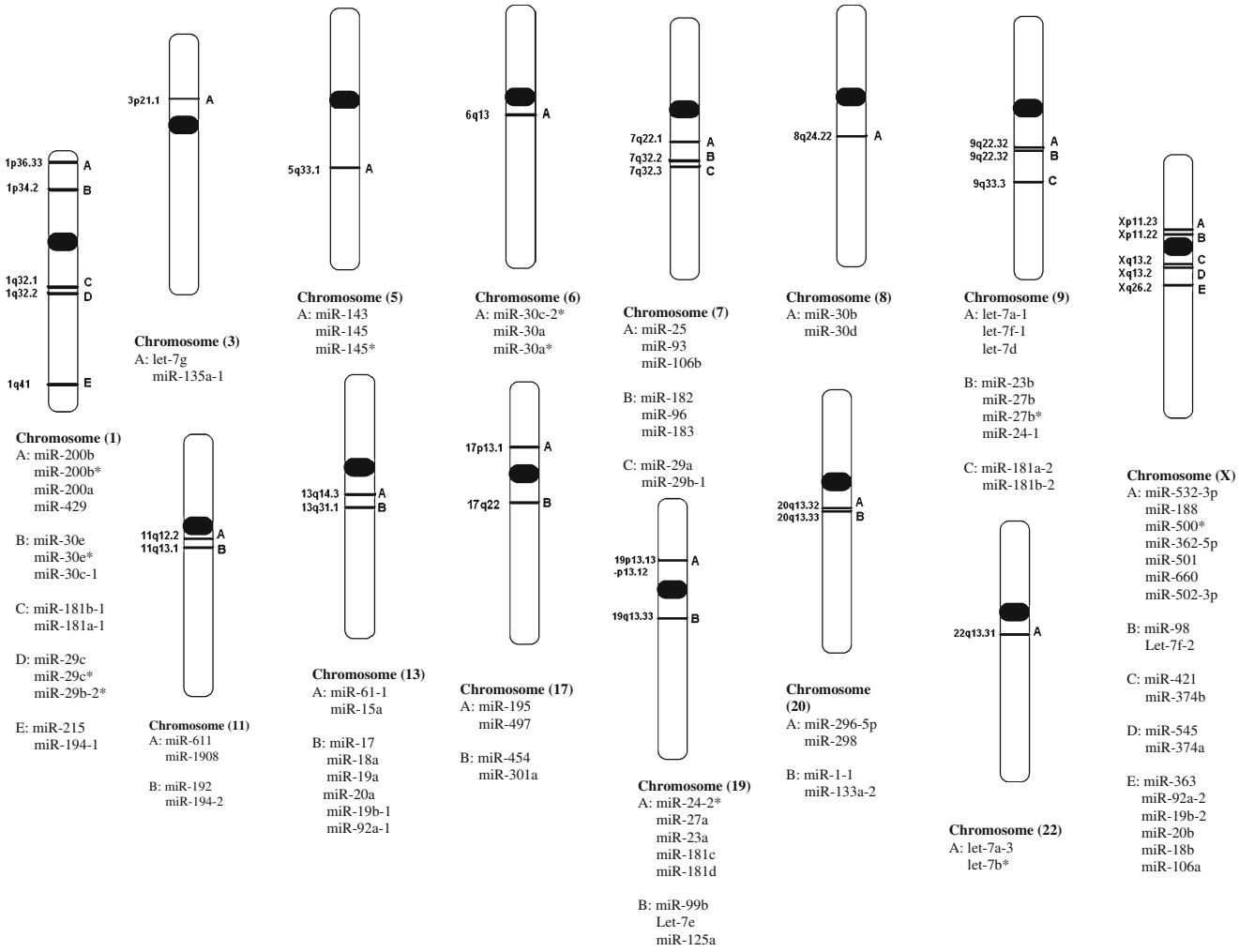

Fig. 3 Representative figure showing the clusters of differentially regulated miRNAs between primary and metastatic ccRCC. Chromosomes are represented by *rectangles* and the centromeres as the *central black ovals*. miRNA clusters in each chromosome are represented by the *horizontal lines*, and members of each cluster are shown *underneath*. The chromosomal location of the cluster is shown to the left of each cluster. Figure is not drawn to scale. Our results indicate that miRNAs that are differentially expressed in metastatic ccRCC tend to occur in clusters, indicating a possible co-regulation mechanism

this negative correlation between miR-126 and VEGF-A, in agreement with previously published data in lung and breast cancers [34, 35].

Another interesting observation is that the miRNAs that were found to be differentially expressed in metastatic ccRCC are predicted to target key molecules that are

Table 3 Intronic miRNAs and their host gene dysregulation in cancer progression

miRNA	Dysregulation	Reference	Host gene	Dysregulation	Reference	Gene function
miR-106b	Up	[46]	MCM7	Up	[46–49]	Minichromosome complex maintenance component. Initiation of genome replication
miR-93	Up	[50]	MCM7	Up	[46–49]	Minichromosome complex maintenance component. Initiation of genome replication
miR-151-5p	Up	[51]	FAK	Up	[51–54]	Focal adhesion kinase.
miR-196a	Up	[55]	HOXB7	Up	[56]	Homeobox domain family of transcription factors
miR-10b	Up	[57]	HOXD3	Up	[58]	Homeobox domain family of transcription factors
miR-126	Down	[59, 60]	EGFL7	Down	[60]	Epidermal growth factor like.
miR-149	Down	[61]	GPC-1	Up	[62]	Cell surface heparan sulfate proteoglycans.

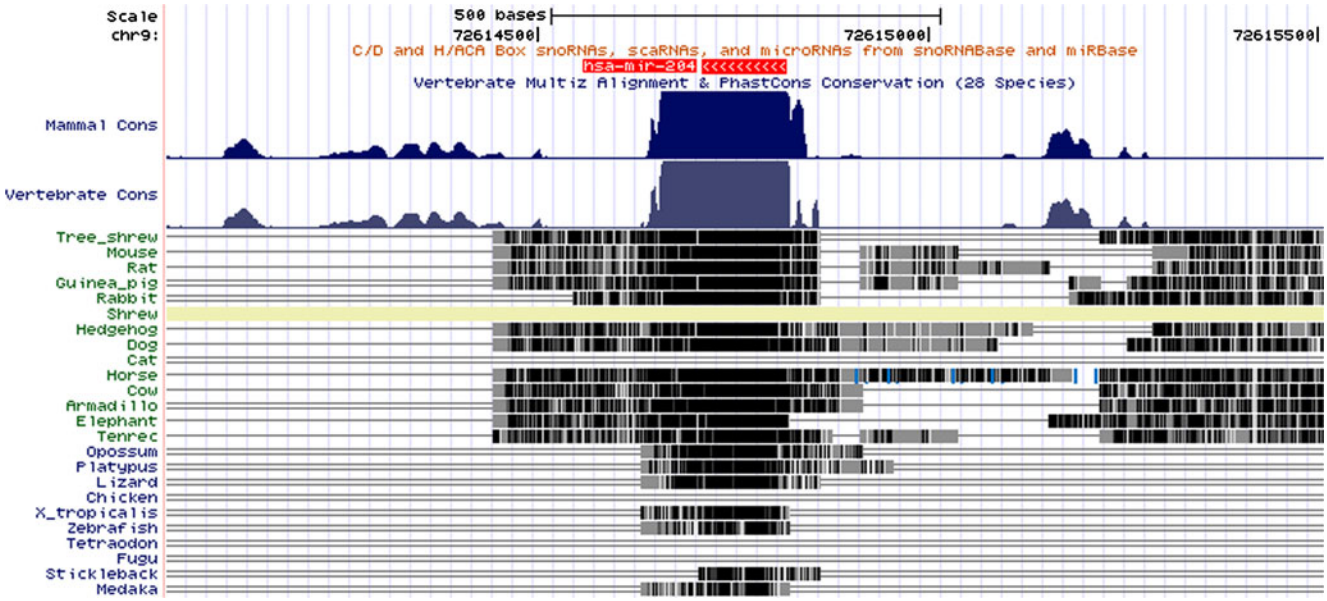

Fig. 4 A representative figure showing that miR-204 is highly conserved among different species. Data were analyzed according to the University of California Santa Cruz Human Genome browser. Many of the differentially regulated miRNAs were shown to be highly conserved among different species

involved in the metastasis of several types of cancer such as breast, hepatocellular and colorectal cancers. This implies the presence of common pathways or mechanisms that are utilized by multiple malignancies to achieve the aggressive metastatic phenotype and opens the possibility of using the same targeting therapy in multiple cancers.

Chuang et al. defined MMP2 to be one of the tumour-derived factors that affect tumour progression in RCC as they reported that MMP2 is secreted by advanced RCC and promote invasion in low grade RCC tumours [36]. Polanski et al. demonstrated that MDM2 increased cell motility and invasiveness in RCC cells [37]. Also, Mizutani et al. showed that TYMS activity was correlated with RCC tumour progression [38].

The mechanisms involved in the regulation of miRNA expression are not fully understood. In the current study, we investigated, in silico, some of these mechanisms. One such mechanism is the co-expression and co-regulation of miRNA clusters. Our results show that about 57% of the significantly dysregulated miRNAs in metastatic ccRCC were organized in clusters. Chhabra et al. reported that about 34% of human miRNAs are found in 64 clusters [39]. Co-expression of miRNA clusters may have vital functional implications. Cluster members can target the same molecule, and this may provide more powerful or synergetic control. Moreover, members of the same cluster can hit multiple targets in the same or related biological pathway. Table 1 shows that miR-29b and miR-29c can target VEGF, PDGFB and PDGFC. Both miRNAs are found in the same cluster on chromosome 1. Pichiorri et al. demonstrated that miR-192, miR-194 and miR-215 target MDM2 [40]. These three miRNAs are found in two clusters on two different chromosomes. miR-215 and miR-194-1 are found in a cluster located on chromosome 1 while miR-192 and miR-194-2 are found in a cluster on chromosome 11 as shown in Supplementary Table 3 and Fig. 3.

We found that 51.8% of the most significantly dysregulated miRNAs in metastatic ccRCC to be intronic (Supplementary Table 4). A recent study demonstrated that about 45% of human miRNAs are located in the intronic regions of protein-coding genes [41]. Our results demonstrated that many of these intronic miRNAs followed the same expression patterns of their host genes. Interestingly, Girijadevi et al. reported that intronic miRNAs can target their host gene [42].

About 88% of the significantly dysregulated miRNAs in metastatic ccRCC are highly conserved among species as demonstrated by our phylogenetic analysis. This conservation indicates functional importance and thus may help more understanding of their role in tumour progression and metastasis.

In conclusion, in this study, we further validated the presence of a distinct miRNA signature in ccRCC metastasis. Our in silico analysis also provide preliminary evidence of some potential mechanisms that can contribute to regulation of miRNAs in metastasis, including the co-expression of the miRNA cluster members and the co-expression of the intronic miRNAs with their host genes. We demonstrated that miRNAs can play an important role in ccRCC metastasis by targeting several key molecules and so may represent a potential kidney cancer metastasis biomarker. Further studies are needed for more understanding of metastatic ccRCC

pathogenesis that may help to offer potential therapeutic targets.

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Conflicts of interest None

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