MicroRNA Expression Signature and Target Prediction in Familial and Sporadic Primary macronodular adrenal hyperplasia

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Abstract

Background: Primary macronodular adrenal hyperplasia (PMAH) is a rare cause of Cushing's syndrome, usually characterized by functioning adrenal macronodules and increased cortisol production.

Methods: To screen and analyze the microRNA profile of PMAH, so as to elucidate the possible pathogenesis of the disease, MiRNA microarray was used to test the tissue samples from familial patients with PMAH, sporadic patients with PMAH and normal controls of other non-tumor adrenocortical tissues, to identify characteristic microRNAs expression signatures. Random selected miRNAs were validated by RT-PCR. Furthermore, the key signal pathways and miRNAs involved in PMAH pathogenesis were analyzed by gene ontology and pathway analysis.

Results: Characteristic microRNA expression signatures were identified for familial patients with PMAH including 16 differentially expressed microRNAs and sporadic patients with PMAH including 8 differentially expressed microRNAs respectively. RT-PCR assay confirmed the chosen miRNAs expression, suggesting the high reliability of miRNA array. Pathway analysis showed that the most enriched pathway was renal cell carcinoma pathway. Overexpression of miR-17, miR-20a and miR-130b may be inhibit glucocorticoid-induced apoptosis in the PMAH pathogenesis.

Conclusion: We have identified the miRNA signature in Familial and Sporadic patients with PMAH, The differentially expressed miRNAs may be involved in the mechanisms of PMAH pathogenesis. Specific miRNAs, such as miR-17, miR-20a and miR-130b may be new potential targets for further functional studies of PMAH.

Background

ACTH INDEPENDENT MACRONODULAR ADRENAL HYPERPLASIA (AIMAH, OMIM #615954), also known as primary bilateral macronodular adrenal hyperplasia, is a rare disorder characterized by bilateral macro-nodular hyperplasia of the adrenal glands with increased cortisol production. Patients typically present in the fifth and sixth decades of life with obvious clinical manifestations of moon face, sanguineous temperament, skin thinning, buffalo hump and thickened supraclavicular fat pad. [1, 2]. PMAH accounts for less than 1% of adrenocorticotrophic hormone independent Cushing's syndrome, and the extent of cortisol excess ranges from the level of sub-clinical to overt Cushing's syndrome[3]. The production of cortisol in patients with PMAH is often aberrantly modulated by some other hormones such as vasopressin, gonadotropins, angiotensin, gastric inhibitory peptide and catecholamines, and the endocrine disturbance is also associated with abnormal adrenocortical sensitivity to various hormonal secretagogues. Furthermore a few hormone receptor abnormal activities have been considered to be causative factors of PMAH [4, 5]. Although the disease phenotype occurs sporadically in majority of the affected patients with PMAH, several reports of familial clustering have been reported [6, 7]. In recent years, with the development of gene sequencing technology, the related gene mutation of PMAH in familial and sporadic were found, such as ARMC5 mutations found by DNA sequencing[8] and whole-

genome sequencing[9], EDNRA mutation found by whole exome sequencing[10]. PMAH presents a bimodal age distribution with rare subset presenting in the first years of life particularly with the McCune-Albright syndrome, most patients with PMAH presents in the fifth and sixth decades, a later age of onset compared with unilateral adenomas or Cushing's syndrome [2, 11]. Although reports for large kindred with PMAH have suggested the phenotype is consistent with monogenetic, probable autosomal dominant inheritance [12], the genetic or epigenetic basis of familial PMAH remains to be undetermined.

MicroRNA associated research has generated great interest owing to their extensively potential effects on the regulation of gene expression both on the level of transcription and that of post-transcription since the first microRNA was identified[13]. MicroRNAs (miRNAs) are a group of single-stranded, non-coding small RNAs with the length of approximately 22 nucleotides[14, 15], MiRNAs are first transcribed by RNA polymerase in hairpin structures and then processed by RNase Drosha to become long pre-miRNAs. And then RNase Dicer is involved in the process from precursors to mature microRNAs, which could bind to 3' untranslated region (3'UTR) of their targeting messenger RNAs in a sequence-specific manner completely or partially complementarily leading to degradation or translation repression, respectively[16]. Only a small fraction of identified miRNAs have been elucidated for their biological functions, these miRNAs could involve into the essential regulation processes such as proliferation, differentiation, apoptosis and development[17]. Although microRNA expression profiles differed with each other in different cancer types, it was demonstrated that several microRNAs might play very specific roles in tumourigenesis through regulating a series of pathways [18-23].

PMAH is rare and generally presents as a sporadic disease. The family clustering of PMAH and normal controls of other non-tumor adrenocortical tissues is more infrequent. In the past decade, the PMAH affected families had been diagnosed in the General Hospital of the People's Liberation Army. Three out of seventeen members were diagnosed with typical or sub-clinical PMAH syndrome (Fig. 1). We have tried our best to collect samples from such a familial affected pedigree patients and adrenal lipoma patients with complete clinical information by adrenalectomy. The aims of the study were to identify via a high-throughput approach the differentially expressed miRNAs in the samples from familial patients with PMAH, sporadic patients with PMAH and normal controls of adrenal lipoma patients, and to conduct bioinformatics analysis to determine the potential functions of the differentially expressed miRNAs and possible pathogenesis of PMAH through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, respectively.

Methods

Clinical samples

All methods were performed in accordance with the relevant guidelines and regulations. Written informed consent for genetic studies was obtained prior to initiating this study in agreement with protocols approved by the institutional review board at the General Hospital of the People's Liberation Army. This research was reviewed and approved by the ethics committee of General Hospital of the People's

Liberation Army. Adrenal nodule samples from patients with familial and sporadic PMAH were collected after the surgeries. The normal controls tissue were collected from the non-tumor part of large adrenal lipoma which were ≥ 2 cm apart from the lipoma after the surgeries. Moreover, the computed tomographic (CT) imaging technique and hematoxylin and eosin (H&E) staining methods were employed to identify the characteristics of nodular hyperplasia. For CT analysis, the images were reviewed by two experienced observers, who arrived at a consensus. Imaging characteristics were recorded. For H&E staining, all surgical specimens were fixed in 10% buffered formaldehyde and routinely processed for histologic diagnosis. The tumor histology was independently confirmed by 2 pathologists (Fig. 2). Samples were snap frozen in liquid nitrogen immediately after resection and stored at -20°C until the extraction of RNA.

Total RNA extraction

Total RNA from the removed adrenal gland tissue of patients with familial and sporadic PMAH as well as the normal controls was extracted according to the standard protocol.

MicroRNA assay

The Affymetrix GeneChip miRNA Array (Affymetrix, Santa Clara, CA) was applied to investigate microRNA differential expression profile in familial and sporadic PMAH compared with normal control samples. This microRNA array contains 46228 probes which comprise 7815 probe sets including controls, and covers 71 organisms including human, mouse, rat, canine and rhesus macaque. The array content is derived from the Sanger miRBase miRNA database v.11 (April 15, 2008). In brief, 1.5 µg of total RNA extracted from different groups of samples is labeled using the 3DNA Array Detection FlashTag™ RNA Labeling Kit according to the recommendations from manufacturers. Firstly, addition of poly A tail was carried out at 37°C for 15 min in a volume of 15 µl reaction mix, which contained 1×Reaction Buffer, 1.5ml MgCl₂, 1µl ATP Mix diluted 1:500 and 1µl PAP enzyme. Second, Flash Tag Ligation was performed at room temperature for 30 min by adding 4µl of 5× Flash Tag Ligation Mix Biotin and 2µl of T4 DNA Ligase into the 15µl of reaction mix. To stop the reaction, 2.5µl of Stop Solution was added. All samples were hybridized, washed and scanned with an Affymetrix Scanner.

miRNA qRT-PCR

miRNA reverse transcription was performed using the TaqMan microRNA Reverse transcription Kit (Applied Biosystems) at 16°C for 30 min, 42°C for 30min, and denaturation of the enzyme at 85°C for 5min. The RT reaction was performed at 37°C for 1 hour followed by 5min at 95°C. TaqMan microRNA assays (Applied Biosystems) were performed using the 7500 Fast Real-Time PCR System at the 9600 emulation run mode. Ct values were converted into copy numbers (copy no. = $2^{(-Ct)}$) and normalized to RNU48.

Bioinformatics analysis

The GO project provides a controlled vocabulary to describe gene and gene product attributes in any organism (<http://www.geneontology.org>). The ontology covers three domains: biological process, cellular component and molecular function. Fisher's exact test was used to determine if there is more overlap between the differentially expressed list and the GO annotation list than would be expected by chance. The P-value denotes the significance of GO terms enrichment in the differentially expressed genes. The lower the P-value, the more significant the GO term (P-value < 0.05 is recommended).

Pathway analysis is a functional analysis mapping genes to KEGG pathways. The P-value (EASEscore, Fisher-P value or hypergeometric-P value) denotes the significance of the pathway correlated to the conditions. Lower the p-value, more significant is the pathway (the recommend P-value cutoff is 0.05).

Statistical analysis

Results are expressed as means $\pm$ SE. Data were compared using paired t-test for hemodynamic parameters. Values of $P < 0.05$ were considered statistically significant ($P < 0.05$ for differentially expressed miRNAs).

When comparing the differentially expressed miRNA profiles between two groups, fold change and P-value were calculated and used to identify significantly differentially expressed miRNAs (based on all-isoform value). Differentially expressed miRNAs between two samples were filtered through fold change (based on all-isoform value), followed by hierarchical clustering. miRNA target prediction was performed by the GO and KEGG pathway analyses were performed based on the differentially expressed miRNAs.

Results

Expression profiles of microRNAs in familial or sporadic PMAH cases compared with normal control

MicroRNA microarray analysis was respectively performed on tissue samples from 3 cases of patients with familial PMAH (Group1), 2 sporadic cases (Group2) as well as 3 normal control tissues (Group3) to compare the difference on microRNA expression levels. The differences on microRNAs expression were analyzed between patients with familial PMAH and normal controls to identify potential microRNA which may play important roles in the pathogenesis of familial PMAH. 16 microRNAs passed the FDR cutoff value of 0.05. 7 miRNAs were upregulated (hsa-miR-4306, hsa-miR-130a, hsa-miR-20b, hsa-miR-20a, hsa-miR-15a, hsa-miR-106a, hsa-miR-17) and 3 miRNAs were downregulated (hsa-miR-197, hsa-miR-3656, hsa-miR-3196) (Table 1). However, the following microRNAs exhibit differentially expression levels with statistical significance but signal intensities were very low, such as: hsa-miR-17*, hsa-miR-18b, hsa-miR-1976, has-miR-454, has-miR-629*, has-miR-629. Although we could detect some difference on microRNAs expression levels between the group of patients with familial PMAH and normal controls, expression levels of these microRNAs were statistically insignificant with p-value more than 0.05. After filtering low-intensity miRNAs, raw signal intensities were normalized by median. The differentially expressed miRNAs passed volcano plot filtering (Fig. 3A).

Similar analysis was performed on the differences of microRNAs expression levels between 2 sporadic patients with PMAH and 3 normal controls. Through filtering according to FDR cutoff value of 0.05 and expression signal intensities, 8 microRNAs were differentially expressed between above 2 groups, 1 miRNAs were upregulated (hsa-miR-3196) and 2 miRNAs were downregulated (hsa-miR-342-3p, hsa-miR-532-3p) (Table 2). However there was 5 microRNAs meeting requirements of statistical significance but their signal intensities were not strong enough, including hsa-miR-1976, hsa-miR-485-3p, hsa-miR-3195, hsa-miR-3180 and hsa-miR-445. After filtering low-intensity miRNAs, raw signal intensities were normalized by median. The differentially expressed miRNAs passed volcano plot filtering (Fig. 3B). The other microRNAs were found no expression difference in sporadic patients with PMAH compared to that of normal controls.

In order to identify the microRNAs which were differentially expressed between patients with familial and sporadic PMAH, we compared the microRNAs expression profiles of these two independent groups of samples. The same criteria of FDR cutoff value and expression intensities were applied to filter the candidate microRNAs. Results showed that, 2 miRNAs were upregulated (hsa-miR-342-3p, hsa-miR-532-3p) and 9 miRNAs were downregulated (hsa-miR-4306, hsa-miR-20b, hsa-miR-20a, hsa-miR-106a, hsa-miR-17, hsa-miR-101, hsa-miR-16, hsa-miR-26a, hsa-miR-106b) (Table 3). After filtering low-intensity miRNAs, raw signal intensities were normalized by median. The differentially expressed miRNAs passed volcano plot filtering (Fig.3C).

Validation of microRNA arrays by qRT-PCR

With the purpose of validating the results from microRNA microarrays, qRT-PCR was performed on total RNA extracted from familial and sporadic PMAH surgical tissues compared with the normal controls respectively. We evaluated microRNA level of has-miR-20b that, based on our microarray data, was differentially expressed in tissues from familial patients with PMAH compared with normal controls. The expression level of has-miR-20b in familial patients with PMAH was 1.56 fold higher than normal controls with p-value of 0.01165 which was in accordance with the results from microRNA arrays (3.11 fold higher with p-value=0.003463). The expression levels of has-miR-342-3p (0.68 fold downregulated with p-value=0.005814) were determined by qRT-PCR respectively in sporadic patients with PMAH and normal control samples to validate the comparison of microRNA expression profile between these 2 groups, which was consistent with the result from microRNA array (hsa-miR-342-3p was 0.21 fold downregulated in sporadic patients with PMAH versus normal controls). Similarly, hsa-miR-342-3p and hsa-miR-101 were selected to validate the different microRNA expression levels between familial and sporadic patients with PMAH, qRT-PCR results showed that, hsa-miR-342-3p was 1.58 fold upregulated while hsa-miR-101 was 0.40 fold downregulated in familial patients with PMAH compared to sporadic patients. These qRT-PCR test results were consistent with those from microRNA arrays in which hsa-miR-342-3p was 3.6 fold upregulated and hsa-miR-101 was 0.24 fold downregulated in familial patients with PMAH versus sporadic patients.

Hierarchical clustering of microRNAs with different expression levels

In order to identify the special microRNAs expression signatures among different groups of patients, hierarchical clustering analysis was performed on all microRNAs expression levels in the form of signal intensities included in microarrays. This hierarchical clustering analysis clustered microRNAs together according to their expression levels and samples from different groups based on similarity of the investigated microRNA expression profiles[24]. Supervised hierarchical pairwise comparison among these 3 groups of samples, we could always identify a special group of microRNAs which could discriminate one group from the other. The 16 differential expressed microRNAs signature from the results in microarray could clearly discriminate between familial patients with PMAH and normal controls including 10 downregulated microRNAs and 6 upregulated microRNAs (Fig.4A). Similarly, the microRNAs signature containing 8 differentially expressed microRNAs could discriminate between the sporadic patients with PMAH and normal controls with statistical significance p-value of 0.05 (Fig.4B). The identified 11 differentially expressed microRNAs could well discriminate between familial and sporadic patients with PMAH (table 3).

Comprehensive target-network prediction of differential expressed microRNAs and Pathway analysis

Functional analysis of the altered microRNAs between the PMAH patients and normal controls revealed that some enriched pathways such as signal transduction, signaling molecules and interaction metabolic, were potentially associated with the pathogenesis of PMAH. When comparison analysis was performed between familial patients with PMAH and normal controls, all of the 16 altered microRNAs were imported into KEGG Pathway analysis software to reveal that Circadian Rhythm pathway was the most affected pathway by these microRNAs with 6 genes predicted as the potential targets, including NPAS2, CRY2, BHLHE40, BHLHE41, CRY1 and CLOCK. At the same time, however, renal cell carcinoma pathway, mTOR signaling pathway, glioma pathway, pancreatic cancer pathway and endocytosis pathway were ranked top most signaling pathways affected by these altered microRNAs (Fig.5A). When KEGG Pathway analysis was similarly performed on the differentially expressed 8 microRNAs between the sporadic patients with PMAH and normal controls, renal cell carcinoma pathway, dilated cardiomyopathy pathway, axon guidance pathway, Ubiquitin mediated proteolysis, endocytosis and MAPK signaling pathway were ranked top most affected pathways on the basis of predicted targets, while renal cell carcinoma pathway contained 5 genes which was predicted as the potential downstream targets for the altered microRNAs, including CDC42, CUL2, EP300, GRB2 and SLC2A1 (Fig.5B).

Interestingly, the renal cell carcinoma pathway was ranked at top 2 most affected pathways among all associated pathways containing potential targets of the altered microRNAs which were differentially expressed in familial or sporadic patients with PMAH compared to normal controls. In comparison of altered microRNAs between familial patients with PMAH and normal controls, renal cell carcinoma pathway contained 25 genes which were targeted by these microRNAs(including EGLN3, EGLN2, EGLN1, PAK6, PAK7, CUL2, CDC42, RAC1, SOS2, GAB1, SLC2A1, TGFA, PAK1, PIK3R1, AKT3, PIK3R2, MAP2K1, MET, RAF1, MAPK1, HIF1A, CRKL, VEGFA, RAP1A and CRK), while 5 genes (including CDC42, CUL2, EP300, GRB2 and SLC2A1) were targeted by microRNAs which were differentially expressed between sporadic patients with PMAH and normal controls. These results indicated that renal cell carcinoma

pathway might play an important role in the pathogenesis of PMAH although PMAH was a benign hereditary endocrine abnormality without malignant characters (Fig.6).

Discussion

Recent researches indicated that microRNA expression signatures might be useful for the characterization and prediction of some certain diseases especially for benign or malignant tumors [25, 26], but the investigation of microRNA expression signatures on familial PMAH and sporadic patients with PMAH whose incidence was very low remained to be unclear. In this study microRNA microarray analysis was applied to identify the differentially expressed microRNAs between familial or sporadic patients with PMAH and normal controls, our results revealed that a series of altered microRNAs found in comparison of different groups could contribute to characterization of PMAH. Out of the differentially expressed microRNAs, hsa-miR-101, hsa-miR-20b and hsa-miR-342-3p were validated by qRT-PCR which showed directly correlated with our microRNA microarray data.

KEGG pathway analysis of the selected microRNAs and further validation by qRT-PCR revealed that these microRNAs had putative downstream targets involved into many important pathways including circadian rhythm pathway, renal cell carcinoma pathway, MAPK signaling pathway and some other signaling pathways.

Our results showed that the renal cell carcinoma pathway was ranked top most affected signaling pathways in both familial patients with PMAH and sporadic patients with PMAH compared to normal controls although PMAH was considered to be a cytologically benign disease without propensity for invasion and metastasis. CUL2, CDC42, GLUT1 and some other proteins, which had been demonstrated to be critical in malignant renal cell carcinoma, were also predicted to be downstream target proteins of microRNAs altered in PMAH patients compared with normal controls. Our findings in this study was consistent with the former researches from other groups, for instance, microRNA expression profile for massive macronodular adrenocortical disease shared differentially microRNAs with renal cell carcinoma which were previously implicated in tumorigenesis or metastasis[27]. In another research, several aberrantly expressed genes which were demonstrated in oncogenic pathways were also identified by gene expression profile in one PMAH patient [28]. Although PMAH were regarded benign for the reason that there had never been reported occurrence of metastasis and invasion in a long term post operative surveillance of patients[29], colossal increase in adrenocortical cell mass turned out be a typical characteristic for PMAH. Based on results mentioned above, it was indicated that there was some kind of abnormality occurred on the control of proliferation in adrenocortical cells which was similar to some extent to the characteristics of malignant tumors. Potential mechanisms underlying abnormality in cell apoptosis might include the following possibilities at least partially: normal cell cycle progression was disrupted through the altered microRNAs.

MiR-17 and miR-20a which were significantly upregulated between familial patients with PMAH and normal controls were two of six miRNAs of the miR-17-92 cluster consists (miR-17, -18a, -19a, -19b-1,

-20a, and 92a-1) generated from a single precursor RNA that is transcribed from chromosome 13[30]. Both miR-17 and miR-20a was reported to contribute to glucocorticoid-induced apoptosis of chondrocytes in lymphoma cells[31-33]. MiR-17 was reported that involvement of miR17 pathway was in glucocorticoid-induced cell death in pediatric acute lymphoblastic leukemia[34-36]. And the NR3C1-targeting miR-130b was more strongly expressed in a glucocorticoid-insensitive multiple myeloma cell line, and its introduction to a glucocorticoid-sensitive line impaired cellular response to glucocorticoid treatment, including the induction of apoptosis [37]. So, we might predict that overexpression of miR-17, miR-20a and miR-130b can inhibit glucocorticoid-induced apoptosis in the PMAH pathogenesis.

Hsa-miR-342-3p, hsa-miR-20b and hsa-miR-101 targeting CDC42, ERK and MEK in the MAPK signaling pathway, or abnormal expression of PI3K/AKT by the differentially expressed microRNAs might interfere the normal apoptosis progression in turn to promote cell survival of adrenocortical cells, in addition, TGF- β signaling pathway and VEGF signaling pathway were also involved into the pathogenesis of PMAH.

In comparison between the familial PMAH and normal controls, circadian rhythm pathway turned out to be the most affected signaling pathway according to downstream targets of the altered microRNAs. Circadian rhythm showed a universally 24-hour oscillation pattern in metabolic, physiological, endocrinal and behavioral functions of almost all species, core circadian genes such as PER, CRY and CLOCK seemed to be important for tissue homeostasis and tumorigenesis [38-40]. In the field of endocrinal diseases, key components of circadian rhythm pathway could interact with the steroid hormone receptors to exert influence on pathogenesis of some endocrinal tumors. Core circadian rhythm proteins such as PER2, CLOCK could be co-expressed with steroid hormone receptors and CLOCK associated genes could regulate glucocorticoid activity in almost all tissues through enhancing the transcriptional activity of glucocorticoid receptors[41, 42]. One of the potential underlying mechanisms was that, the acetylation of several lysine residues of glucocorticoid receptors and concomitant attenuation of GR binding to glucocorticoid response elements [43]. Due to the fact that PMAH patients were characterized by bilateral massive enlargement with dysfunctions of glucocorticoid hormone as well as their response receptors, circadian rhythm pathways might play an important role during the development of this disease through their interactions with glucocorticoid receptors.

Our results could only determine the differentially expressed microRNAs through comparing familial or sporadic patients with PMAH to normal controls, but the mechanism underlying the difference on microRNA expression profiles deserved to be further researched. We had collected clinical tissue samples from PMAH affected family members and sporadic cases, which provided us with sufficient materials to performed the next step of mechanism research. The microRNA expression signatures identified in comparison among different groups of patients need to be validated their effectiveness in other independent test sets of patients. In addition, it was remained to be determined whether the differentially expressed microRNAs in familial and sporadic patients with PMAH were directly involved into the pathogenesis. The microRNA expression profiles of familial patients with PMAH and sporadic patients with PMAH identified in this study turned out to be different, indicating that the mechanism underlying the pathogenesis familial patients with PMAH differed from that of sporadic patients with PMAH.

However, results from functional pathway analysis found that renal cell carcinoma pathway was associated with both familial and sporadic PMAH although different pathways were predicted to be involved into these two groups of patients respectively, for instance, circadian rhythm was ranked the first among associated pathways in familial patients with PMAH while mTOR signaling pathway might take critical part in sporadic PMAH pathogenesis.

Conclusion

In this study we used microRNA microarray to identify characteristic microRNA expression signatures for familial patients with PMAH including 16 differentially expressed microRNAs and sporadic patients with PMAH including 8 differentially expressed microRNAs respectively which were followed by validation of qRT-PCR. Hierarchical clustering analysis using these differentially expressed microRNAs could discriminate familial and sporadic patients with PMAH from normal controls therefore characteristic microRNA expression signatures for familial and sporadic patients with PMAH were identified. Pathway analysis on target proteins of these altered microRNAs indicated that renal cell carcinoma pathway and circadian rhythm might be involved into the pathogenesis of PMAH. Further elucidation of the exact roles that these altered microRNA, such as miR-17, miR-20a and miR-130b, play in the pathogenesis and progress of PMAH would contribute not only to our better understanding of this benign disease but also to development of new therapeutic and preventative strategies for PMAH.

Abbreviations

PMAH: Primary macronodular adrenal hyperplasia; miRNA: microRNA; RT-PCR: reverse transcription-polymerase chain reaction; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; CT: the computed tomographic; H&E staining: hematoxylin-eosin staining

Declarations

Ethics approval and consent to participate

This research was reviewed and approved by the ethics committee of General Hospital of the People's Liberation Army. Written informed consent for genetic studies was obtained prior to initiating this study in agreement with protocols approved by the institutional review board at the General Hospital of the People's Liberation Army.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

LC conceived the study, and developed its design; contributed in patients' assessment, sampling and biochemical testing. Also, participated in the analysis of molecular studies and patients' counseling and manuscript writing.

XG T performed molecular testing, data collection and participated in results analysis and manuscript writing.

JZ was involved in the study design, methods validation, data analysis, manuscript writing and review.

All authors read and approved the final manuscript

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Tables

Table 1 MiRNAs with significantly different expression ($P < .05$) between familial patients with PMAH (Group1) and normal control tissues (Group3)

miRNAs	Fold change	P value	Case/control
hsa-miR-4306	1.43	0.003223	Up
hsa-miR-130a	3.05	0.03120	Up
hsa-miR-20b	3.11	0.003463	Up
hsa-miR-20a	2.07	0.005487	Up
hsa-miR-15a	1.97	0.04733	Up
hsa-miR-106a	1.98	0.01258	Up
hsa-miR-17	1.98	0.01607	Up
hsa-miR-197	0.52	0.02572	Down
hsa-miR-3656	0.53	0.04458	Down
hsa-miR-3196	0.51	0.006570	Down
hsa-miR-17*		0.01237	Up
hsa-miR-18b		0.02489	Up
hsa-miR-1976		0.03086	Down
has-miR-629*		0.03518	Down
has-miR-629		0.03823	Up
has-miR-454		0.03496	Down

Table 2 MiRNAs with significantly different expression (P<.05) between sporadic cases (Group2) and normal control tissues (Group3)

miRNAs	Fold change	P value	Case/control
hsa-miR-342-3p	0.21	0.009685	Down
hsa-miR-532-3p	0.65	0.04271	Down
hsa-miR-3196	1.41	0.02156	Up
hsa-miR-1976		0.0125	Down
hsa-miR-485-3p		0.0241	Down
hsa-miR-3195		0.0272	Up
hsa-miR-3180		0.0288	Up
hsa-miR-445		0.0370	Up

Table 3 MiRNAs with significantly different expression (P<.05) between familial patients with PMAH(Group1) and sporadic cases (Group2)

miRNAs	Fold change	P value	Case/control
hsa-miR-342-3p	3.6	0.01188	Up
hsa-miR-532-3p	2.1	0.03911	Up
hsa-miR-4306	0.71	0.006598	Down
hsa-miR-20b	0.35	0.009508	Down
hsa-miR-20a	0.46	0.006258	Down
hsa-miR-106a	0.44	0.04284	Down
hsa-miR-17	0.44	0.02335	Down
hsa-miR-101	0.24	0.01947	Down
hsa-miR-16	0.64	0.02747	Down
hsa-miR-26a	0.81	0.02877	Down
hsa-miR-106b	0.46	0.02046	Down

Figures

Figure 1

Pedigree of the familial patients with PMAH. Arrow: proband

Figure 2

H&E staining $\times 200\times$ and CT imaging A $\square$ left adrenal nodule of familial PMAH H&E staining imaging B $\square$ left adrenal nodule of sporadic PMAH H&E staining imaging C $\square$ left adrenal lipoma of the normal control H&E staining imaging D $\square$ left adrenal nodule of familial PMAH CT imaging E $\square$ left adrenal nodule of sporadic PMAH CT imaging F $\square$ left adrenal lipoma of the normal control CT imaging

Figure 3

Volcano plot demonstrating differences in expression levels of microRNAs in 2 groups of PMAH patients and normal controls based on the microarray study. The log-fold changes were plotted based on the log

odds of differential expression. The microRNAs with significant differences in expression levels ($p \leq 0.05$) between familial patients with PMAH and normal controls after Benjamini-Hochberg correction are indicated in A. The microRNAs with significant differences in expression levels ($p \leq 0.05$) between sporadic patients with PMAH and normal controls after correction are indicated in B. The microRNAs with significant differences in expression levels ($p \leq 0.05$) between familial and sporadic patients with PMAH after correction are indicated in C.

Figure 4

microRNAs expression signature consisting of diffrentially expressed microRNAs between familial or sporadic PMAH and normal controls. A. Differentially expressed microRNAs ($p \leq 0.05$) were clustered and the results showed that the expression profiles of these 16 microRNAs could be used to separate between the familial PMAH and normal controls. B. Differentially expressed microRNAs ($p \leq 0.05$) were clustered and the results showed that the expression profiles of these 8 microRNAs could be used to separate between the sporadic PMAH and normal controls.

Figure 5

KEGG pathway analysis on differentially expressed microRNAs between familial or sporadic PMAH and normal controls. A. KEGG Pathway analysis was performed on 16 altered microRNAs to reveal that Circadian Rhythm pathway was the most affected pathway. B. KEGG Pathway analysis was performed on 8 altered microRNAs to reveal that renal cell carcinoma pathway was ranked the most affected pathway.

Figure 6

Renal cell carcinoma pathway was predicted to be target of microRNAs differentially expressed between PMAH patients and normal controls. The pink rectangles were indicated to be targets of microRNAs differentially expressed between familial patients with PMAH and normal controls, the blue rectangles were targets of microRNAs differentially expressed between sporadic patients with PMAH and normal controls, the purple rectangles were common targets of the 2 groups of differentially expressed microRNAs.