Differential microRNA expression in aristolochic acid-induced upper urothelial tract cancers *ex vivo*

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Received November 26, 2014; Accepted August 17, 2015

DOI: 10.3892/mmr.2015.4330

Abstract. Aristolochic acid (AA) is a carcinogenic, mutagenic and nephrotoxic compound commonly isolated from members of the plant family of Aristolochiaceae (such as *Aristolochia* and *Asarum*) and used in Chinese herbal medicine. Use of AA and AA-containing plants causes chronic kidney disease (CKD) and upper urinary tract carcinoma (UUC); however, the underlying mechanism remains to be defined. miRNAs regulate a number of biological processes, including cell proliferation, differentiation and metabolism. This study explored differentially expressed miRNAs between AA-induced upper urothelial tract cancer (AAN-UUC) and non-AAN-UUC tissues. Patients with AAN-UUC and non-AAN-UUC (n=20/group) were recruited in the present study. Five tissue samples from each group were used for miRNA microarray profiling and the rest of the tissue samples were subjected to reverse transcription-quantitative polymerase chain reaction analysis including seven selected miRNAs for confirmation. A total of 29 miRNAs were differentially expressed between AAN-UUC and non-AAN-UUC tissues ($P<0.05$). TargenScan and Gene ontology analyses predicted the functions and targeted genes of these differentially expressed miRNAs, i.e. Akt3, FGFR3, PSEN1, VEGFa and AR. Subsequently, expression of the selected differentially expressed miRNAs (Hsa-miR-4795-5p, Hsa-miR-488, Hsa-miR-4784, Hsa-miR-330, Hsa-miR-3916, Hsa-miR-4274 and Hsa-miR-181c) was validated in another set of tissue samples. A total of 29 miRNAs were identified to be differentially expressed between AAN-UUC and non-AAN-UUC tissues and these miRNA target genes in FGFR3 and Akt pathways, which regulate cell growth and tumor progression, respectively.

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Key words: aristolochic acid, microRNA, upper urothelial tract cancer, microRNA-488, microRNA-181c

Introduction

Aristolochic acid (AA) is a carcinogenic, mutagenic and nephrotoxic compound, which is commonly present in members of the plant family of Aristolochiaceae and is widely used in Chinese herbal medicine. Structurally, AA is related to nitrophenanthrene carboxylic acid, principally aristolochic acid I (AA-I) and aristolochic acid II (AA-II) (1). Consumption or administration of AA or AA-containing plants leads to nephrotoxicity and carcinogenesis, resulting in chronic kidney disease (2) and upper urinary tract carcinoma (UUC) (3). Balkan endemic nephropathy (BEN) and Chinese Herb Nephropathy (CHN) share the same etiology (4). Specifically, BEN is a chronic tubulointerstitial disease, affecting individuals living in the alluvial plains along the tributaries of the Danube River. BEN is closely associated with urothelial cell carcinoma of the upper urinary tract (5). The etiology of BEN was hypothesized to be due to ingestion of a toxic component of Aristolochia in bread prepared from flour with contaminated grain (6); however, due to the limitation of coeval methodology and technology, the etiology was not confirmed at that time and was unknown for >50 years (7). Another study suggested that Ochratoxin A (OTA) could be the cause of BEN due to the fact that individuals livings in regions where that had been an epidemic of BEN were exposed to relatively high OTA concentrations (8); however, Clark and Snedeker (9) demonstrated that high OTA levels in the blood and urine can occasionally be found in individuals not suffering from BEN (9). An epidemiologic and experimental study conducted by Grollman and Jelaković (10) denied the correlation between OTA and BEN, and instead demonstrated the presence of dA-aristolactam (AL) and dG-AL DNA adducts in the renal cortex of patients with BEN but not in patients with other chronic renal diseases using (32) P-post labeling/PAGE and authentic standards (11), which confirmed that AA is the cause of BEN. Furthermore, CHN gained attention in 1991 when nephrologists noticed an increase in the number of otherwise healthy females with different degrees of renal failure. These females all visited the same private clinic for weight control and had ingested extracts of

Chinese herbs containing *Aristolochia fangchi* (12), ~100 of these women developed chronic renal deficiency. Thereafter, Vanherweghem *et al* was the first to identify that the Chinese herbs were associated with chronic renal deficiency (12,13). Cosyns *et al* suggested that AA is the most likely cause of the renal injury and later development of urothelial-cell atypia and carcinoma (3). A number of studies also subsequently confirmed this hypothesis (2,14-16). Although AA is found primarily in members of the genus *Aristolochia*, it may be also present in other plant types (17). AA binds to genomic DNA after metabolic activation and forms AL-DNA adducts, generating a unique TP53 mutational spectrum in the urothelium. The AL-DNA adducts are concentrated in the renal cortex, which could serve as a biomarker for AA exposure (10,11,18). AA also specifically induces TP53 A:T→T:A mutation and is considered as the 'TP53 mutation signature' of BEN and AAN-UUC (18,19). However, the precise molecular mechanism underlying AA-induced BEN or UUC remains to be defined.

MicroRNAs (miRNAs) are a class of highly conserved small RNA molecules, which regulate key biological processes, including cell proliferation, differentiation, development and metabolism (20). Dysregulation of miRNA expression contributes to human cancer development; for example, miRNAs regulate all hallmarks of cancer in cell growth, cell cycle control, apoptosis, tumor invasion, metastasis and angiogenesis (21,22). Molecularly, miRNAs regulate the expression of various signal transduction pathway genes, such as transforming growth factor-β (TGFβ), WNT, Notch and epidermal growth factor (EGF) (23). A previous study showed aberrant expression of miRNAs in kidney, bladder and prostate cancer (24). Izquierdo *et al* (25) reported a differential miRNA expression pattern between patients with progressing and non-progressing UUC. Clinically, patients with BEN and CHN have an apparent higher risk of developing UUC than the normal population. Therefore, the present study aimed to investigate whether there is any difference in miRNA expression between AAN-induced UUC and common UUC using miRNA microarray analysis. The results validated the differentially expressed miRNAs using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Materials and methods

Patient samples. In the present study, paraffin-embedded tissue samples were collected from 20 patients with AA nephropathy (AAN-UUC) and 20 non-AAN-UUC patients, who had UUC but not associated with AA, treated in Shanghai Jiao Tong University-Affiliated First Hospital (Shanghai, China) between 2005 and 2010. All the patients were diagnosed according to medical history and pathology of tumor lesions. All the patients with AAN-UUC had a clear AA-containing drug intake history, and received cadaveric renal transplant between 2005 and 2010. Non-AAN-UUC patients did not have a history of AA contact, transplantation, and immunosuppressive drugs. Five samples from each group (AAN group, two males and three females; non-AAN group, four males and one female) were subjected to an miRNA microarray analysis and the rest of tissue samples (11 females and nine males in the AAN group, seven females and 13 males in the non-AAN group) were utilized as a set of samples for verification by RT-qPCR analysis. A protocol for the use of human surgical samples was approved by the Medical Ethics Committee of Shanghai First People's Hospital of Shanghai Jiao Tong University and each participant signed a written consent form for using their data in the present study. The patients were aged between 52 and 78 years.

miRNA microarray analysis. The miRNA microarray profiling was performed using Affymetrix GeneChip miRNA arrays (Affymetrix, Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was labeled by polyA polymerase addition using the Genisphere FlashTag HSR kit according to the manufacturer's instructions (Genisphere, Hatfield, PA, USA). The labeled RNA was hybridized as a probe to the Affymetrix miRNA array, according to the manufacturer's details. Standard Affymetrix array cassette staining, washing and scanning was performed using the post-hybridization kit (cat. no. 900720; Affymetrix) and GeneChip Scanner 3000 (Affymetrix, Inc.). Feature extraction was performed using Affymetrix Command Console software (v.1.2). The raw data were treated by the following workflow: Background detection, RMA global background correlation, quartile normalization, median polish and log2-transformation with miRNA QC tool software (Affymetrix).

Gene ontology (GO) and gene pathway analyses. The gene GO analysis was performed to evaluate differential expression. Pathway analysis was used to sort out the significant pathways of the differential genes according to KEGG, Biocarta and Reatome (26-28).

RNA isolation and RT-qPCR. Total cellular RNA was isolated from tissue samples and subjected to RT-qPCR analysis using an ABI 7900 HT Real-time PCR system in a 384-well plate format (cat. no. 4366596; Applied Biosystems, Foster City, CA, USA). Reverse transcription was performed, according to the manufacturer's instructions. Brifely, 1 μg RNA was reverse-transcribed into cDNA using a stem-loop RT primer. The reaction conditions were as follows: 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min. RNA was isolated from all samples using an mirVanaTM miRNA Isolation kit (cat. no. AM1560; Ambion Life Technologies, Foster City, CA, USA), according to the manufacturer's instructions. The PCR reaction volume of 5 μl contained 2.5 μl TaqMan PCR Master Mix-UNG (2X), 0.25 μl each TaqMan assay probe (20X), 1.25 μl of diluted cDNA and 1 μl H_2O. qPCR was performed at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min using the specific miRNA primers (Table I). The data were analyzed using ABI RQ Manager software (Applied Biosystems) after exportation as a SDS file.

Statistical analysis. An random variance model t-test was performed to analyze the microarray data by comparing the differentially expressed genes between the control and experimental group (29-31). The two-side Fisher's exact test was used to analyze the GO category data and the false discovery rate (FDR) was used to calculate the P-value as

Table I. MicroRNA kits used for reverse transcription-quantitative polymerase chain reaction.

Assay ID	Target sequence	Assay name
463364_mat	AGAAGUGGCUAAUAAUAUUGA	hsa-miR-4795-5p
002357	UUGAAAGGCUAUUUCUUGGUC	hsa-miR-488
463501_mat	UGAGGAGAUGCUGGGACUGA	hsa-miR-4784
000544	GCAAAGCACACGGCCUGCAGAGA	hsa-miR-330
464679_mat	AAGAGGAAGAAAUGGCUGGUUCUCAG	hsa-miR-3916
243075_mat	CAGCAGUCCCUCCCCCUG	hsa-miR-4274
000482	AACAUUCAACCUGUCGGUGAGU	hsa-miR-181c
Customer design	AGGAGAAGUAAAGUAGAA	has-miR-4434

Table II. Characteristics of patients with UUC.

Clinicopathological feature	AAN-UUC N	Non-AAN-UUC N	P-value
Age (mean ± SEM)	63.9±1.64	65.6±1.66	0.497
Gender			0.341
Male	9	13	
Female	11	7	
Tumor differentiation			0.240
Well	4	9	
Moderate	7	5	
Poor	9	6	
Tumor stage			0.519
I-II	9	7	
III-IV	11	13	
Lymph node metastasis			0.465
Yes	4	6	
No	16	14	
Distant metastasis			N/A
Yes	0	0	
No	20	20	
Tumor size (cm)			0.057
<3	14	8	
>3	6	12	

AAN, aristolochic acid; UUC, upper urinary tract carcinoma; SEM, standard error of the mean.

mentioned by Dupuy *et al* (32). The smaller the FDR, the lower the error in judgment of the P-value. The FDR was defined, according to the following equation:

$$FDR = 1 - \frac{N_k}{T}$$

N_k refers to the number of Fisher's test P-values that were below the χ^2 test P-values (32). T refers tot he total number of tests. The χ^2 test was used to evaluate patient characteristics (IBM SPSS version 19, IBM, Armonk, NY, USA). The unpaired 2-tailed Student's t-test was used to evaluate the association between miRNA expression and clinicopathological data from the tumor stage/size. The statistical analyses were performed either by SPSS software or Graphpad Prism 5 (Graphpad Software, Inc., San Diego, CA, USA).

Results

Characteristics of patients with UUC. A total of 20 samples each from patients with AAN-UUC and non-AAN-UUC were collected for miRNA microarray profiling of differentially expressed miRNAs. The clinical characteristics of these patients are listed in Table II. Specifically, all the patients with AAN-UUC had clear AA-containing drug intake history, and received cadaveric renal transplant between 2005 and 2010. A standard immunosuppressive regimen was administered to

Table III. Differential expression of microRNAs between AAN-UUC and non-AAN-UUC using miRNA microarray profiling analysis.

Name of miRNA	P-value	FDR	GMI (AAN-UUC)	GMI (UUC)	Fold-change	Change
hsa-miR-488-3p	0.0202204	<0.05	2.75	1.91	1.44	Up
hsa-miR-4434	0.0236167	<0.05	1.96	1.41	1.39	Up
hsa-miR-4274	0.0435406	<0.05	4.12	2.91	1.42	Up
hsa-miR-224-3p	0.0511639	<0.05	5.46	3.24	1.68	Up
hsa-miR-548x-3p	0.0578456	<0.05	4.26	3.8	1.12	Up
hsa-miR-890	0.0726481	<0.05	3.6	2.32	1.55	Up
hsa-miR-452-5p	0.0822436	<0.05	4.98	3.17	1.57	Up
hsa-miR-1272	0.0857332	<0.05	4.34	3.2	1.35	Up
hsa-miR-1294	0.1048154	<0.05	3.51	2.58	1.36	Up
hsa-miR-32-5p	0.1187305	<0.05	3.09	2.34	1.32	Up
hsa-miR-3910	0.1374783	<0.05	4.89	3.76	1.3	Up
hsa-miR-4795-5p	0.0064733	<0.05	1.29	1.74	0.74	Down
hsa-miR-4784	0.0231033	<0.05	2.8	3.29	0.85	Down
hsa-miR-330-3p	0.0280638	<0.05	4.52	5.66	0.8	Down
hsa-miR-3916	0.0409906	<0.05	3.62	4.79	0.76	Down
hsa-miR-181c-5p	0.047703	<0.05	3.65	5.17	0.71	Down
hsa-miR-342-5p	0.0551755	<0.05	5.43	6.75	0.8	Down
hsa-miR-4736	0.0581968	<0.05	1.73	2.58	0.67	Down
hsa-miR-15a-5p	0.0679372	<0.05	7.75	8.59	0.9	Down
hsa-miR-10a-5p	0.0930021	<0.05	7.33	8.93	0.82	Down
hsa-miR-4310	0.0981875	<0.05	2.18	3.12	0.7	Down
hsa-miR-4647	0.0987192	<0.05	2.43	3.11	0.78	Down
hsa-miR-4490	0.100964	<0.05	4.24	3.18	1.33	Down
hsa-miR-4695-3p	0.1017726	<0.05	3.14	4.13	0.76	Down
hsa-miR-3607-5p	0.1307393	<0.05	4.52	5.97	0.76	Down
hsa-miR-875-3p	0.1310434	<0.05	1.7	2.2	0.77	Down
hsa-miR-4499	0.1325093	<0.05	2.87	3.71	0.77	Down
hsa-miR-200c-3p	0.1352006	<0.05	13.34	14.01	0.95	Down
hsa-miR-3064-5p	0.1389973	<0.05	2.24	2.89	0.77	Down

GMI (AAN-UUC), geom mean of intensities in the AAN-UUC group; GMI (UUC), geom mean of intensities in the non-AAN-UUC group; FDR, false discovery rate; AAN, aristolochic acid; UUC, upper urinary tract carcinoma.

these patients, which included cyclosporine A, mycophenolate mofetil and prednisone with or without anti-lymphocyte antibody-induction therapy. All the enrolled patients were diagnosed with UUC during the follow-up, according to symptoms, including hematuria and pain, and CT scanning. Whereas, non-AAN-UUC patients had no history of contact with AA and did not undergo transplantation.

Differential expression of miRNAs in AAN-UUC tissues. The differential expression of miRNAs was profiled in AAN-UUC tissues using miRNA microarray analysis of five samples of AAN and non-AAN UUC tissues. The 29 most differentially expressed miRNAs were identified between AAN-UUC and non-AAN-UUC tissues (FDR<0.05, P<0.05; Table III and Fig. 1). In Fig. 1, a heat map is shown for the eight most significant differentially expressed miRNAs using GeneChip 3.0; each column represents a tissue sample, and each row represents an miRNA. The dendrograms of clustering analysis for samples and miRNAs are displayed on the top and left, respectively. Signals 1-5 represent AAN-UUC samples and signals 6-10 represent non-AAN-UUC tissue samples. Furthermore, TargetScan analyses were performed to predict the functions and targeted genes of these differentially expressed miRNAs. It was found that the mTOR, MAPK, focal adhesion, long-term potentiation and protein processing in endoplasmic signaling pathways were upregulated, whereas PI3K-Akt, HTLV-I infection, and the proteoglycan pathways were downregulated (Fig. 2). Among upregulated genes, VEGFA, RPS6KA6, IGF1, RPS6KA3 and FGFR3 were frequently upregulated in UUC tissues, whereas E2F3, FGFR1, IGF1R, AR and RAS were downregulated (Table. IV and V).

To further validate the microarray results, the eight genes with the greatest difference in expression compared with the non-AAN-UUC samples (P<0.05; hsa-miR-488-3p, hsa-miR-4434, hsa-miR-4274, hsa-miR-4795-5p,

Table IV. Upregulated genes by altered microRNAs in AAN-UUC tissues.

Path ID	Pathway name	Enrichment	P-value	FDR	Gene ID	Gene name
04150	mTOR signaling pathway	18.279	3.94E-06	3.75E-04	7422	VEGFA
04150	mTOR signaling pathway	18.279	3.94E-06	3.75E-04	27330	RPS6KA6
04150	mTOR signaling pathway	18.279	3.94E-06	3.75E-04	3479	IGF1
04150	mTOR signaling pathway	18.279	3.94E-06	3.75E-04	6197	RPS6KA3
04150	mTOR signaling pathway	18.279	3.94E-06	3.75E-04	1975	EIF4B
04150	mTOR signaling pathway	18.279	3.94E-06	3.75E-04	7248	TSC1
04010	MAPK signaling pathway	7.030	7.89E-06	3.75E-04	5908	RAP1B
04010	MAPK signaling pathway	7.030	7.89E-06	3.75E-04	5923	RASGRF1
04010	MAPK signaling pathway	7.030	7.89E-06	3.75E-04	6197	RPS6KA3
04010	MAPK signaling pathway	7.030	7.89E-06	3.75E-04	5906	RAP1A
04010	MAPK signaling pathway	7.030	7.89E-06	3.75E-04	2261	FGFR3
04010	MAPK signaling pathway	7.030	7.89E-06	3.75E-04	27330	RPS6KA6
04010	MAPK signaling pathway	7.030	7.89E-06	3.75E-04	9693	RAPGEF2
04010	MAPK signaling pathway	7.030	7.89E-06	3.75E-04	2122	MECOM
04010	MAPK signaling pathway	7.030	7.89E-06	3.75E-04	5534	PPP3R1
04010	MAPK signaling pathway	7.030	7.89E-06	3.75E-04	5601	MAPK9
04510	Focal adhesion	7.986	9.16E-06	3.75E-04	5908	RAP1B
04510	Focal adhesion	7.986	9.16E-06	3.75E-04	5923	RASGRF1
04510	Focal adhesion	7.986	9.16E-06	3.75E-04	5601	MAPK9
04510	Focal adhesion	7.986	9.16E-06	3.75E-04	5906	RAP1A
04510	Focal adhesion	7.986	9.16E-06	3.75E-04	7422	VEGFA
04510	Focal adhesion	7.986	9.16E-06	3.75E-04	3680	ITGA9
04510	Focal adhesion	7.986	9.16E-06	3.75E-04	4660	PPP1R12B
04510	Focal adhesion	7.986	9.16E-06	3.75E-04	2335	FN1
04510	Focal adhesion	7.986	9.16E-06	3.75E-04	3479	IGF1
04720	Long-term potentiation	15.447	1.07E-05	3.75E-04	5906	RAP1A
04720	Long-term potentiation	15.447	1.07E-05	3.75E-04	5908	RAP1B
04720	Long-term potentiation	15.447	1.07E-05	3.75E-04	6197	RPS6KA3
04720	Long-term potentiation	15.447	1.07E-05	3.75E-04	4660	PPP1R12B
04720	Long-term potentiation	15.447	1.07E-05	3.75E-04	5534	PPP3R1
04720	Long-term potentiation	15.447	1.07E-05	3.75E-04	27330	RPS6KA6
04141	Protein processing in endoplasmic reticulum	7.662	1.62E-04	4.53E-03	1965	EIF2S1
04141	Protein processing in endoplasmic reticulum	7.662	1.62E-04	4.53E-03	11231	SEC63
04141	Protein processing in endoplasmic reticulum	7.662	1.62E-04	4.53E-03	27248	ERLEC1
04141	Protein processing in endoplasmic reticulum	7.662	1.62E-04	4.53E-03	5601	MAPK9
04141	Protein processing in endoplasmic reticulum	7.662	1.62E-04	4.53E-03	10130	PDIA6
04141	Protein processing in endoplasmic reticulum	7.662	1.62E-04	4.53E-03	4287	ATXN3
04141	Protein processing in endoplasmic reticulum	7.662	1.62E-04	4.53E-03	7322	UBE2D2
04722	Neurotrophin signaling pathway	9.139	2.19E-04	5.10E-03	5906	RAP1A
04722	Neurotrophin signaling pathway	9.139	2.19E-04	5.10E-03	6197	RPS6KA3
04722	Neurotrophin signaling pathway	9.139	2.19E-04	5.10E-03	5601	MAPK9
04722	Neurotrophin signaling pathway	9.139	2.19E-04	5.10E-03	5663	PSEN1
04722	Neurotrophin signaling pathway	9.139	2.19E-04	5.10E-03	5908	RAP1B
04722	Neurotrophin signaling pathway	9.139	2.19E-04	5.10E-03	27330	RPS6KA6
04914	Progesterone-mediated oocyte maturation	10.627	4.56E-04	9.13E-03	27330	RPS6KA6
04914	Progesterone-mediated oocyte maturation	10.627	4.56E-04	9.13E-03	2771	GNAI2
04914	Progesterone-mediated oocyte maturation	10.627	4.56E-04	9.13E-03	3479	IGF1
04914	Progesterone-mediated oocyte maturation	10.627	4.56E-04	9.13E-03	6197	RPS6KA3
04914	Progesterone-mediated oocyte maturation	10.627	4.56E-04	9.13E-03	5601	MAPK9
04340	Hedgehog pathway	14.336	7.14E-04	1.25E-02	53944	CSNK1G1
04340	Hedgehog pathway	14.336	7.14E-04	1.25E-02	5727	PTCH1
04340	Hedgehog pathway	14.336	7.14E-04	1.25E-02	8945	BTRC

Table IV. Continued.

Path ID	Pathway name	Enrichment	P-value	FDR	Gene ID	Gene name
04340	Hedgehog pathway	14.336	7.14E-04	1.25E-02	51715	RAB23
04723	Retrograde endocannabinoid signaling	8.873	1.06E-03	1.66E-02	57030	SLC17A7
04723	Retrograde endocannabinoid signaling	8.873	1.06E-03	1.66E-02	5601	MAPK9
04723	Retrograde endocannabinoid signaling	8.873	1.06E-03	1.66E-02	2771	GNAI2
04723	Retrograde endocannabinoid signaling	8.873	1.06E-03	1.66E-02	2892	GRIA3
04723	Retrograde endocannabinoid signaling	8.873	1.06E-03	1.66E-02	222236	NAPEPLD
04114	Oocyte meiosis	8.160	1.57E-03	2.19E-02	27330	RPS6KA6
04114	Oocyte meiosis	8.160	1.57E-03	2.19E-02	8945	BTRC
04114	Oocyte meiosis	8.160	1.57E-03	2.19E-02	3479	IGF1
04114	Oocyte meiosis	8.160	1.57E-03	2.19E-02	6197	RPS6KA3
04114	Oocyte meiosis	8.160	1.57E-03	2.19E-02	5534	PPP3R1
05200	Pathways in cancer	4.472	1.91E-03	2.43E-02	2261	FGFR3
05200	Pathways in cancer	4.472	1.91E-03	2.43E-02	2335	FN1
05200	Pathways in cancer	4.472	1.91E-03	2.43E-02	7422	VEGFA
05200	Pathways in cancer	4.472	1.91E-03	2.43E-02	5727	PTCH1
05200	Pathways in cancer	4.472	1.91E-03	2.43E-02	2122	MECOM
05200	Pathways in cancer	4.472	1.91E-03	2.43E-02	3479	IGF1
05200	Pathways in cancer	4.472	1.91E-03	2.43E-02	4824	NKX3-1
05200	Pathways in cancer	4.472	1.91E-03	2.43E-02	5601	MAPK9
05031	Amphetamine addiction	10.445	2.41E-03	2.82E-02	9586	CREB5
05031	Amphetamine addiction	10.445	2.41E-03	2.82E-02	5534	PPP3R1
05031	Amphetamine addiction	10.445	2.41E-03	2.82E-02	2892	GRIA3
05031	Amphetamine addiction	10.445	2.41E-03	2.82E-02	6571	SLC18A2
04151	PI3K-Akt signaling pathway	4.214	2.81E-03	3.03E-02	3479	IGF1
04151	PI3K-Akt signaling pathway	4.214	2.81E-03	3.03E-02	3680	ITGA9
04151	PI3K-Akt signaling pathway	4.214	2.81E-03	3.03E-02	7248	TSC1
04151	PI3K-Akt signaling pathway	4.214	2.81E-03	3.03E-02	2335	FN1
04151	PI3K-Akt signaling pathway	4.214	2.81E-03	3.03E-02	2261	FGFR3
04151	PI3K-Akt signaling pathway	4.214	2.81E-03	3.03E-02	9586	CREB5
04151	PI3K-Akt signaling pathway	4.214	2.81E-03	3.03E-02	7422	VEGFA
04151	PI3K-Akt signaling pathway	4.214	2.81E-03	3.03E-02	1975	EIF4B
04728	Dopaminergic synapse	6.977	3.20E-03	3.20E-02	2771	GNAI2
04728	Dopaminergic synapse	6.977	3.20E-03	3.20E-02	5601	MAPK9
04728	Dopaminergic synapse	6.977	3.20E-03	3.20E-02	9586	CREB5
04728	Dopaminergic synapse	6.977	3.20E-03	3.20E-02	2892	GRIA3
04728	Dopaminergic synapse	6.977	3.20E-03	3.20E-02	6571	SLC18A2
04144	Endocytosis	5.376	3.81E-03	3.54E-02	2261	FGFR3
04144	Endocytosis	5.376	3.81E-03	3.54E-02	9525	VPS4B
04144	Endocytosis	5.376	3.81E-03	3.54E-02	9135	RABEP1
04144	Endocytosis	5.376	3.81E-03	3.54E-02	26052	DNM3
04144	Endocytosis	5.376	3.81E-03	3.54E-02	80223	RAB1-1FIP1
04144	Endocytosis	5.376	3.81E-03	3.54E-02	8027	STAM
04130	SNARE interactions in vesicular transport	15.232	4.04E-03	3.54E-02	9527	GOSR1
04130	SNARE interactions in vesicular transport	15.232	4.04E-03	3.54E-02	8417	STX7
04130	SNARE interactions in vesicular transport	15.232	4.04E-03	3.54E-02	8674	VAMP4
00565	Ether lipid metabolism	13.056	6.34E-03	5.11E-02	85465	EPT1
00565	Ether lipid metabolism	13.056	6.34E-03	5.11E-02	5048	PAFA-H1B1
00565	Ether lipid metabolism	13.056	6.34E-03	5.11E-02	8613	PPAP2B
05205	Proteoglycans in cancer	4.831	6.56E-03	5.11E-02	7422	VEGFA
05205	Proteoglycans in cancer	4.831	6.56E-03	5.11E-02	5727	PTCH1

Table IV. Continued.

Path ID	Pathway name	Enrichment	P-value	FDR	Gene ID	Gene name
05205	Proteoglycans in cancer	4.831	6.56E-03	5.11E-02	4660	PPP1R12B
05205	Proteoglycans in cancer	4.831	6.56E-03	5.11E-02	3479	IGF1
05205	Proteoglycans in cancer	4.831	6.56E-03	5.11E-02	1975	EIF4B
05205	Proteoglycans in cancer	4.831	6.56E-03	5.11E-02	2335	FN1
03013	RNA transport	5.539	8.87E-03	6.53E-02	1975	EIF4B
03013	RNA transport	5.539	8.87E-03	6.53E-02	8669	EIF3J
03013	RNA transport	5.539	8.87E-03	6.53E-02	1965	EIF2S1
03013	RNA transport	5.539	8.87E-03	6.53E-02	8661	EIF3A
03013	RNA transport	5.539	8.87E-03	6.53E-02	4686	NCBP1

FDR, false discovery rate.

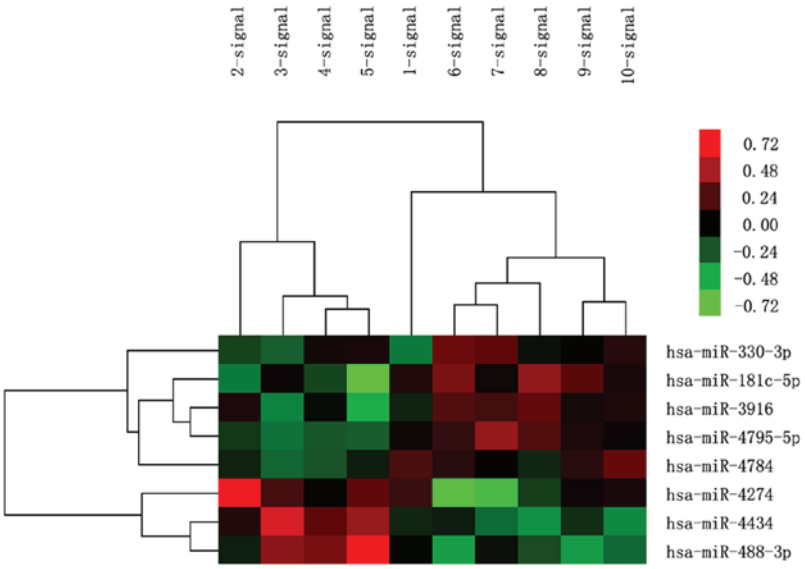

Figure 1. Heat-map of microarray analysis. Heat map shows up- (red spot) and down- (green spot) regulated miRNAs. Signal 1-5, AAN-UUC specimens; Signal 6-10, non-AAN-UUC specimens. AAN, aristolochic acid; UUC, upper urinary tract carcinoma.

hsa-miR-4784, hsa-miR-330-3p, hsa-miR-3916 and hsa-miR-181c-5p) were analyzed using qPCR. As a result, only expression of miR-488 and miR-181c was found to be significantly different ($P<0.05$; Fig. 3).

Furthermore, the expression of miR-488 was higher in stage I and II than stage III and IV tumors (mean ± standard error, $P=0.038$; Fig. 4), while miR-181c was highly expressed in tumors >3 cm than in those <3 cm (mean ± standard error, $P=0.049$; Fig. 5). However, these results in patients with non-AAN-UUC ($P=0.207$ and 0.127, respectively) were not validated. In addition, no other correlation was identified between miRNA expression and tumor behavior or prognosis.

Discussion

In the present study, the expression of miRNAs in AAN-UUC tissues was compared with that in the non-AAN-UUC tissues in order to identify the unique gene alterations for AAN-UUC in order to improve the understanding of this pathogenesis. The 29 most differentially expressed miRNAs were revealed between AAN-UUC and non-AAN-UUC tissues, which could regulate the most frequently altered genes in AAN-UUC, such as VEGFA, RPS6KA6, IGF1, RPS6KA3, FGFR3, E2F3, FGFR1, IGF1R, AR and RAS. As, miRNAs can regulate cellular growth, cell cycle control, apoptosis, invasion, metastasis, tumor angiogenesis (22) and carcinogenesis (33), their expression may be important in AAN-UUC development.

The present study used formalin-fixed and paraffin embedded (FFPE) tissue samples for miRNA microarray and RT-qPCR analyses. To date, there is no specific AAN-UUC-derived cell line available commercially. However, the FFPE tissue samples, are the most widely available clinical specimens for histological and pathological analysis (34), but contain fragmented nucleic acids. miRNA molecules are less prone to degradation for miRNA analysis in contrast to mRNA. Leite *et al* (35) demonstrated non-significant differences in miRNA expression between FFPE and fresh tissue samples. Moreover, qPCR is considered a gold standard

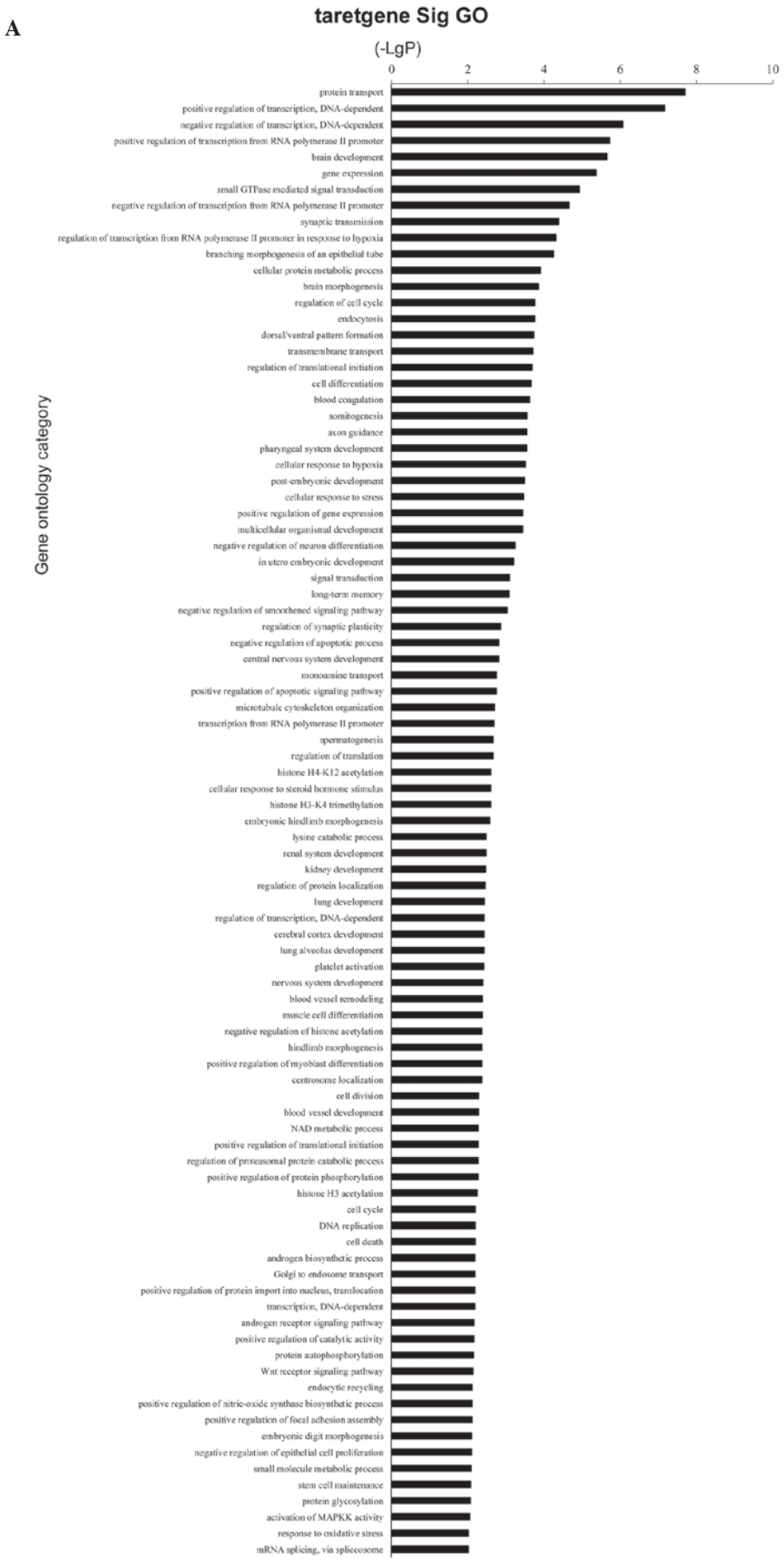

Figure 2. GO analysis of gene pathways that may be regulated by differentially expressed miRNAs in aristolochic acid-induced upper urinary tract carcinoma.tissues. (A) Upregulated gene pathways.

Figure 2. Continued. (B) Downregulated gene pathways. GO, gene ontology; AAN, aristolochic acid; UUC, upper urinary tract carcinoma.

Table V. Downregulated genes by altered miRNA in AAN-UUC tissues.

Path ID	Pathway name	Enrichment	P-value	FDR	Gene ID	Gene name
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	1871	E2F3
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	2260	FGFR1
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	2308	FOXO1
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	2932	GSK3B
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	3480	IGF1R
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	3551	IKBKB
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	367	AR
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	3845	KRAS
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	5156	PDGFRA
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	5594	MAPK1
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	5604	MAP2K1
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	64764	CREB3L2
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	6654	SOS1
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	6655	SOS2
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	6934	TCF7L2
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	7184	HSP90B1
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	8503	PIK3R3
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	1387	CREBBP
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	1869	E2F1
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	10000	AKT3
05215	Prostate cancer	10.050	5.74E-15	1.21E-12	1385	CREB1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	23678	SGK3
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	2932	GSK3B
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	3480	IGF1R
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	3551	IKBKB
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	3558	IL2
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	3696	ITGB8
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	5529	PPP2R5E
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	5586	PKN2
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	5594	MAPK1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	5604	MAP2K1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	5618	PRLR
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	5747	PTK2
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	59345	GNB4
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	6198	RPS6KB1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	6256	RXRA
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	3845	KRAS
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	4254	KITLG
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	5156	PDGFRA
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	54541	DDIT4
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	672	BRCA1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	7184	HSP90B1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	7532	YWHAG
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	8503	PIK3R3
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	896	CCND3
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	2321	FLT1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	10000	AKT3
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	10053-3105	C8orf-44-SGK3
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	1293	COL6A3
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	1385	CREB1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	1977	EIF4E

Table V. Continued.

Path ID	Pathway name	Enrichment	P-value	FDR	Gene ID	Gene name
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	2260	FGFR1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	23035	PHLPP2
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	2309	FOXO3
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	6696	SPP1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	64764	CREB3L2
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	6654	SOS1
04151	PI3K-Akt signaling	4.541	1.06E-13	1.11E-11	6655	SOS2
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	2122	MECOM
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	2260	FGFR1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	2308	FOXO1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	25	ABL1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	2113	ETS1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	51684	SUFU
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	5579	PRKCB
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	5594	MAPK1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	5604	MAP2K1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	6934	TCF7L2
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	7046	TGFBR1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	7184	HSP90B1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	7428	VHL
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	7976	FZD3
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	8503	PIK3R3
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	862	RUNX1T1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	868	CBLB
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	6655	SOS2
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	5747	PTK2
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	6256	RXRA
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	6654	SOS1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	1869	E2F1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	3480	IGF1R
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	10000	AKT3
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	1387	CREBBP
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	2932	GSK3B
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	1871	E2F3
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	5156	PDGFRA
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	26060	APPL1
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	367	AR
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	324	APC
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	4254	KITLG
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	3551	IKBKB
05200	Pathways in cancer	4.428	2.42E-12	1.70E-10	3845	KRAS

FDR, false discovery rate.

for quantification of gene expression and has been widely employed as a validation method for microarray studies (36). Thus, the present study demonstrated novel and reliable results, however, these are preliminary data and more in depth studies are required to understand the role of miRNA in the pathogenesis of AAN-UUC. In this regard, we aim to validate the current data by collecting more fresh AAN-UUC tissues and generate a primary cell culture to investigate how these miRNAs are altered and involved in the regulation of tumor cell growth, apoptosis, invasion, metastasis and angiogenesis.

In the present miRNA microarray study, the most down-regulated miRNAs were hsa-miR-4795-5p, hsa-miR-4784, hsa-miR-330-3p, hsa-miR-15a-5p, hsa-miR-10a-5p, hsa-miR-181c and hsa-miR-200c-3p, whereas the most

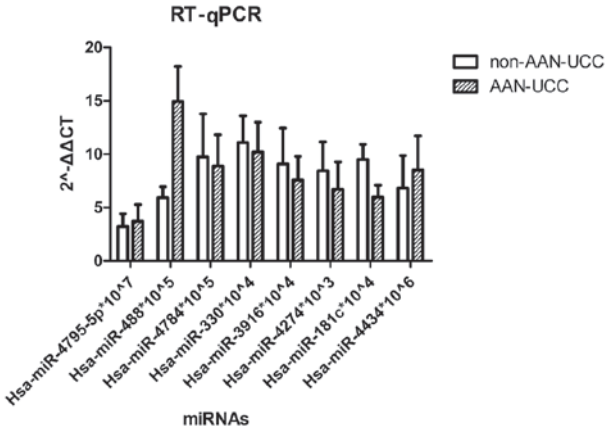

Figure 3. Reverse transcription-quantitative polymerase chain reaction confirmation of differentially expressed miRNAs analyzed by the miRNA microarray between AAN-UUC and non-AAN-UUC tissue samples. miRNA, microRNA; AAN, aristolochic acid; UUC upper urinary tract carcinoma.

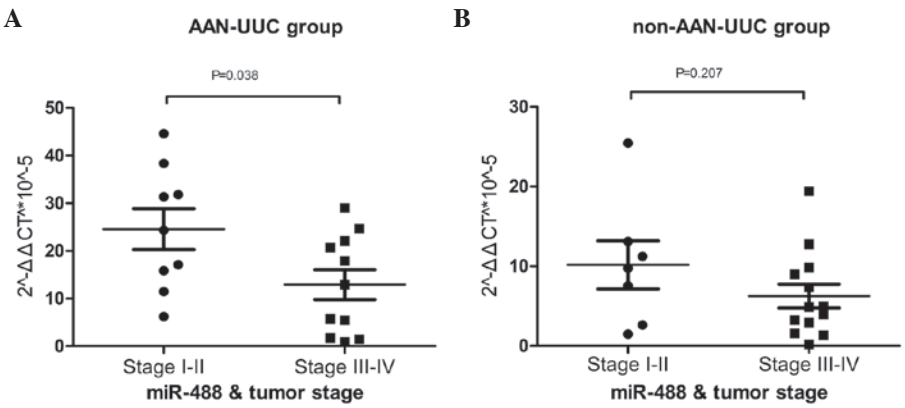

Figure 4. Differential expression of miR-488 in early vs. late tumor stages. (A) AAN-UUC and (B) non-AAN-UCC. miRNA, microRNA; AAN, aristolochic acid; UUC upper urinary tract carcinoma.

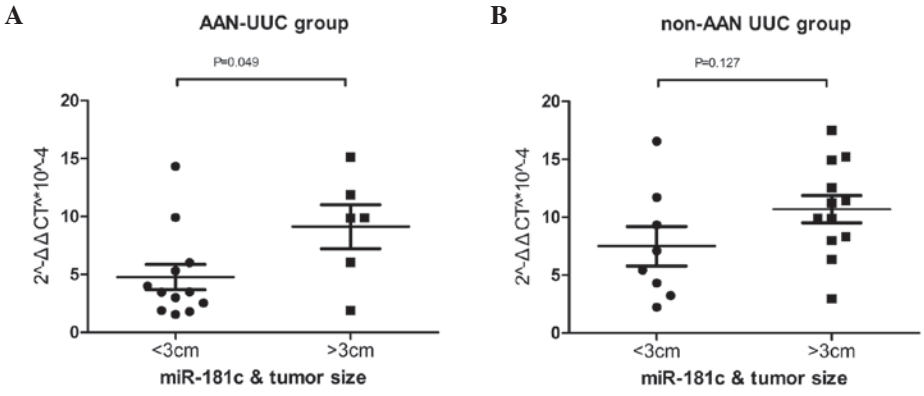

Figure 5. Differential expression of miR-181c in small vs. large tumors. (A) AAN-UUC and (B) non-AAN-UCC. miRNA, microRNA; AAN, aristolochic acid; UUC upper urinary tract carcinoma.

upregulated miRNAs were hsa-miR-488-3p, hsa-miR-4434, hsa-miR-4274 and hsa-miR-224-3p. These miRNAs were previously reported to be associated with the development and progression of different types of human cancer (37-39). For example, 5-fluorouracil treatment upregulated miR-4795-5p in nasopharyngeal carcinoma cell lines (37). In addition, miR-4795-5p is also downregulated in stage II colorectal cancer (38). Similarly, miR-200c expression was found to be lost in pancreatic cancer, and patients with high levels of miR-200c expression had significantly longer survival rates than those with low levels (39). Expression of miR-200c has also been shown to be associated with upregulation of the expression of E-cadherin and downregulation of ZEB1 and ZEB2 in bladder cancer cell-lines (39-41). In addition,

miR-181c was also shown to be highly expressed in gastric cancer tissues compared with gastric ulcer and chronic gastritis tissues (42). By contrast, the present study showed that miR-488-3p was upregulated in AAN-UUC compared with non-ANN-UUC tissues. However, in the majority of published studies (40,41), miR-488-3p was downregulated in different types of human cancer. miR-488 is able to inhibit the expression of androgen receptor (AR) in prostate cancer cells (40). Sikand *et al* (43) showed that overexpression of miR-488 downregulated the transcriptional activity of AR and inhibited the endogenous AR protein production in androgen-dependent and androgen-independent prostate cancer cells. Moreover, a study by Li *et al* (44) demonstrated that suppression of miR-448 expression induced epithelial-mesenchymal transition by directly targeting SATB1 mRNA, and the latter promoter could be bound by activated NF-κB. It is unknown why this discrepancy occurred, however, it may be due to high levels of miR-488-3p expression in non-AAN-UUC compared with that of AAN-UUC tissues. Further investigation using larger sample sizes is therefore required to confirm these results. Meng *et al* (45) recently published a study showing the effects of miR-21 and miR-34a levels in an AA-induced rat model; however, these two miRNAs were not identified in the present study. The discrepancy may be due to the differences in the nature of the design of the studies as Meng *et al* conducted an *in vitro* study and the present study was *ex vivo*.

Cancer is a group of human diseases with various heterogeneity, which could limit the reproducibility of changes in microRNA expression profiles; even the same tumor lesion may have different gene alterations. For instance, in bladder cancers, low-grade tumors exhibited downregulation of numerous miRNAs, and the most downregulated were miRs-99a/100, which were demonstrated to target FGFR3. Accoring to the literature, high-grade bladder cancer often exhibits upregulated levels of miR-21, and miR-21 can target P53. High-grade bladder cancer is characterized by marked miRNA upregulation (46,47), whereas low-grade bladder cancer often exhibits miRNA downregulation. Compared with non-AAN-UUC, AAN-UUC has a distinctive gene alteration pattern, such as AL-DNA adducts and a unique TP53 mutational spectrum A:T→T:A, which implies the presence of a distinctive pathway. Following metabolic activation, AA reacts with genomic DNA to form AL-DNA adducts that generate a unique TP53 mutational spectrum in the urothelium (A:T→T:A). Transcription factor p53 protein is a tumor suppressor. It is the most commonly mutated gene in human cancer and is associated with the alteration of cellular bioactivity (48). p53 protein not only regulates the expression of miRNAs, but is also a target of these miRNAs. For example, miR-34, miR-200 family, miR-192 family, miR-107, miR-145, miR-15a, and miR-16-1 have been identified to be modulated by p53; while miR-504, miR-33, miR-125b, miR-1285 and miR-380-5p have been reported to directly target p53 (49).

Certain classical Aristolochic herbs, such as fangchi and mutong have been banned in a number of countries (17,50). However, AA intake still occurs via contaminated grain in some Balkan regions. Moreover, certain AA-containing herbs could still be used due to lack of recognition (17). Species of *Aristolochia* are widely distributed worldwide, with the exception if Australia, where only few species are known. Since ethnobotanical investigations have indicated that AA family members are frequently used in traditional medicine, it is likely that certain individuals take AA-containing herbs or combinations without being aware of it (4). Furthermore, due to the prevalence of Chinese traditional medicine in China and other Asian countries, these herbal remedies are readily available via the internet. A survey conducted in Taiwan (51) showed that approximately one-third of the population of Taiwan has been exposed to herbs containing AA. In traditional Chinese medicine, a phenomenon termed the 'Jun-Chen-Zou-Shi' principle entails constructing a remedial herbal formula to mitigate the toxicity of the main ingredient. Recently Tsai *et al* (52) performed metabolic analysis using ^{1}H-NMR spectroscopy to validate whether Bu-Fei-A-Jiao-Tang, a compound remedy based on this principle, could decrease the toxicity of AA-containing herbs; however, the result did not support this claim. Thus, the function of the Jun-Chen-Zou-Shi principle was not able to ensure a reduction in AA nephrotoxicity. Despite increasing control of the use of AA by different countries, it is still accessible in numerous ways.

In conclusion, the current study showed that AAN-UUC may have unique miRNA alterations compared with non-AAN-UUC. Further studies using larger sample sizes are proposed to better understand the molecular mechanism underlying AAN-UUC development.

Acknowledgements

This study was supported in part by a grant from the Shanghai Municipal Health Bureau (grant no. XBR 2011038) and from the Medical Induction Promotion Project of Shanghai Natural Science Foundation (grant no. 134119a2801).

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