Identification and characterization of miR-96, a potential biomarker of NSCLC, through bioinformatic analysis

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Received November 8, 2016; Accepted June 6, 2017

DOI: 10.3892/or.2017.5754

Abstract. Lung cancer is the leading cause of cancer-related death worldwide. The poor prognosis is partly due to lack of efficient methods for early diagnosis. MicroRNAs play roles in almost all aspects of cancer biology, and can be secreted into the circulation and serve as molecular biomarkers for the early diagnosis of cancer. In the present study, we determined the expression of miR-96 and the function of its target genes in lung cancer through bioinformatic analysis. Four microRNA expression profiles of lung cancer were downloaded from Gene Expression Omnibus and the data were analyzed using SPSS 16.0 software. Compared to the control group, expression of miR-96 was significantly increased in non-small cell lung cancer (NSCLC) (GSE51855), lung adenocarcinoma (GSE48414), stage I adenocarcinoma tissues (GSE63805) and the plasma of lung cancer patients (GSE68951). miR-96 was also elevated in six different NSCLC cell lines. However, the expression level of miR-96 was not related to the age, gender, clinical stage and histological subtype of the NSCLC patients. GO analysis of 78 predicted target genes of miR-96 showed that 42 of the obtained GO terms are highly associated with specific cellular processes including response to stimulus, signaling pathway, cell division, cell communication, cell migration and calcium signaling. KEGG results indicated that the miR-96 targets are mainly involved in the GnRH signaling pathway, long-term potentiation and insulin signaling pathway. In conclusion, miR-96, functioning as an oncogene, may play an important role in the development and progression of lung cancer. miR-96 may have the potential to serve as a molecular biomarker for the early diagnosis of NSCLC.

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Key words: non-small cell lung cancer, miR-96, bioinformatics

Introduction

Based on GIOBALCAN, an estimated 1.8 million new lung cancer cases and 1.6 million deaths occurred in 2012. This makes lung cancer the leading cause of cancer-related death among males worldwide and females in developed countries (1). The poor prognosis is attributed to the lack of efficient methods for early diagnosis and lack of successful treatment for metastasis. Since non-small cell lung cancer (NSCLC), which accounts for approximately 85% of all lung cancer cases, is not very sensitive to chemotherapy and/or radiation, surgery remains the treatment of choice. However, most newly diagnosed NSCLC patients cannot undergo surgery due to local invasion or distant metastasis. Therefore, it is particularly important to study the molecular mechanisms underlying NSCLC, which may provide novel molecular targets for the early diagnosis of lung cancer.

MicroRNAs (miRNAs) are a group of non-coding RNAs (~22 nucleotides) that can degrade target mRNA transcripts directly or suppress their translation through complete or partial complementarity recognizing the 3'UTR of target mRNAs (2). miRNAs have been proven to play an important role in the post-transcriptional regulation of gene expression and are involved in almost all aspects of cancer biology such as tumor transformation, growth, angiogenesis and epithelial-mesenchymal transition by inhibiting specific oncogenes or tumor-suppressor genes. Accumulating data indicate that miRNAs are present in body fluids including blood plasma and serum, urine, saliva and semen (3,4) and circulating miRNA levels are more accurate than the protein-coding gene profiles in tumor typing (5). Therefore, miRNAs are more likely to be novel molecular biomarkers in the screening and monitoring of cancer patients (6).

In our previous study, we found that DAL-1 (differentially expressed in adenocarcinoma of the lung-1; also known as EPB41L3, 4.1B) has an important role in the invasion and metastasis of NSCLC (7). By using microRNA.org, TargetScan and PicTar, we predicted four miRNAs, miR-26a, miR-26b, miR-96 and miR-223, that regulate DAL-1. Data from several studies previously showed that miR-223 does not only promote the invasion of lung cancer cells but also the metastasis of gastric cancer via targeting tumor suppressor DAL-1 (8,9). Our previous study demonstrated that both miR-26a and DAL-1 gene expression are decreased in NSCLC, and DAL-1

is not a real target gene of miR-26a (10). Both miR-26b and miR-26a belong to the miR-26 family, and miR-26b has low expression levels in many types of cancer, such as epithelial ovarian (EOC) (11), hepatocellular carcinoma (HCC) (12), as well as colorectal cancer (13). In this study, we chose miR-96 as our research target.

MicroRNA-96 (hsa-miR-96, miR-96), located on chromosome 7 (7q31~34), belongs to the miR-183 gene family, which is the first gene cluster to be reported in the development and function of ciliated ectodermal cells and organs and is essential for the development and function of animal sensory organs (14,15). With the growing interest in the miR-183 gene family, miR-96 has been detected to be highly expressed in various human tumors and involved in cancer development by regulating key genes in tumor cell division and apoptosis (16-18). Although studies have shown that miR-96 is overexpressed in lung cancer (19,20), it still remains unclear whether miR-96 could be used for early diagnosis and how miR-96 affects the progression of lung cancer. Herein, we determined the expression of miR-96 and the function of its target genes in lung cancer through bioinformatic analysis, aiming to ascertain whether it is a potential molecular biomarker for the early diagnosis of NSCLC and to obtain clues for the pathogenesis of lung cancer.

Materials and methods

Affymetrix microarray. The microRNA expression profiles of lung cancer (GSE51855, GSE48414, GSE63805, GSE68951) were downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), which are based on the platform of Affymetrix Human Genome U133 Plus 2.0 Array.

Probe re-annotation. Four TEX texts (GPL7341, GPL16770, GPL18410, GPL16770) were downloaded from GEO public data platform, to find the probe number of the hsa-miR-96 gene in GSE51855, GSE48414, GSE63805, GSE68951, respectively.

Cell culture. The following cell lines were cultured individually in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA): human lung adenocarcinoma (A549, NCI-H1299 and pAa), human lung large cell carcinoma (NCI-H460), human lung squamous cell carcinoma (NCI-H520), human lung small cell carcinoma (NCI-H446), human lung giant-cell carcinoma (95D) and human bronchial epithelial (16HBE) cell lines. The medium was supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). Cells were maintained in 5% CO_2 at 37˚C.

Real-time quantitative PCR. Specific RT primers and TaqMan probe (American ABI Company) were used for quantitative detection of hsa-miR-96 (cat no. A25576) and reference gene U6 (cat no. 4426961). Total RNAs in cells were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA yield and the ratio of absorbance at 260 to 280 nm (A260/A280 ratio) were determined with the NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). The cDNA synthesis and qRT-PCR were carried out using the TaqMan MicroRNA Reverse Transcription kit and TaqMan MicroRNA assays and TaqMan® Universal Master Mix, No AmpErase® UNG (all from ABI, USA), respectively, according to the manufacturer's protocol. qRT-PCR was carried out using Applied Biosystems® 7500 real-time PCR systems (Applied Biosystems, Foster City, CA, USA). The experiment was repeated 3 times. The relative quantitative analysis was carried out using the ΔΔCt method and the control was used for normalization of miRNA expression.

Bioinformatic analysis of miR-96 target genes. The target genes of miR-96 were predicted using miRecords. The intersection prediction results from at least 6 miRNA target gene prediction databases were analyzed to reduce the false-positive rate. To explore the functional annotation and pathway enrichment of those predicted genes, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database analyses were conducted using a Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 online analysis tool with $P<0.05$ as the significant threshold to obtain significant gene sets.

Statistical analysis. All data are presented as mean ± SD and statistical analyses were processed using SPSS 16.0 statistical software. Wilcoxon's rank-sum test was used to compare the expression of miR-96 between lung cancer and normal lung tissues/plasma in GSE51855, GSE48414 and GSE68951. Wilcoxon matched-pairs signed ranks sum test was used to analyzed the miR-96 expression in GSE63805. Wilcoxon's rank-sum test and Kruskal-Wallis test were conducted to analyze the correlation of miR-96 expression with the clinicopathological features in GSE48414 and GSE51855. Independent-sample t-test was conducted to evaluate the miR-96 expression in lung cancer cell lines and 16HBE cells. A P-value of <0.05 was considered statistically significant.

Results

Expression of miR-96 in lung cancer tissues, plasma and cell lines. We analyzed four microRNA expression profiling datasets to explore the expression pattern of miR-96 in the tissues and plasma of lung cancer patients. The result indicate that, compared with the normal lung tissues, miR-96 was significantly increased in NSCLC (GSE51855, Fig. 1A and Table I, $P<0.001$), lung adenocarcinoma (GSE48414, Fig. 1B and Table II, $P<0.001$) and stage I adenocarcinoma tissues (GSE63805, Fig. 1C and Table III, $P<0.001$). In addition, the expression level of miR-96 in the plasma (GSE68951) of the lung cancer patients was significantly higher compared to that of the non-cancer lung disease patients (Fig. 1D and Table IV, $P<0.05$).

We subsequently examined the level of miR-96 in different types of lung cancer cell lines and bronchial epithelial 16HBE cells using qRT-PCR. As shown in Fig. 2, the expression level of miR-96 was elevated in all the 6 NSCLC cell lines but downregulated in the small cell lung cancer NCI-H446 cells. The highest expression levels for miR-96 were found in squamous cell carcinoma NCI-H520, adenocarcinoma NCI-H1299 and pAa cells ($P<0.001$ for each).

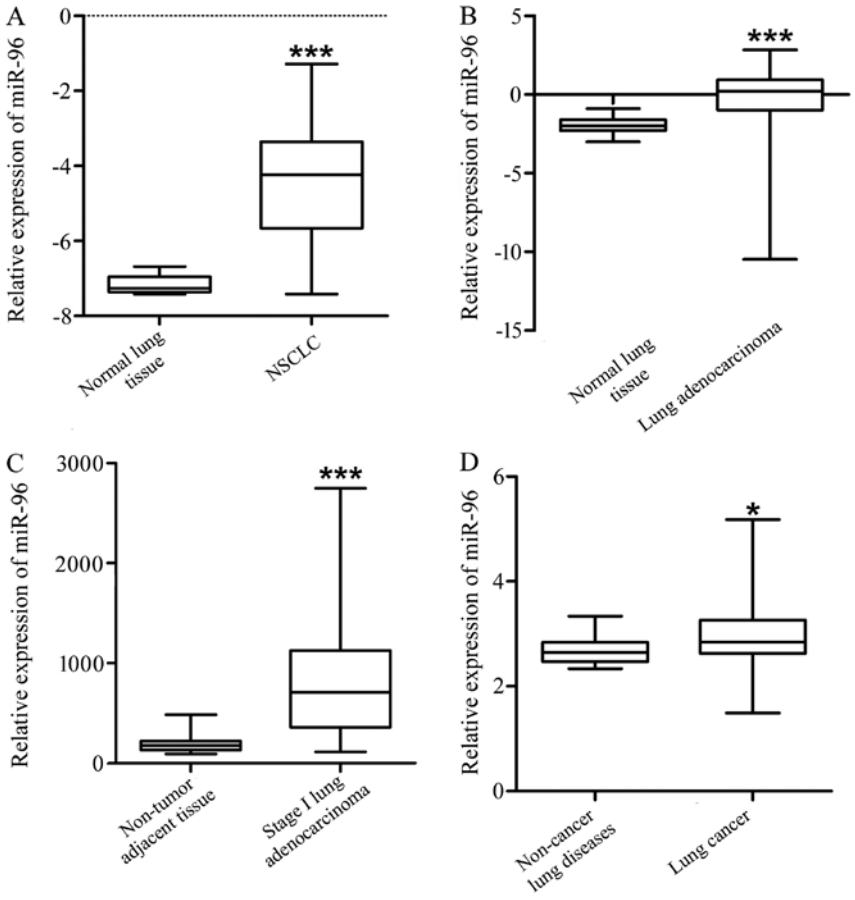

Figure 1. Expression of miR-96 in tissues and plasma from patients with lung cancer. (A) Dataset GSE51855 showed the miRNA profiling of 126 NSCLC and 5 normal lung tissues. miR-96 was overexpressed in the NSCLC tissues (P=0.000). (B) Dataset GSE48414 showed the miRNA profiling of 154 lung adenocarcinoma and 20 paired normal lung tissues. miR-96 was overexpressed in the lung adenocarcinoma tissues (P=0.000). (C) Dataset GSE63805 showed the miRNA profiling of 32 stage I lung adenocarcinoma and 30 matched non-tumor adjacent tissues. miR-96 was overexpressed in the stage I lung adenocarcinoma tissues (P=0.000). (D) Dataset GSE68951 showed the plasma miRNA profiling of 26 lung cancer and 12 non-cancer lung diseases. miR-96 was overexpressed in the plasma of the lung cancer patients (P=0.031). NSCLC, non-small cell lung cancer.

Table I. miR-96 expression in NSCLC and normal lung tissues (GSE51855).

Variables	N	Expression of miR-96	χ^2/X	P-value
Total	131			
NSCLC	126	-4.3623±0.12467	-3.699	0.000
NA	5	-7.1807±0.12815		

NSCLC, non-small cell lung cancer; NA, normal lung tissues.

Table II. miR-96 expression in lung adenocarcinoma and normal lung tissues (GSE48414).

Variables	N	Expression of miR-96	χ^2/Z	P-value
Total	174			
ADC	154	-0.548±0.12819	-5.804	0.000
NA	20	-1.9477±0.11705		

NSCLC, non-small cell lung cancer; NA, normal lung tissues.

Table III. miR-96 expression in adenocarcinoma and adjacent non-tumor lung tissues (GSE63805).

Variables	N	Expression of miR-96	χ^2/Z	P-value
Total	62			
Stage I ADC	32	849.71±114.63	-4.638	0.000
NA	30	184.93±13.41056		

ADC, adenocarcinoma; NA, adjacent non-tumor lung tissues.

Table IV. miR-96 expression in the serum of NSCLC and non-cancerous pulmonary disease patients (GSE68951).

Variables	N	Expression of miR-96	χ^2/Z	P-value
Total	38			
Lung cancer	26	2.99409±0.0028	-2.159	0.031
Non-cancerous lung diseases	12	2.687812±0.0226		

NSCLC, non-small cell lung cancer.

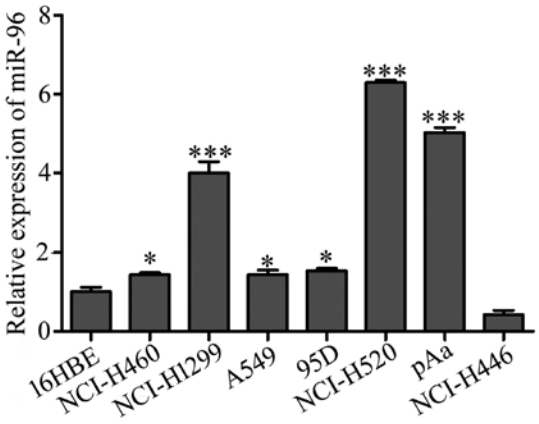

Figure 2. Expression of miR-96 in lung cancer cell lines. miR-96 expression in 7 lung cancer cell lines and human bronchinal epithelial 16HBE cells was determined by qRT-PCR. miR-96 was markedly increased in 6 NSCLC cell lines. *P<0.05, ***P<0.001 vs. 16HBE cells.

Correlation between miR-96 expression and clinicopathological features of NSCLC. We then analyzed the correlation between miR-96 expression and the clinicopathological features of NSCLC to further explore the potential role of miR-96 in the development and progression of lung cancer. Our results showed that the expression level of miR-96 in the tumors was not related to the age (P=0.631), sex (P=0.678), clinical stage (P=0.841) and histological subtype (P=0.051) of the NSCLC patients (GSE48414 and GSE51855, Tables V and VI).

Bioinformation analysis of miR-96

Prediction of miR-96 targets. Next, we used miRecords database to investigate miR-96 targets. miRecords does not only provide the target gene prediction of miRNAs but also the exact target genes regulated by miRNAs, which have already been experimentally validated. As shown in Table VII, a total of 71 target genes of miR-96 were predicted by at least six prediction softwares involved in miRecords. Ten miR-96 target genes were found and experimentally validated in the miRecords database, among which ADCY6, IRS1 and MYRIP were also in the prediction list. Finally, 71 predicted and 7 validated miR-96 targets (Table VII) were involved in the GO and KEGG analysis.

Gene ontology and KEGG pathway enrichment analysis of miR-96 target genes. Gene ontology enrichment analysis was performed to analyze 78 miR-96 target genes (Table VII). In total, 42 GO terms were obtained, which included 24 biological processes, 15 cellular components and 3 molecular functions. These 42 GO terms were sorted by P-values for further analysis and are listed in Tables VIII and IX.

Among the 24 biological process GO terms, the top 10 terms were: GO:0009725 (response to hormone stimulus), GO:0009719 (response to endogenous stimulus), GO:0010033 (response to organic substance), GO:0016197 (endosome transport), GO:0032228 (regulation of synaptic transmission, GABAergic), GO:0016055 (Wnt receptor signaling pathway), GO:0032868 (response to insulin stimulus), GO:0044057 (regulation of system process), GO:0007169 (transmembrane receptor protein tyrosine kinase signaling pathway) and GO:0042325 (regulation of phosphorylation) (Table VIII).

Table V. Correlation of miR-96 expression and the clinicopathological characteristics of the lung adenocarcinoma cases (GSE48414).

Variables	N	Expression of miR-96	χ^2/Z	P-value
Total	154			
Age (years)				
<65	68	-0.0922±0.15804	-0.481	0.631
>65	86	-0.0244±0.14538		
Sex				
Male	67	0.0161±0.18784	-0.415	0.678
Female	87	-0.1094±0.12377		
TNM classification				
I+II	126	-0.069±0.14867	0.04	0.841
III+IV	28	0.067259±0.04541		

Table VI. Correlation of miR-96 expression and the different histological types of NSCLC (GSE51855).

Variables	N	Expression of miR-96	χ^2/X	P-value
Total	126			
ADC	76	-4.1497±0.15738	7.75	0.051
SQC	29	-4.8942±0.21236		
ASC	4	-4.8262±0.74010		
LCC	17	-4.3103±0.40235		

NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SQC, squamous cell carcinoma; ASC, adenosquamous carcinoma, LCC, large cell carcinoma.

The 15 cellular component GO terms were: GO:0042995 (cell projection), GO:0005815 (microtubule organizing center), GO:0005624 (membrane fraction), GO:0005626 (insoluble fraction), GO:0031095 (platelet dense tubular network membrane), GO:0005813 (centrosome), GO:0031094 (platelet dense tubular network), GO:0000267 (cell fraction), GO:0044463 (cell projection part), GO:0045202 (synapse), GO:0012505 (endomembrane system), GO:0005955 (calcineurin complex), GO: 0044430 (cytoskeletal part), GO:0045121 (membrane raft) and GO:0005856 (cytoskeleton) (Table IX).

In regards to the molecular function of the GO terms, GO:0005220 (inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity), GO:0008095 (inositol-1,4,5-trisphosphate receptor activity) and GO:0005516 (calmodulin binding) were the highest presented terms (Table X).

KEGG pathway analysis indicated that miR-96 target genes are mainly enriched in 9 pathways (Table XI). Among these pathways, hsa04912 (GnRH signaling pathway) (Fig. 3), hsa04114 (oocyte meiosis), hsa04720 (long-term potentiation) (Fig. 4), hsa04910 (insulin signaling pathway) (Fig. 5), hsa05215 (prostate cancer) and hsa04540 (gap junction) showed significantly higher enrichment, followed by hsa04916

Table VII. The target genes of hsa-miR-96 investigated by miRecords database.

No.	miRNA ID	Refseq	Symbol	Description	Note
1	hsa-miR-96	NM_015270	ADCY6	Adenylate cyclase 6	Predicted
2	hsa-miR-96	NM_198715	PTGER3	Prostaglandin E receptor 3 (subtype EP3)	Predicted
3	hsa-miR-96	NM_016623	FAM49B	Family with sequence similarity 49, member B	Predicted
4	hsa-miR-96	NM_016565	CHCHD8	Coiled-coil-helix-coiled-coil-helix domain containing 8	Predicted
5	hsa-miR-96	NM_015516	TSKU	Tsukushin	Predicted
6	hsa-miR-96	NM_015460	MYRIP	Myosin VIIA and Rab interacting protein	Predicted
7	hsa-miR-96	NM_015215	CAMTA1	Calmodulin binding transcription activator 1	Predicted
8	hsa-miR-96	NM_014946	SPAST	Spastin	Predicted
9	hsa-miR-96	NM_014943	ZHX2	Zinc fingers and homeoboxes 2	Predicted
10	hsa-miR-96	NM_014839	LPPR4	Plasticity related gene 1	Predicted
11	hsa-miR-96	NM_012300	FBXW11	F-box and WD repeat domain containing 11	Predicted
12	hsa-miR-96	NM_012257	HBP1	HMG-box transcription factor 1	Predicted
13	hsa-miR-96	NM_007198	PROSC	Proline synthetase co-transcribed homolog (bacterial)	Predicted
14	hsa-miR-96	NM_006940	SOX5	SRY (sex determining region Y)-box 5	Predicted
15	hsa-miR-96	NM_006861	RAB35	RAB35, member RAS oncogene family	Predicted
16	hsa-miR-96	NM_006791	MORF4L1	Mortality factor 4 like 1	Predicted
17	hsa-miR-96	NM_017974	ATG16L1	ATG16 autophagy related 16-like 1 (*S. cerevisiae*)	Predicted
18	hsa-miR-96	NM_018018	SLC38A4	Solute carrier family 38, member 4	Predicted
19	hsa-miR-96	NM_018243	11-Sep	Septin 11	Predicted
20	hsa-miR-96	NM_198459	DENND2C	DENN/MADD domain containing 2C	Predicted
21	hsa-miR-96	NM_194071	CREB3L2	cAMP responsive element binding protein 3-like 2	Predicted
22	hsa-miR-96	NM_033505	SELI	Selenoprotein I	Predicted
23	hsa-miR-96	NM_033260	FOXQ1	Forkhead box Q1	Predicted
24	hsa-miR-96	NM_032560	SMEK1	SMEK homolog 1, suppressor of mek1 (*Dictyostelium*)	Predicted
25	hsa-miR-96	NM_032373	PCGF5	Polycomb group ring finger 5	Predicted
26	hsa-miR-96	NM_032139	ANKRD27	Ankyrin repeat domain 27 (VPS9 domain)	Predicted
27	hsa-miR-96	NM_024915	GRHL2	Grainyhead-like 2 (*Drosophila*)	Predicted
28	hsa-miR-96	NM_024811	FLJ12529	Pre-mRNA cleavage factor I, 59 kDa subunit	Predicted
29	hsa-miR-96	NM_022041	GAN	Giant axonal neuropathy (gigaxonin)	Predicted
30	hsa-miR-96	NM_021229	NTN4	Netrin 4	Predicted
31	hsa-miR-96	NM_020871	LRCH2	Leucine-rich repeats and calponin homology (CH) domain containing 2	Predicted
32	hsa-miR-96	NM_020795	NLGN2	Neuroligin 2	Predicted
33	hsa-miR-96	NM_020423	SCYL3	SCY1-like 3 (*S. cerevisiae*)	Predicted
34	hsa-miR-96	NM_020182	PMEPA1	Prostate transmembrane protein, androgen induced 1	Predicted
35	hsa-miR-96	NM_006373	VAT1	Vesicle amine transport protein 1 homolog (*T. californica*)	Predicted
36	hsa-miR-96	NM_006283	TACC1	Transforming, acidic coiled-coil containing protein 1	Predicted
37	hsa-miR-96	NM_006016	CD164	CD164 molecule, sialomucin	Predicted
38	hsa-miR-96	NM_002959	SORT1	Sortilin 1	Predicted
39	hsa-miR-96	NM_002923	RGS2	Regulator of G-protein signaling 2, 24 kDa	Predicted
40	hsa-miR-96	NM_002833	PTPN9	Protein tyrosine phosphatase, non-receptor type 9	Predicted
41	hsa-miR-96	NM_002734	PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, α (tissue specific extinguisher 1)	Predicted
42	hsa-miR-96	NM_002515	NOVA1	Neuro-oncological ventral antigen 1	Predicted
43	hsa-miR-96	NM_002265	KPNB1	Karyopherin (importin) β 1	Predicted
44	hsa-miR-96	NM_002223	ITPR2	Inositol 1,4,5-triphosphate receptor, type 2	Predicted
45	hsa-miR-96	NM_002222	ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	Predicted
46	hsa-miR-96	NM_002015	FOXO1	Forkhead box O1	Predicted
47	hsa-miR-96	NM_001945	HBEGF	Heparin-binding EGF-like growth factor	Predicted

Table VII. Continued.

No.	miRNA ID	Refseq	Symbol	Description	Note
47	hsa-miR-96	NM_001945	HBEGF	Heparin-binding EGF-like growth factor	Predicted
48	hsa-miR-96	NM_001931	DLAT	Dihydrolipoamide S-acetyltransferase	Predicted
49	hsa-miR-96	NM_001839	CNN3	Calponin 3, acidic	Predicted
50	hsa-miR-96	NM_000945	PPP3R1	Protein phosphatase 3 (formerly 2B), regulatory subunit B, α isoform	Predicted
51	hsa-miR-96	NM_000935	PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	Predicted
52	hsa-miR-96	NM_000663	ABAT	4-aminobutyrate aminotransferase	Predicted
53	hsa-miR-96	NM_002998	SDC2	Syndecan 2	Predicted
54	hsa-miR-96	NM_003060	SLC22A5	Solute carrier family 22 (organic cation/carnitine transporter), member 5	Predicted
55	hsa-miR-96	NM_003182	TAC1	Tachykinin, precursor 1	Predicted
56	hsa-miR-96	NM_006007	ZFAND5	Zinc finger, AN1-type domain 5	Predicted
57	hsa-miR-96	NM_005766	FARP1	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derived)	Predicted
58	hsa-miR-96	NM_005544	IRS1	Insulin receptor substrate 1	Predicted
59	hsa-miR-96	NM_005502	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	Predicted
60	hsa-miR-96	NM_005400	PRKCE	Protein kinase C, ε	Predicted
61	hsa-miR-96	NM_005277	GPM6A	Glycoprotein M6A	Predicted
62	hsa-miR-96	NM_004985	KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Predicted
63	hsa-miR-96	NM_004958	FRAP1	FK506 binding protein 12-rapamycin associated protein 1	Predicted
64	hsa-miR-96	NM_004926	ZFP36L1	Zinc finger protein 36, C3H type-like 1	Predicted
65	hsa-miR-96	NM_004731	SLC16A7	Solute carrier family 16, member 7 (monocarboxylic acid transporter 2)	Predicted
66	hsa-miR-96	NM_004514	FOXK2	Forkhead box K2	Predicted
67	hsa-miR-96	NM_004481	GALNT2	UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2)	Predicted
68	hsa-miR-96	NM_003654	CHST1	Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	Predicted
69	hsa-miR-96	NM_003379	EZR	Ezrin	Predicted
70	hsa-miR-96	NM_003342	UBE2G1	Ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, yeast)	Predicted
71	hsa-miR-96	NM_000332	ATXN1	Ataxin 1	Predicted
72		NM_000863	HTR1B		Validated
73		NM_198159	MITF		Validated
74		NM_002015.3	Foxo1		Validated
75		NM_0016513	AQp5		Validated
76		NM_001408	CELSR2		Validated
77		NM_153437	ODF2		Validated
78		NM_001005861	RYK		Validated

(melanogenesis), hsa04270 (vascular smooth muscle contraction) and hsa04930 (Type II diabetes mellitus).

Discussion

Owing to its elevated expression, much effort has been dedicated to study the role of miR-96 in various types of cancers (21-23). In the majority of the tumors, miR-96 acts as an oncogene to promote the proliferation and invasion of cancer cells by inhibiting transcription factor FOXO1 (24), FOXO3a (25), tumor suppressor protein RECK (26) and metastasis suppressor protein MTSS1 (27). However, in pancreatic cancer, miR-96 functions as a tumor suppressor by targeting HERG1 and NUAK1 (28,29).

There is no explicit conclusion whether miR-96 could affect the development and progression of lung cancer and serve as a molecular biomarker for the clinical diagnosis of lung cancer. By analyzing four microRNA expression profiles and qRT-PCR, we showed that miR-96 was markedly increased in NSCLC, lung adenocarcinoma, stage I adenocarcinoma tissues and NSCLC cell lines. Consistent with our result, Ma *et al* reported that miR-96 was significantly upregulated in six NSCLC tissues and its expression was then validated in an independent set of 35 pairs of tumors and their

Table VIII. GO gene function (biological_process) analysis of the miR-96 targets.

GO ID	GO ontology	GO term	Counts	P-value
GO:0009725	Biological_process	Response to hormone stimulus	9	3.95E-04
GO:0009719	Biological_process	Response to endogenous stimulus	9	7.57E-04
GO:0010033	Biological_process	Response to organic substance	11	0.002442
GO:0016197	Biological_process	Endosome transport	4	0.002826
GO:0032228	Biological_process	Regulation of synaptic transmission, GABAergic	3	0.003041
GO:0016055	Biological_process	Wnt receptor signaling pathway	5	0.004019
GO:0032868	Biological_process	Response to insulin stimulus	4	0.012812
GO:0044057	Biological_process	Regulation of system process	6	0.0173
GO:0007169	Biological_process	Transmembrane receptor protein tyrosine kinase signaling pathway	5	0.023729
GO:0042325	Biological_process	Regulation of phosphorylation	7	0.025784
GO:0032870	Biological_process	Cellular response to hormone stimulus	4	0.027124
GO:0001666	Biological_process	Response to hypoxia	4	0.027651
GO:0043279	Biological_process	Response to alkaloid	3	0.028506
GO:0050804	Biological_process	Regulation of synaptic transmission	4	0.028721
GO:0051174	Biological_process	Regulation of phosphorus metabolic process	7	0.030557
GO:0019220	Biological_process	Regulation of phosphate metabolic process	7	0.030557
GO:0070482	Biological_process	Response to oxygen levels	4	0.03149
GO:0010648	Biological_process	Negative regulation of cell communication	5	0.032803
GO:0007612	Biological_process	Learning	3	0.03461
GO:0051969	Biological_process	Regulation of transmission of nerve impulse	4	0.034993
GO:0031998	Biological_process	Regulation of fatty acid beta-oxidation	2	0.03838
GO:0031644	Biological_process	Regulation of neurological system process	4	0.038689
GO:0043434	Biological_process	Response to peptide hormone stimulus	4	0.039324
GO:0046907	Biological_process	Intracellular transport	8	0.040338

GO, Gene Ontology.

Table IX. GO gene function (cellular_component) analysis of the miR-96 targets.

GO ID	GO ontology	Term	Count	P-value
GO:0042995	Cellular_component	Cell projection	11	9.15E-04
GO:0005815	Cellular_component	Microtubule organizing center	6	0.005289516
GO:0005624	Cellular_component	Membrane fraction	10	0.009055844
GO:0005626	Cellular_component	Insoluble fraction	10	0.011347551
GO:0031095	Cellular_component	Platelet dense tubular network membrane	2	0.013319656
GO:0005813	Cellular_component	Centrosome	5	0.017582227
GO:0031094	Cellular_component	Platelet dense tubular network	2	0.017720687
GO:0000267	Cellular_component	Cell fraction	11	0.020267702
GO:0044463	Cellular_component	Cell projection part	5	0.02029103
GO:0045202	Cellular_component	Synapse	6	0.020676761
GO:0012505	Cellular_component	Endomembrane system	9	0.021888271
GO:0005955	Cellular_component	Calcineurin complex	2	0.022102431
GO:0044430	Cellular_component	Cytoskeletal part	10	0.024057867
GO:0045121	Cellular_component	Membrane raft	4	0.025855924
GO:0005856	Cellular_component	Cytoskeleton	12	0.039405823

GO, Gene Ontology.

Table X. GO gene function (molecular_function) analysis of the miR-96 targets.

GO ID	GO ontology	Term	Count	P-value
GO:0005220	Molecular_function	Inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity	2	0.01449
GO:0008095	Molecular_function	Inositol-1,4,5-trisphosphate receptor activity	2	0.01927
GO:0005516	Molecular_function	Calmodulin binding	4	0.03047

GO, Gene Ontology.

Table XI. Pathways enrichment analysis of the miR-96 tagets (KEGG).

GO ID	Name of pathway	Count	P-value	Genes
hsa04912	GnRH signaling pathway	5	0.001862149	NM_004985, NM_002223, NM_002222, NM_015270, NM_001945
hsa04114	Oocyte meiosis	5	0.002843987	NM_012300, NM_002223, NM_002222, NM_015270, NM_000945
hsa04720	Long-term potentiation	4	0.005899778	NM_004985, NM_002223, NM_002222, NM_000945
hsa04910	Insulin signaling pathway	5	0.005930538	NM_004958, NM_002015, NM_004985, NM_005544, NM_002734
hsa05215	Prostate cancer	4	0.01237797	NM_004958, NM_002015, NM_004985, NM_194071
hsa04540	Gap junction	4	0.01237797	NM_004985, NM_002223, NM_002222, NM_015270
hsa04916	Melanogenesis	4	0.016481296	NM_004985, NM_015270, NM_198159, NM_194071
hsa04270	Vascular smooth muscle contraction	4	0.02283774	NM_002223, NM_002222, NM_015270, NM_005400
hsa04930	Type II diabetes mellitus	3	0.027134231	NM_004958, NM_005544, NM_005400

KEGG, Kyoto Encyclopedia of Genes and Genomes.

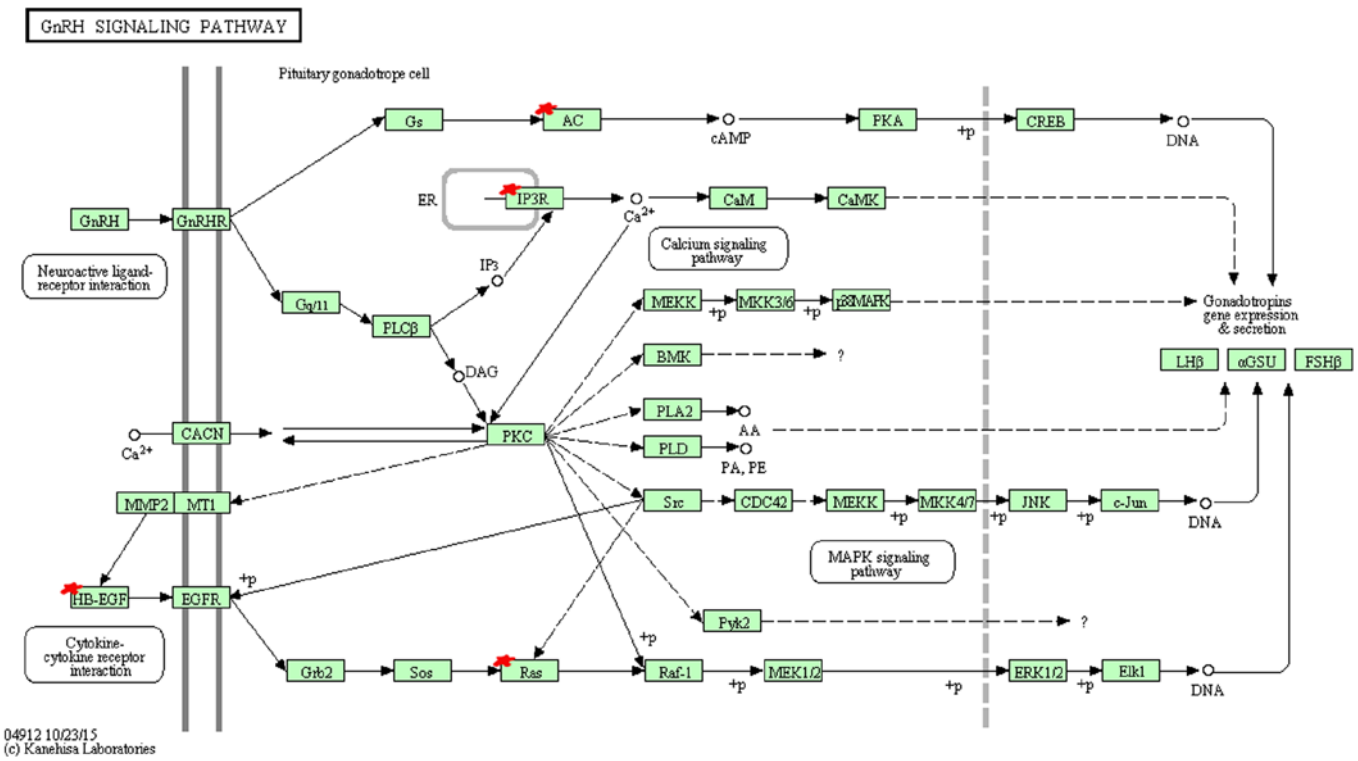

Figure 3. Five miR-96 target genes were enriched in the GnRH signaling pathway, including ADCY6 (AC), HBEGF, ITPR1 and ITPR2 (IP3R), and KRAS (Ras).

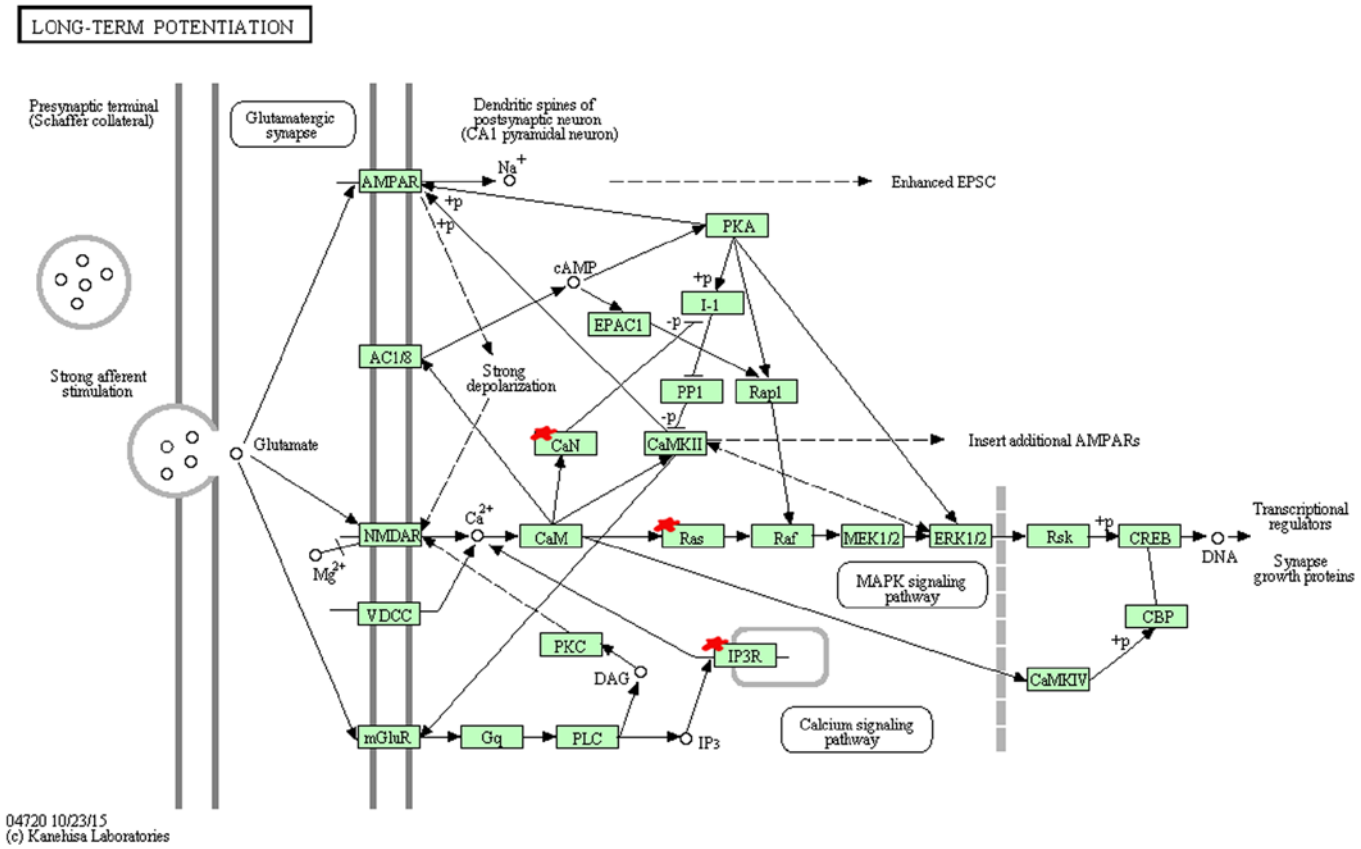

Figure 4. Four miR-96 target genes were enriched in the long-term potentiation signaling pathway, including ITPR1 and ITPR2 (IP3R), PPP3R1 (CaN), KRAS (Ras).

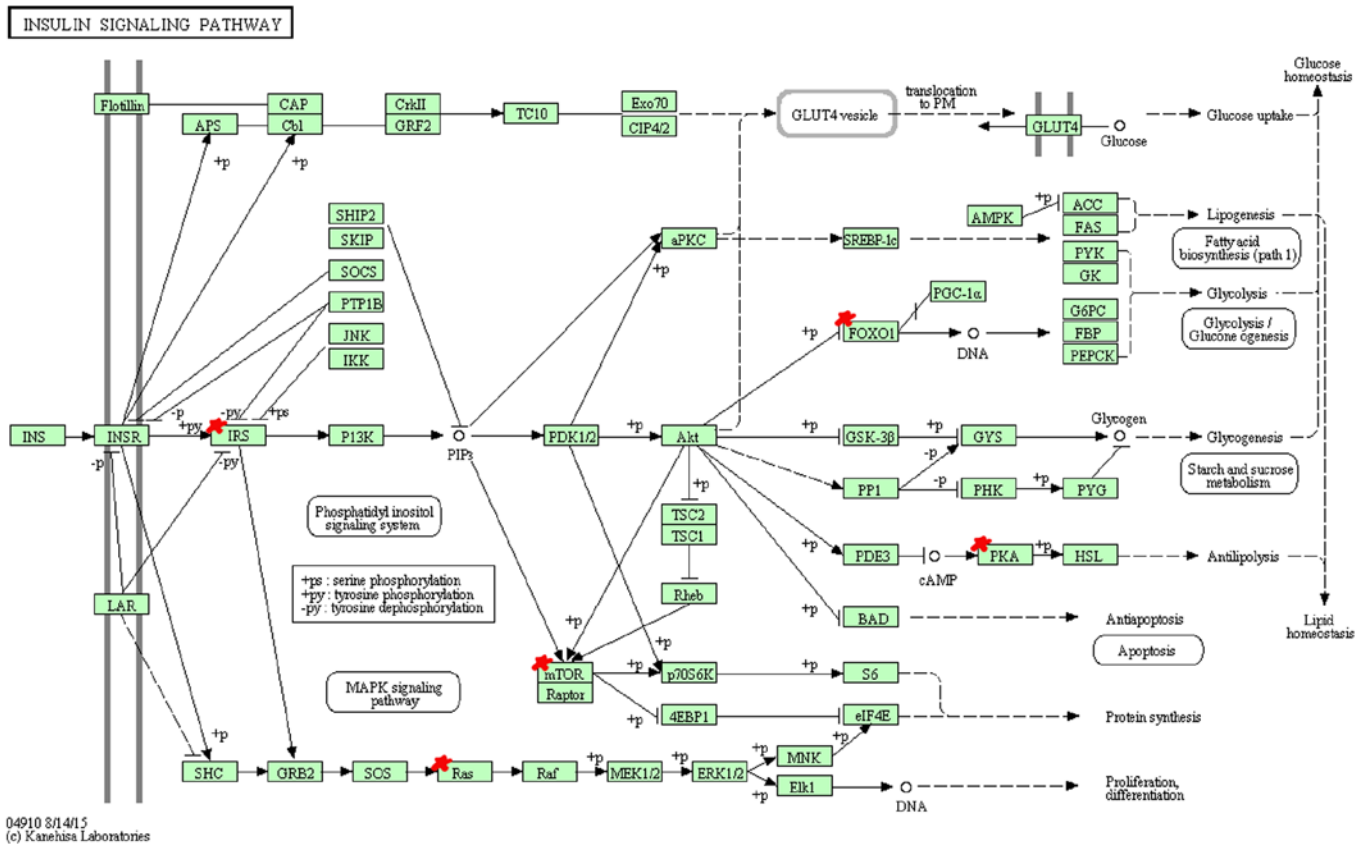

Figure 5. Five miR-96 target genes were enriched in the insulin signaling pathway, including FOXO1, IRS1 (IRS), FRAP1 (mTOR), PRKAR1A (PKA) and KRAS (Ras). mTOR, mammalian target of rapamycin.

adjacent normal tissues as well as in the serum of patients with NSCLC (19). To verify the microRNA expression signatures of lung cancer, Vosa *et al* performed a comprehensive meta-analysis of 20 published microRNA expression studies in lung cancer and identified a statistically significant microRNA meta-signature of seven upregulated microRNAs, including miR-21, miR-210, miR-182, miR-183, miR-31, miR-200b and miR-205. Since miR-182, miR-183 and miR-96 all belong to the miR-183 family, in conjunction with our results, miR-96 may serve as a novel molecular biomarker to distinguish early NSCLC patients from healthy individuals.

miRNAs are present not only in tissues but also in body fluids, such as blood, plasma, serum and sputum. Shen *et al* conducted several studies to assess the function of miRNAs in the sputum and plasma of lung cancer patients (30). They showed that the expression profile of plasma miR-21, miR-126, miR-210 and miR-486-5p produce high sensitivity and specificity in identifying stage I NSCLC patients. Zhu *et al* examined 70 pairs of lung cancer and non-cancerous tissues as well as serum samples. They found that miR-96 expression in tumors was positively associated with its expression in serum (31). Our data revealed that miR-96 expression in the plasma of lung cancer was significantly higher compared to that of non-cancer lung disease patients, suggesting that miR-96 may serve as a potential non-invasive marker for lung cancer diagnosis.

Although studies have shown that miR-96 is associated with poor overall survival in patients with pancreatic cancer (32), liver cancer (33) and colorectal cancer (34), our results did not demonstrate any significant correlation between the expression level of miR-96 and clinical stage as well as the histological subtype of the NSCLC patients. These discrepancies may be due to the different samples and databases that were used. Further studies are needed to confirm whether miR-96 could serve as a prognostic biomarker for lung cancer.

To date, computational methods have been widely used for the prediction of miRNAs and their target genes. However, the most commonly used miRNA target prediction websites, such as TargetScan, microRNA.org and PicTar, could not yield consistent results due to their different algorithm. miRecords is an integrated microRNA target database which includes a total of 11 established prediction programs. In this study, we selected the results predicted by at least six softwares in miRecords as the putative miR-96 target gene set and a collection of 78 predicted target genes were involved in GO/KEGG functional enrichment analysis. Since the GO hierarchy contains an added complexity by allowing terms to have multiple parents or ascendants, we used Fisher's exact 0.01 to reduce the redundancy in lists of enriched GO terms. Our data showed that among the 24 biological process GO terms obtained, the top 10 terms could be roughly grouped into several different categories including response to the stimulus (GO:0009725, GO:0009719, GO:0010033 and GO:0032868), signaling pathway (GO:0016055, GO:0032868, GO:0007169) and neurotransmission (GO:0032228). Tyrosine kinase signaling (GO:0007169) is currently known as the most successful molecular-targeted therapeutic approach for lung cancer (35). The canonical Wnt signaling pathway (GO:0016055), is another important regulator of proliferation (36) and metastasis (37) of non-small lung cancer cells. In addition, the 15 cellular component GO terms were significantly enriched in various specific processes with high frequency, such as cell division (GO:0005815, GO:0005813), cell communication (GO:0042995, GO:0044463) and cell migration (GO:0042995, GO:0044463, GO:0044430, GO:0005856), indicating that miR-96 may function as a regulator for the motility, migration and invasion of tumor cells. Moreover, three highly enriched molecular function GO terms (GO: 0005220, GO:0008095, and GO:0005516) suggest a potential new role of miR-96 in regulating calcium signaling important for tumor cell proliferation, apoptosis and migration.

In the KEGG annotation, GnRH signaling pathway (hsa04912), oocyte meiosis (hsa04114), long-term potentiation (hsa04720), insulin signaling pathway (hsa04910) and prostate cancer (hsa05215) showed the highest enrichment. GnRH has been reported to participate in the self-renewal of A549-derived lung cancer stem-like cells by upregulating the JNK signaling pathway (38). Insulin, bound to insulin receptor, promotes cell proliferation through the RAS-RAF-MAP kinase signaling pathway and regulates cell survival process through (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway, playing an important role in the clinical treatment of NSCLC (39). Long-term potentiation and prostate cancer pathway, related to transcription regulation, cancer cell survival and proliferation respectively, suggest the potential function for miR-96 in cancer growth.

Although DAL-1 was not in the list of the 78 targets, DAL-1 was predicted as the target gene of miR-96 by 5 predicted databases of miRecords: MirTarget2, PicTar, PITA, RNAhybird, and TargetScan/TargetScanS (data not shown). For future studies, comprehensive screening, confirmation experiments and further bioinformatic analysis using available web tools such as Ingenuity Pathway Analysis (IPA) and STRINGProtein-Protein Interaction Networks need to be carried out on the predicted targets to explore the novel regulatory mechanism of miR-96 in cancer metastasis.

In conclusion, our results showed that miR-96, functioning as an oncogene, may play an important role in the development and progression of lung cancer. Both in tissue and plasma, miR-96 may have the potential to serve as a molecular biomarker for the early diagnosis of NSCLC.

Acknowledgements

This study was funded by the National Nature Science Foundation of China (no. 81401391), Ph.D. Programs Foundation of the Ministry of Education of China (no. 20134423110001); National Nature Science Foundation of Guangdong Province (no.S2012010010181); Science and Technology Project of Guangzhou City (no. 2014Y2-00171) and Education System Innovative Academic Team of Guangzhou City (no. 13C06); Guangzhou City-Belonged Universities Scientific Research Program (no. 2012C130); National Natural Science Foundation of Guangdong Province (no. 2015A030313452).

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