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Genome-wide analysis of microRNA signature in lung adenocarcinoma with EGFR exon 19 deletion

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Running Title: Genome-wide microRNA signature of EGFR exon 19 deletion

Keywords: microRNA, EGFR, exon 19 deletion, non-small cell lung cancer

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ABSTRACT

The findings of EGFR mutations and the development of targeted therapies have significantly improved the overall survival of lung cancer patients. Still, the prognosis remains poor, so we need to know more about the genetic alterations in lung cancer. MicroRNAs are dysregulated in lung cancer, and microRNAs can regulate EGFR. So it is important to predict the candidate microRNAs that target mutated EGFR and to investigate the availability of these candidate microRNAs regulators in lung cancer. In this study, we investigated the difference of microRNAs expression in lung adenocarcinoma cell lines with EGFR exon 19 deletion (H1650 and PC9) and wild-type (H1299 and A549) using the Phalanx Human Whole Genome Microarray. Then the expression of individual microRNAs was validated by qRT-PCR assays. Moreover, we have detected microRNAs expression in serum of lung adenocarcinoma patients with EGFR exon 19 deletion and wide-type. The expression of 1,732

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microRNAs was evaluated, and we found that microRNAs expression was different between these two groups. hsa-miR-141-3p, hsa-miR-200c-3p, hsa-miR-203, hsa-miR-3182, hsa-miR-934 were up-regulated and hsa-miR-3196 was down-regulated in the EGFR exon 19 deletion group compared with wide-type group. The detection of circulating microRNAs also showed that miR-3196 was down-regulated in patients with EGFR exon 19 deletion compared with wide-type lung adenocarcinoma patients. It is suggested that these microRNAs associated with EGFR mutation can be further explored for potential predictors and targeted markers when it's difficult to get the tumors.

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INTRODUCTION

Lung cancer is one of the most common malignancies (Ferlay et al. 2010) and the leading cause of cancer death in the world. The prevalence and mortality of the cancer is still rising. In all diagnosed lung cancer, non-small cell lung cancer (NSCLC) accounts for approximately 85%, which varies both in molecular and clinical presentation. Despite years of research, the survival of NSCLC remains dismal, and the 5-year survival is only about 10% (Jemal et al. 2008). The discovery of key oncogenic drivers has led to a more personalized approach in the treatment of advanced disease. The activating mutations in the EGFR gene implicated sensitivity to EGFR tyrosine kinase inhibitors. It has been extensively proved that the sensitive EGFR mutations mainly refer to exon 19 deletion or L858R substitution in exon 21. However, the efficacy of EGFR-TKIs varies among different sensitive EGFR mutations. Several studies have reported that advanced NSCLC patients with EGFR exon 19 deletion had a longer overall survival (OS) and/or progression-free survival (PFS) following treatment with gefitinib or erlotinib compared with those with the L858R mutation (Jackman et al. 2006; Riely et al. 2006; Goto et al. 2013; Zhang et al. 2014).

MicroRNAs are evolutionarily conserved, endogenous small non-coding RNAs with 18–25 nucleotides that have important functions in diverse biological processes, including cell proliferation, differentiation, and apoptosis (Ambros et al. 2003; Galasso et al. 2010; Lu et al. 2005). Furthermore, microRNAs play an essential role in behaving either as oncogenes or tumor suppressor genes. Increasing evidence indicates that dysregulation of specific microRNAs contributes to the development and progression of cancer, including lung cancer (Chen et al. 2008 ; Gao et al. 2011; Shen et al. 2011). Moreover, microRNAs can regulate EGFR (Wang et al. 2011). So it

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is important to make it clear that the candidate microRNAs regulating EGFR exon 19 deletion because it might spark the design of novel therapeutics to combat the development of resistance to EGFR-TKIs or develop new targeted therapy.

In this study, we conducted an explorative microRNAs expression study in two groups of lung adenocarcinoma cell lines, including EGFR exon 19 deletion group (H1650 and PC-9) and EGFR wide-type group (A549 and H1299), using microRNA microarrays. Our main focus was the different microRNA expression in two groups. The selected microRNAs were confirmed using qRT-PCR in the cell lines and then we measured 3 microRNAs expressed differently in cell lines in the serum of 14 non-smoking female lung adenocarcinoma patients with wt EGFR and 13 patients with EGFR 19 del. Accordingly we discussed the association of microRNAs and EGFR exon 19 deletion.

MATERIALS and METHODS

Cell lines and cell culture

Human adenocarcinoma cell line PC-9 and H1650 (harboring EGFR exon 19 deletion) and A549 and H1299 (harboring wide-type EGFR) was provided by Cancer Institute of Tongji University Medical School, China. All these cells were cultured at 37°C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin.

Patient enrollment and serum samples

All patients in the study were recruited form Shanghai Pulmonary Hospital, Tongji University Medical School, between January 2012 and June 2014. The patients were newly diagnosed and histologically confirmed primary lung adenocarcinoma. Patients with a previous medical history of cancer, radiotherapy or chemotherapy

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were excluded. The study was approved by an ethical review committee at Tongji University Institutional Care and Use Committee. Written consent was obtained from all participants. All blood serum samples were collected and put into a liquid nitrogen tank for long-term storage until microRNAs extraction.

Total RNA isolation

Total RNA was extracted from cells using TRIZOL Reagent (Invitrogen, USA). The RNA concentration and purity were checked by OD260/OD280 (□1.6) and OD260/OD230 (□1.0), and the RNA yield and quality were accessed (RIN□5.0) using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Human microRNAs OneArray®

Human microRNA OneArray® v3 (Phalanx Biotech Group, Taiwan) contains triplicated 1,711 unique microRNA probes from Human (miRBase Release v17) , each printed in technical triplicate, and 189 experimental control probes.

Microarray analysis

Small RNA was pre-enriched by Nanoseplook (Pall Corporation, USA) from 2.5 μg total RNA samples and labeled with microRNAs ULSTM Labeling Kit (Kreatech Diagnostics, The Netherlands). Labeled targets were hybridized to the Human microRNA OneArray® v3 with OneArray® Hybridization System. After 16 hours hybridization at 37 □, non-specific binding targets were washed away by three different washing steps (Wash□37 □ 5 mins ;Wash□37 □, 5 mins 25 □ 5 mins; Wash III rinse 20 times), and the slides were dried by centrifugation and scanned by an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). The Cy5 fluorescent intensities of each probe were analyzed by GenePix 4.1 software (Molecular Devices). The raw intensity of each probe was processed by R program.

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Probes that passed the criteria were normalized by 75% median scaling normalization method. Normalized spot intensities were transformed to gene expression log2 ratios between the mutation and wide-type group. The spots with log2 ratio ≥ 1 or log2 ratio ≤ -1 and P-value < 0.05 are tested for further analysis.

Validation by qRT-PCR

Quantitative real time-PCR (qRT-PCR) was carried out on the 7900HT thermocycler (Applied Biosystems, Foster City, CA). The data were managed using the Applied Biosystems software RQ Manager v1.2.1. Relative expression was calculated by using the comparative Ct method and obtaining the fold-change value ($2^{-\Delta\Delta Ct}$). Data analyses were performed via GraphPad Prism v6.00.

EGFR mutation analyses

Mutation analyses of EGFR exons 18–21 were performed on 27 of the tumor samples using the ARMS. Data analyses were performed by employing the LightCycler Adapt software (LightCycler 480 Software, v. 1.5).

Digital PCR

Digital PCR was performed in parallel for the measurement of microRNAs in the serially-diluted oligonucleotides. 30 μL of the reaction mixture containing 15 μL QuantStudio™ 3D Digital PCR Master Mix, 2X (Life technologies), 2 μL of cDNA solution, and 1.5 μL of TaqManR Assay, 20X (primer/probe mix) (Life technologies) and 11.5 μL water. The droplets generated from each sample were transferred to a 96-well PCR plate (Eppendorf, Germany).PCR amplification was carried on a T100 thermal cycler (QuantStudio™ 3D Digital PCR System) at 96°C for 10 min, followed by 39 cycles 60°C for 2 min, then 98°C for 0.5 min. For Final extension , followed by 60°C for 2 min, Last ,Storage is at 10°C for 100min. The following figure shows the

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workflow for running a single QuantStudio™ 3D Digital PCR 20K Chip on the QuantStudio™ 3D Digital PCR Instrument (Sun et al. 2014).

RESULTS

Microarray analysis of the different microRNAs according to mutation status

PCA Analysis

Principle Component Analysis (PCA) was performed to evaluate any differences among biological replicates and their treatment conditions (Figure1). PCA uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of uncorrelated variables called principal components.

Clustering analysis of microRNAs in four cell lines

For advanced data analysis, all biological replicates were pooled and calculated to identify differentially expressed genes based on the threshold of fold change and p-value. The correlation of expression profiles between biological replicates and treatment conditions was demonstrated by unsupervised hierarchical clustering analysis. A subset of genes was selected for clustering analysis. An intensity filter was used to select genes where the difference between the maximum and minimum intensity values exceeds 200 among all microarrays. For this microarray project, the number of genes clustered was 260 (Figure 2).

Microarrays analysis of cell lines with EGFR exon 19 deletion compared with wide-type

To identify microRNAs that were differentially expressed between cell lines with EGFR exon 19 deletion and the wide-type adenocarcinoma cell lines, the expression profiles of microRNAs (1,711 microRNAs) were assessed using microRNA

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microarrays. Standard selection criteria to identify differentially expressed genes are established at log2 |Fold change| ≥ 0.8 and P < 0.05. The microRNAs differentially expressed between EGFR exon 19 deletion and wide-type are shown in Table 1, ($p < 0.05$). In total, let-7d-3p, miR-1307-5p, miR-141-3p, miR-200c-3p, miR-203, miR-3182, miR-4510, miR-934 were up-regulated in EGFR exon 19 deletion group compared with the wide-type group. On the contrary, miR-3196, miR-4450, miR-4649-5p were down-regulated.

Validation of the microarrays results using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The eleven microRNAs, which differently expressed in the two cell groups in microarray analyses, were validated by qRT-PCR. As a result, six microRNAs were identified and differentially expressed between the two cell groups with EGFR exon 19 deletion and wide-type (see Table 2). Hsa-miR-141-3p, hsa-miR-200c-3p, hsa-miR-203, hsa-miR-3182, hsa-miR-934 were up-regulated, and hsa-miR-3196 was down-regulated (Figure 3).

Pathway analysis

Using the microRNA target filter in TargetScan Release 6.2, we searched for genes involved in the signaling pathways that are experimentally observed or highly predicted to be regulated by the selected microRNAs. The six microRNAs that were differentially expressed between EGFR exon 19 deletion and EGFR wide-type cell lines were associated with 3181 mRNA targets. Two microRNAs (hsa-miR-200c-3p, hsa-miR-203) were experimentally confirmed to target EGFR, and be associated with EGFR-TKI resistance (Li et al. 2014; Siu et al. 2014).

Circulating microRNAs in relation to EGFR exon 19 deletion

This study was conducted on 27 participants stratified into EGFR exon 19

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deletion group and wide-type group. Selected candidates were non-smoking lung adenocarcinomas. It is well known that the sensitivity of qPCR for the detection of a low copy number in genes is not high enough, as it only resolves ~1.5-fold changes of nucleic acids (Guan et al. 2012). Given that a proportion of the cancer-associated microRNAs is derived from primary tumor and could be 'diluted' in a background of normal microRNAs (Chen et al. 2008; Mitchell et al. 2008; Pritchard et al. 2012), the microRNAs presenting at low levels in serum could be undetectable by qPCR. Droplet digital PCR is a direct method for quantitatively detecting nucleic acids as it depends on limiting partition of the PCR volume, where a positive result of a large number of microreactions indicates the presence of a single molecule in a given reaction (Hayden et al. 2013; Day et al. 2013). The number of positive reactions, together with Poisson's distribution, can be used to produce a straight and high-confidence measurement of the original target concentration (Vogelstein et al. 1999).

In our study, digital PCR was used to detect three circulating microRNAs (hsa-miR-200c-3p, hsa-miR-203, hsa-miR-3196) of 27 patients, 13 patients with EGFR exon 19 deletion and 14 wide-type EGFR. In the results, we found that hsa-miR-3196 was down-regulated in the group of EGFR exon 19 deletion compared with wide-type, which was consistent with our results of cell study (Figure 4).

DISCUSSION

In the present work，we found eleven microRNAs differentially expressed between EGFR exon 19 deletion cell lines and EGFR wide-type lung adenocarcinoma cell lines in the microarray analysis. Then the PCR study identified that

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hsa-miR-141-3p, hsa-miR-200c-3p, hsa-miR-203, hsa-miR-3182, and hsa-miR-934 were up-regulated, and hsa-miR-3196 was down-regulated in EGFR exon 19 deletion group compared with wide-type group. Moreover, the detection of circulating microRNAs found that miR-3196 was down-regulated in patients with EGFR exon 19 deletion compared with wide-type lung adenocarcinoma patients.

Presently, not much is known about the microRNAs expression in EGFR exon 19 deletion versus wide-type lung adenocarcinomas. Using microRNA microarrays, Dacic et al. analyzed six lung adenocarcinomas and found that miR-155 was up-regulated only in EGFR/KRAS-negative group, miR-25 was up-regulated only in EGFR-positive group and miR-495 was up-regulated only in KRAS-positive adenocarcinoma. In opposite, let-7g was down-regulated in all three groups, with more significant downregulation in EGFR/KRAS-negative adenocarcinoma (Dacic et al. 2010). Seike et al. identified 12 microRNAs differentially expressed between 6 EGFR-mutated tumors and 22 wild-type tumors. Zhang et al. found that miR-122 were differently expressed between wild and mutant EGFR carriers (P=0.018) (Seike et al. 2009). But all these have not accurately make the relation of microRNAs and EGFR exon 19 deletion clear.

In our results, Hsa-miR-141-3p and hsa-miR-200c-3p were up-regulated in exon 19 deletion versus wide-type. MiR-141-3p is a miR-200 family member, which consists of five microRNAs located in two different clusters (miRs-200b/a/429 and miRs-200c/141) on chromosome 1 and 12 in humans (Gregory et al. 2008). Previous studies have shown that miR-141/miR-200 is involved in cancer development and metastasis.

Tejero R et al. reported that high miR-141 and miR-200c expression are associated with shorter OS in NSCLC patients with adenocarcinoma through MET

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and angiogenesis (Tejero et al. 2014). MiR-141 and miR-200c expression was significantly up-regulated in NSCLC tissues, and its overexpression accelerated NSCLC cell proliferation in vitro and tumor growth in vivo (Mei et al. 2014; Baffa et al. 2009). In line with our results, miR-203 overexpression resulted in increased sensitivity to gefitinib-induced apoptosis in nude mice after two weeks of treatment (Garofalo et al. 2011).

The function of hsa-miR-934 is unknown, but it is located in intron 4 of the vestigial-like 1 (VGLL1) gene. The miR-934 was the most strongly up-regulated microRNA in triple-negative IDCs (61.5-fold increase with respect to ER+ breast carcinomas) (Castilla et al. 2014). Though studies of hsa-miR-3196 are rare, it was found that miR-3196 was down-regulated in basal cell carcinoma compared with nonlesional skin (Sand et al. 2012), and was also down-regulated in PTC patients with non-(131) I-avid lung metastases versus (131)I-avid lung metastases (Qiu et al. 2015).Regarding hsa-miR-3182, there is still no published study.

A large number of microRNAs have been found to be stably expressed in human serum and plasma. Circulating microRNAs and their expression profiles have been proposed to be useful biomarkers in the diagnosis and prognosis of cancer. Overexpression of serum miR-21 was linked to the poor survival of NSCLC and associated with lymph node metastasis and advanced stage (Liu et al. 2012).

As previously stated, very little is known about miR-3196, but the data from the next two previous published studies point to the possibility that EGFR exon 19 deletion could have a distinct molecular identity. Bjaanaes MM et.al examined microRNA expression in 154 surgically resected lung adenocarcinomas and 20 corresponding normal lung tissue samples using Agilent microarrays and found that 17 microRNAs were differentially expressed. between EGFR-mutated

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and EGFR wildtype tumors (Bjaanaes et al. 2014). Recently, Zhang et al. reported that circulating miR-195 and miR-122 may have prognostic values in predicting the overall survival as well as predicting EGFR mutation status in non-smoking female patients with lung adenocarcinoma. Measuring plasma levels of miR-195 and miR-122 may especially be useful in EGFR mutant patients with lung adenocarcinoma (Zhang et al. 2013). In this regard, we selected hsa-miR-200c-3p, hsa-miR-203, and hsa-miR-3196 to measure the expression level in serum of patients. The cause of our selection was the high expression level in cell lines. Lastly, hsa-miR-3196 was significantly differentially expressed between EGFR exon 19 deletion compared with wide-type lung adenocarcinomas. It is consistent with our result of cell lines.

Therefore, the circulating microRNAs may potentially provide a noninvasive strategy for predicting response to EGFR TKIs when it's difficult to get the tumor tissues. Moreover, we identified that microRNAs regulate EGFR exon 19 deletion and which may represent a clinically useful modality to treat TKI resistance in NCSLC patients.

ACKNOWLEDGEMENTS

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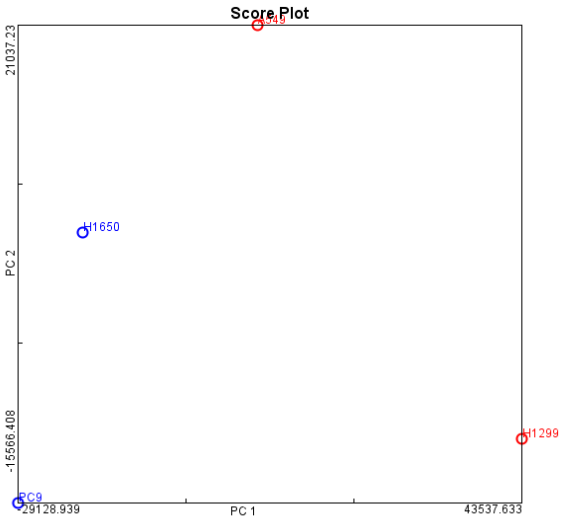

Figure 1. The variable of the first three principal components (PC1, PC2, PC3) for this study are 68.95%, 17.98% and 13.07% respectively.

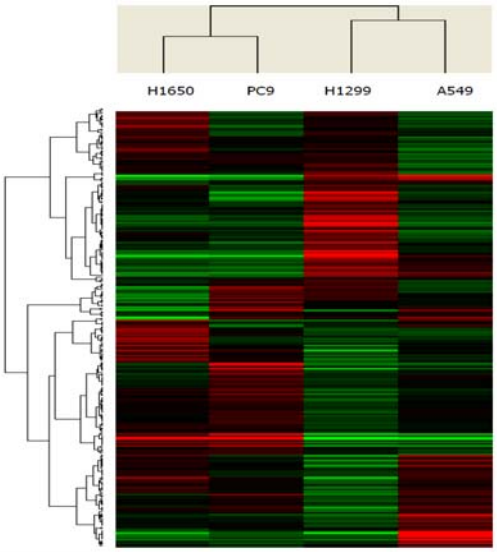

Figure 2. Unsupervised clustering of 260 microRNAs (rows) in 4 lung adenocarcinoma cell lines (columns). Up- and down-regulated genes are represented in red and green colors, respectively.

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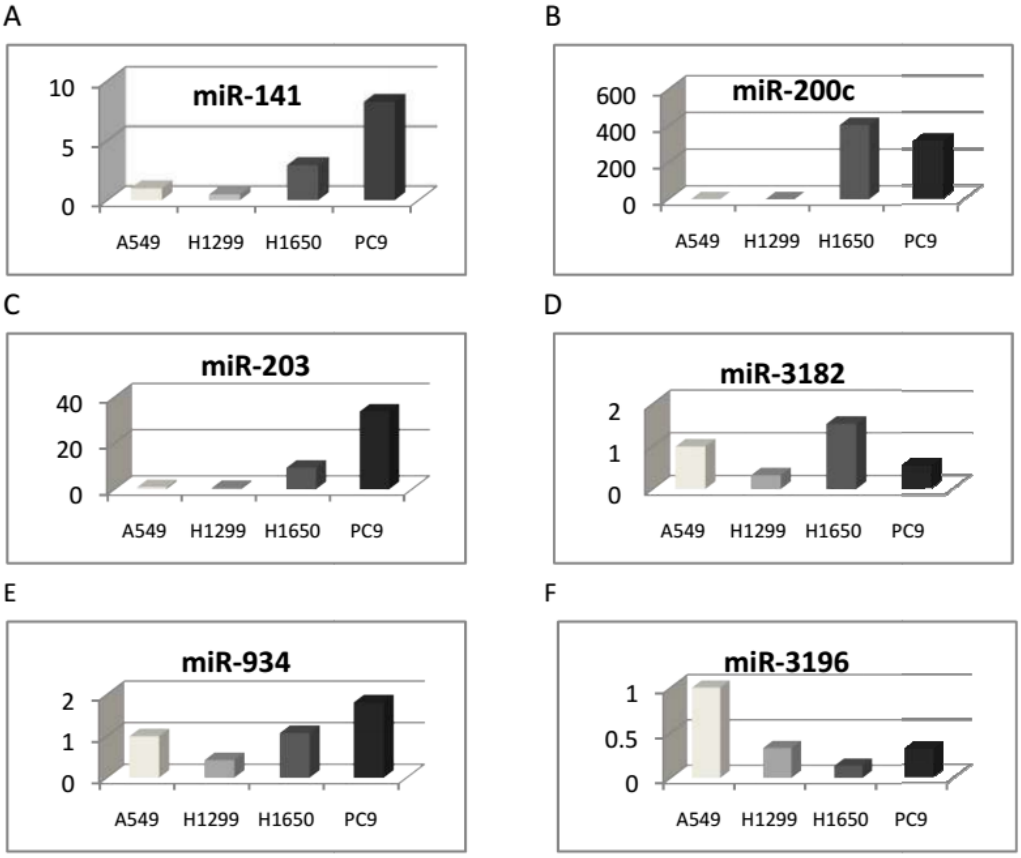

Figure 3. Six microRNAs were identified and differentially expressed between the two cell groups with EGFR exon 19 deletion and wide-type using qRT-PCR. A: Hsa-miR-141-3p, B: hsa-miR-200c-3p, C: hsa-miR-203, D: hsa-miR-3182, E: hsa-miR-934 was up-regulated, and F: hsa-miR-3196 was down-regulated.

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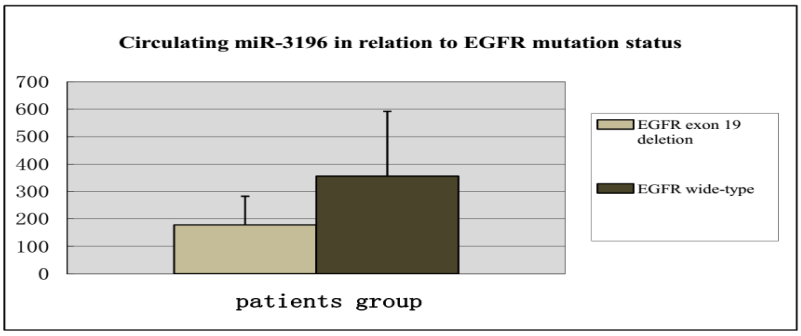

Figure 4. Digital PCR results showed that circulating hsa-miR-3196 was down-regulated in serum of lung adenocarcinoma patients with EGFR exon 19 deletion compared with wide-type.

Table 1. MicroRNAs that were significantly differentially expressed between EGFR exon 19 deletion compared with wide-type cell lines

EGFR exon 19 deletion vs. wide-type lung adenocarcinoma cell lines (up-regulated)			
	MicroRNA	log2 (Ratio) Mutant/WT	P-value
1	hsa-let-7d-3p	0.647145	0.040585
2	hsa-miR-1307-5p	0.927271	0.040504
3	hsa-miR-141-3p	4.568754	0.025595
4	hsa-miR-200c-3p	5.145011	0.00012
5	hsa-miR-203	1.932793	0.01194
6	hsa-miR-3182	0.92985	0.027437
7	hsa-miR-4510	1.097083	0.041041
8	hsa-miR-934	2.629921	0.040343
EGFR exon 19 deletion vs. wide-type lung adenocarcinoma cell lines (down-regulated)			
1	hsa-miR-3196	-1.042712	0.02772
2	hsa-miR-4450	-2.423635	0.016643
3	hsa-miR-4649-5p	-1.662239	0.047984

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Table 2. Validation of the microRNAs that were significantly differentially expressed between EGFR exon 19 deletion compared with wide-type cell lines (qRT-PCR)

EGFR exon 19 deletion vs. wide-type lung adenocarcinoma cell lines (up-regulated)					
	MicroRNA	$2^{-\Delta\Delta CT}$			
		A549	H1299	H1650	PC9
1	hsa-miR-141-3p	1	0.501157	2.941734	8.224911
2	hsa-miR-200c-3p	1	0.203063	405.4368	314.4456
3	hsa-miR-203	1	0.66742	9.426137	33.74652
4	hsa-miR-3182	1	0.326842	1.52979	0.532185
5	hsa-miR-934	1	0.429283	1.076738	1.815038
EGFR exon 19 deletion vs. wide-type lung adenocarcinoma cell lines (down-regulated)					
6	hsa-miR-3196	1	0.33371	0.13742	0.314253

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