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ORIGINAL ARTICLE

Detection and Analysis of Wnt Pathway Related lncRNAs Expression Profile in Lung Adenocarcinoma

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Abstract Studies have shown that the expression profile of Wnt signaling pathway is very important in lung adenocarcinoma (LAD) and some lncRNAs can regulate the expression of key molecules of Wnt pathway. However, Wnt pathway related lncRNAs are not systematically analyzed and detected in lung adenocarcinoma. We used a high-throughput microarray to compare the lncRNA expression profiles in LAD and corresponding normal tissue (NT) samples. Several candidate Wnt pathway related lncRNAs were verified by real-time quantitative reverse transcription polymerase chain reaction (PCR) analysis. We found that 232 Wnt pathway related lncRNAs were obviously expressed (≥2-fold change) in lung adenocarcinoma samples and 13 Wnt pathway related lncRNAs were aberrantly expressed in lung adenocarcinoma compared with matched histologically normal lung tissues by qPCR. Among these, RP11-181G12.2 and RP11-89 K21.1 were the most aberrantly expressed lncRNAs. Our study ascertained the expression of Wnt pathway related lncRNAs in lung adenocarcinoma. The results revealed that many Wnt pathway related lncRNAs were differentially expressed in lung adenocarcinoma tissues, suggesting that they may play a key role in tumor development.

Keywords Lung adenocarcinoma · LncRNAs · Wnt pathway · Expression profile

Introduction

Lung cancer has the highest mortality rate of all cancers, and its incidence is gradually increasing [1]. Lung adenocarcinoma (LAD) is the most common type of lung cancer (about 40 %) and more prone to women and non-smokers, also found in smoking patients. In recent years, a growing proportion of lung adenocarcinoma is attributable to causes such as passive smoking, environmental pollution, diet, hormone levels and genetic predisposition and so on. However, the mechanisms underlying LAD development has not been elucidated; thus, the study of LAD remains extremely important.

Wnt signaling pathway plays an important role in cancer and in embryonic development and regulate cell proliferation, differentiation and apoptosis. Therefore Wnt signaling pathway has been recognized as an important anti-cancer and drug intervention targets [2, 3]. Studies have shown that the expression of Wnt signaling pathway was significantly increased and very important to invasion, lymph node metastasis and drug in lung adenocarcinoma [4–6].

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Table 1 The clinical data of 3 cases LAD patients for high-throughput lncRNA array

No	sex	age(years)	TMN stage	differentiation	tumor volume	metastasis	smoking
FZ142	female	55	T2aN0MO	Well	3.0*3.0	no	no
FZ143	female	78	T1aN0MO	Moderate	5.0*5.0	no	no
FZ144	male	76	T2bN2MO	Poor	1.5*1.5	no	no

LncRNAs have been shown to be involved in the development and progression of LAD [7, 8]. Some studies confirmed lncRNAs can regulate the expression of key molecules of Wnt signaling pathway in some cancers [9–14]. However, Wnt pathway related lncRNAs are not systematically analyzed and detected in lung adenocarcinoma. We will use a high-throughput lncPathTM human Wnt pathway array to obtain Wnt pathway related lncRNAs expression profiles in lung adenocarcinoma and several candidate Wnt pathway related lncRNAs were verified by real-time quantitative reverse transcription polymerase chain reaction (PCR) analysis in tissue samples of LAD.

Materials and Methods

Patient Samples

The LAD tissue samples and corresponding NT samples were prospectively collected from 33 patients of the First Affiliated Hospital of Wenzhou Medical University, China, from April 2013 to August 2015. Of these patients, three (the basic medical records see Table 1) were used for lncPathTM human Wnt pathway array and 30 were used for clinical validation. The diagnosis of LAD was confirmed by histopathology. The LAD and matched NT samples were snap-frozen in liquid nitrogen immediately after resection. This study was approved by the Institutional Ethics Review Board of the First Affiliated

Table 2 Wnt pathway related LncRNA gene primers for qPCR

LncRNA gene	Sense primer(5′-3′)	Antisense primer(5′-3′)	PCR production(bp)
RP11-50B3.2	ATAACTCCGCAAAGCATA	TGATGGATAACCGATACG	148
XLOC_003,147	GAGGTTGACACCCTGTGC	GGAGATGGCAGCAATCCA	103
RP11-89 K21.1	AAAGGGACTTCAGCAAAG	AAGGGTCTCACCTAACAAAA	169
RP13-39P12.2	GGGTGTCAATGTTGTAGTCTTG	GAGCGTCCATCAGGTTCTT	152
RP11-60H5.1	ATCACCAGCCTCACAGAC	AGTCATGGCACCTTACCT	215
RP11-94C24.10	GTGGAATCCCATCATCGC	GGGTGCCCTTGGTGTCTT	103
LOC390483	GGGTGTCAATGTTGTAGTCTTG	GAGCGTCCATCAGGTTCTT	152
RP11-305O4.1	AATGTAAACTTTGCCACTG	CACAATAACCACATATCCC	155
RP11-680F20.5	AGCTTCAATGGGCCATCTT	GGGTGGTGGTTGGTGTTTC	136
RP11-982 M15.2	GAAGAATTTGGAGGGAAGG	GGACAGAGGAGCAAGGTTT	113
RP11-44I10.5	CCTCTGATTTCCCCTTTT	TTCTGTTGGTCCTTTTGT	132
RP11-181G12.2	TGTATAGGCATCGGCTTCA	CGATTCCGTTCCCAGTTT	172
XLOC_002680	ACAATCATTCTCATGCCACC	GCACCCAGCAACCACTTA	182
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA	121

Table 3 RNA quantification and quality assurance of six samples

Sample ID	OD260/280 Ratio	OD260/230 Ratio	Conc. (ng/μl)	Volume (μl)	Quantity (ng)
FZ142	2.03	2.26	965.16	40	38,606.40
FZ143	1.97	2.41	617.61	40	24,704.40
FZ144	2.01	2.37	1295.47	10	12,954.70
AP142	1.89	1.96	80.20	40	3208.00
AP143	1.98	2.12	406.75	40	16,270.00
AP144	1.99	2.31	628.11	40	25,124.40

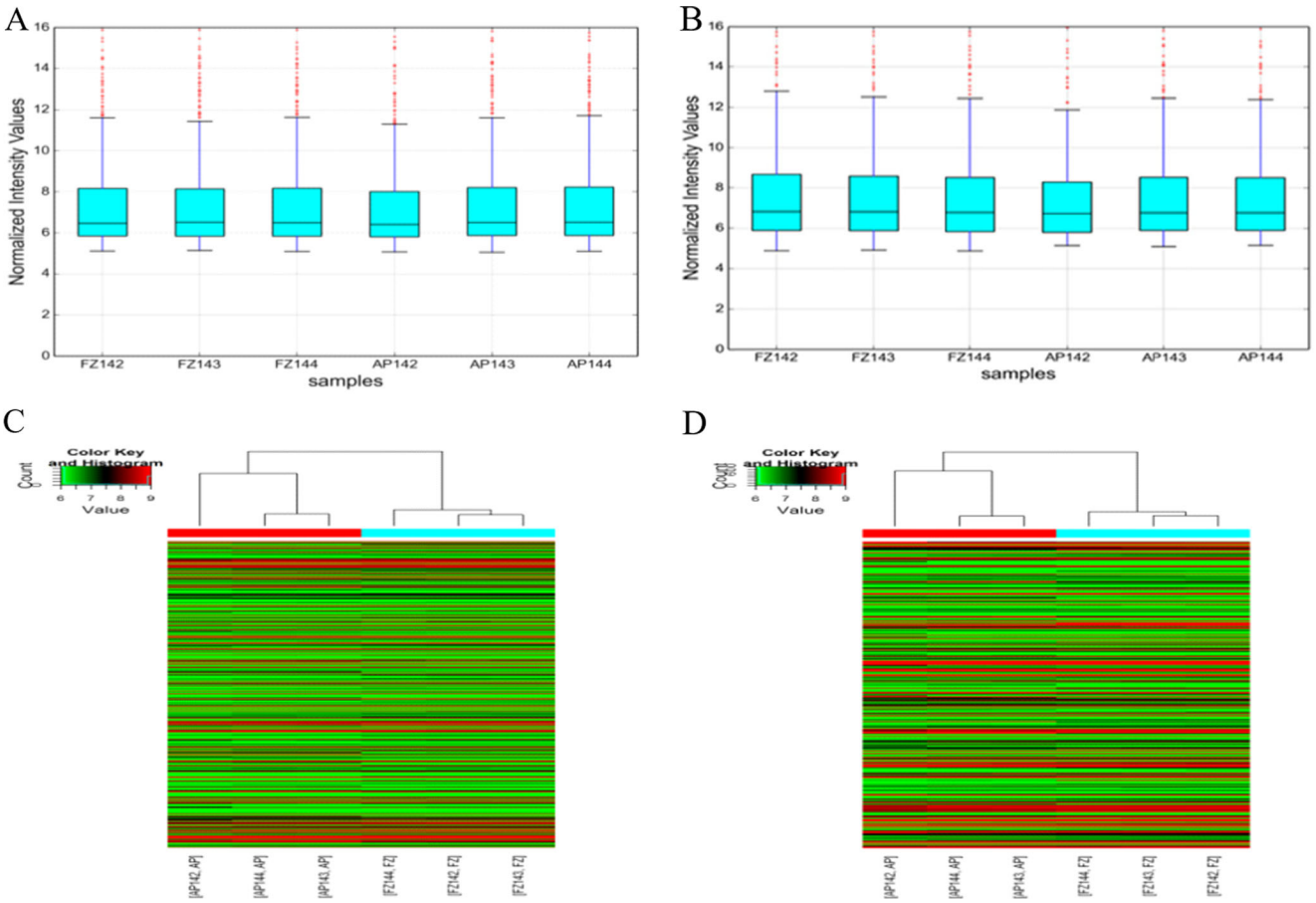

Fig. 1 Box plot and Hierarchical Clustering of LncRNA and mRNA. **a** lncRNA Box plot. **b** mRNA Box plot. **c** lncRNA Hierarchical Clustering. **d** mRNA Hierarchical Clustering

Hospital of Wenzhou Medical University, and all patients provided written informed consent for this study.

RNA Extraction

According to the manufacturer's protocol, total RNA come from tissue samples and cell lines of LAD was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The integrity of the RNA was assessed by denaturing agarose gel electrophoresis. A NanoDrop ND-1000 spectrophotometer was used for evaluating RNA concentration (OD_{260}), protein contamination (OD_{260}/OD_{280} ratio), and organic compound contamination (OD_{260}/OD_{230} ratio).

Microarray and Computational Analysis

For microarray analysis, Agilent Array platform was employed. The sample preparation and microarray

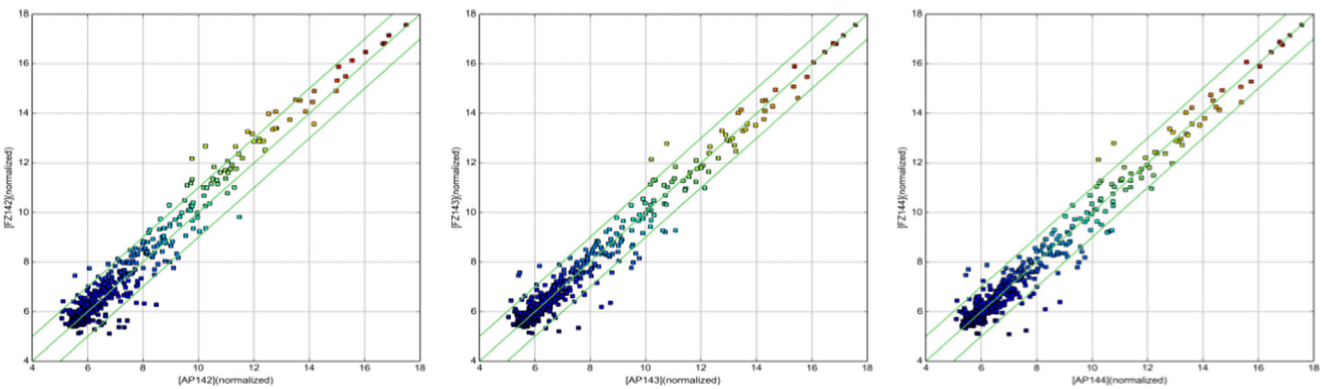

Fig. 2 lncRNA Scatter Plots

Table 4 some Wnt pathway related lncRNAs were lower or raised in LAD

probeID	Fold Change	Regulation	seqname
ASPWP0000123	12.043502	up	ENST00000333854
ASPWP0001165	13.216247	up	ENST00000508920
ASPWP0002775	8.448925	up	ENST00000557223
ASPWP0006337	15.259001	up	ENST00000578935
ASPWP0008660	9.356259	up	NR_037843
ASPWP0049239	10.140124	up	uc003jcd.1
ASPWP0067449	5.959143	up	ENST00000555315
ASPWP0090287	14.545314	up	ENST00000530834
ASPWP0097154	9.023115	up	TCONS_00006040
ASPWP0008505	−14.64322	down	NR_033320
ASPWP0008597	−14.05234	down	NR_002227
ASPWP0008644	−7.3677	down	uc.338+
ASPWP0090292	−8.57732	down	ENST00000532357
ASPWP0096142	−8.88421	down	TCONS_00006524
ASPWP0129497	−16.17989	down	ENST00000354756
ASPWP0133645	−6.46174	down	ENST00000429825
ASPWP0144419	−6.28293	down	ENST00000453828

hybridization were performed based on the manufacturer's standard protocols. Briefly, total RNA from each sample was amplified and transcribed into fluorescent cRNA with using Arraystar Flash RNA Labeling protocol (Arraystar). The labeled cRNAs were hybridized onto the LncPathTM Human Wnt Pathway Array (6x7K, Arraystar). After having washed the slides, the arrays were scanned by the Agilent Scanner G2505C.Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the R software package. Differentially expressed LncRNAs with statistical significance between two groups were identified through Volcano Plot filtering. Differentially expressed LncRNAs between two samples were identified through Fold Change filtering. Hierarchical Clustering was performed to show the distinguishable LncRNAs expression pattern among samples. The microarray work was performed by KangChen Bio-tech, Shanghai, and People's Republic of China.

Quantitative PCR

Total RNA was extracted from frozen LAD tissues by using Trizol reagent (Invitrogen) and then reverse-transcribed using an RT Reagent Kit (Thermo Scientific), according to the manufacturer's instructions. LncRNA expression in LAD tissues was measured by quantitative PCR by using SYBR Premix Ex Taq and an ABI 7000 instrument. Some candidate lncRNAs were validated by SYBRP PCR, these gene primers in the study for Q-PCR see Table 2. Total RNA (2 mg) was transcribed to cDNA. PCR was performed in a total reaction volume of 20 μl, including 10 μl of SYBR Premix (2×), 2 μl of cDNA template, 1 μl of PCR forward primer (10 mM), 1 μl of PCR reverse primer (10 mM), and 6 μl of double-distilled water. The quantitative real-time PCR reaction included an initial denaturation step of 10 min at 95 °C; 40 cycles of 5 s at 95 °C, 30 s at 60 °C; and a final extension step of 5 min at 72 °C. All experiments were performed in triplicate, and all samples were normalized to GAPDH. The median in each triplicate was used to calculate relative lncRNA concentrations (△Ct = Ct median lncRNA-Ct median GAPDH), and the fold changes in expression were calculated [15].

Statistical Methods

Statistical analysis was performed for the comparison of two groups in the microarray, and analysis of variance

Table 5 some Wnt pathway related lncRNAs were predicted_ regulation of mRNA gene in LAD

GeneSymbol	Log Fold change	Regulation	Genomic Relationship	mRNA symbol
RP11-50B3.2	−5.1945	down	upstream	CDON
XLOC_003,147	−5.95762	down	downstream	PRICKLE2
RP11-60H5.1	4.904409	up	upstream	TCF7L1
RP11-94C24.10	7.685378	up	upstream	CACNA1G
RP11-89 K21.1	−4.66898	down	upstream	SIX3
LOC390483	4.565179	up	downstream	PPM1A
RP11-305O4.1	5.458088	up	upstream	PPP2R3A
RP11-680F20.5	4.347843	up	downstream	CDON
RP11-982 M15.2	4.292149	up	overlapping	AKT1
RP13-39P12.2	−8.03954	down	upstream	DLG5
RP11-44I10.5	3.036824	up	downstream	SIAH1
RP11-181G12.2	4.031044	up	overlapping	PRKCZ
XLOC_002680	3.016579	up	upstream	FHIT

Table 6 some Wnt pathway related mRNAs were lower or raised in LAD

probeID	Fold Change	Regulation	seqname
ASPWP0010983	15.384457	up	GDNF
ASPWP0011649	6.111724	up	PRKAR1A
ASPWP0011888	8.11319	up	MAPK11
ASPWP0012092	4.315084	up	HDAC2
ASPWP0012198	10.724534	up	SDC2
ASPWP0012201	12.757905	up	FZD6
ASPWP0000009	−6.77146	down	PAF1
ASPWP0000688	−7.10176	down	TCF7L1
ASPWP0002191	−9.45949	down	PXN
ASPWP0004859	−4.07037	down	HDAC11
ASPWP0005656	−12.12072	down	EDN1

for multiple comparisons was performed using the Student's t-test or Mann-Whitney U test. Differences with $P < 0.05$ were considered statistically significant in both cases. The fold change and the Student's *t*-test were used to analyze the statistical significance of the microarray results. The false discovery rate (FDR) was calculated to correct the *P*-value. The threshold value used to designate differentially expressed lncRNAs and mRNAs was a fold change of ≥2.0 or ≤0.5 ($P < 0.05$).

Results

RNA Quantification and Quality Assurance

For spectrophotometer, the O.D. A260/A280 ratio should be close to 2.0 for pure RNA (ratios between 1.8 and 2.1 are acceptable). The O.D. A260/A230 ratio should be more than 1.8. Ours result shown that RNA quantification and quality assurance of six samples is passed from Table 3.

Overview of lncRNA Profiles

We obtained the Wnt pathway related lncRNA expression profiles in human LAD through microarray analysis (Figs. 1a, c and 2). The expression profiles of 232 lncRNAs indicated that they were differentially expressed (fold change ≥2.0 or ≤0.5; $P < 0.05$) between LAD and normal lung samples. Among these, 152 lncRNAs were found to be upregulated more than two-fold in the LAD group compared to the normal lung group, while 80 lncRNAs were downregulated more than two-fold ($P < 0.05$; Table 4). Oherwise, we found that wnt pathway related lncRNAs were predicted_regulation of mRNA gene in LAD (Table 5).

Overview of mRNA Profiles from Wnt Pathway

In total, 100 mRNAs were found to be differentially expressed between LAD and normal lung samples, including 71 upregulated mRNAs and 29 downregulated mRNAs (Fig. 1c, d, Table 6 and Fig. 3).

Real-Time Quantitative PCR Validation

According to fold difference, gene locus, nearby encoding gene, and so on. We initially identified a number of interesting candidate lncRNAs (including RP11-50B3.2, XLOC_003,147, RP11-89 K21.1, RP13-39P12.2, RP11-60H5.1, RP11-94C24.10, LOC390483, RP11-305O4.1, RP11-680F20.5, RP11-982 M15.2, RP11-44I10.5, RP11-181G12.2, XLOC_002680), and verified the expression of lncRNAs by real-time quantitative RT-PCR with GAPDH as reference gene, by calculating the $2^{-\Delta\Delta CT}$. We found that multiple lncRNAs of microarray are consistent to results of RT-PCR, see Fig. 4. The RP11-181G12.2 and RP11-89 K21.1 was most significantly changed lncRNA of these candidate

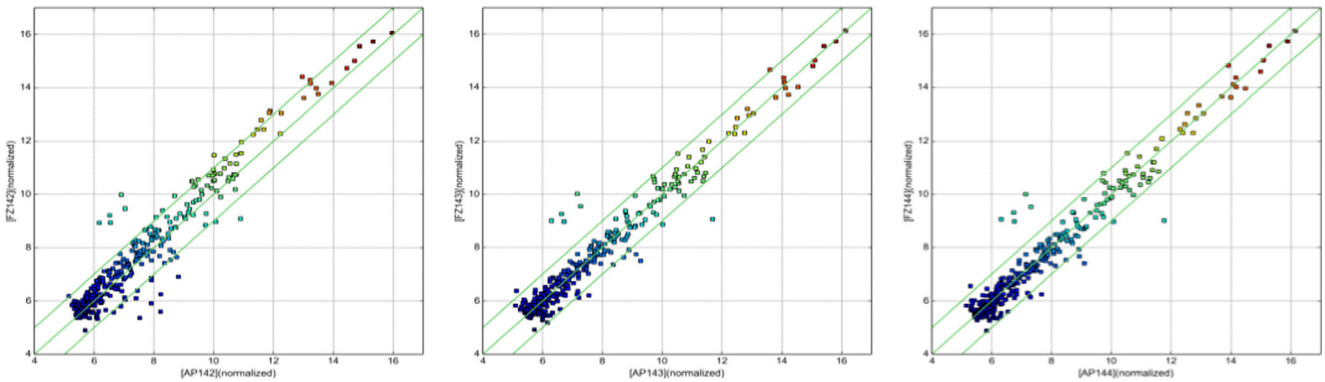

Fig. 3 mRNA Scatter Plots

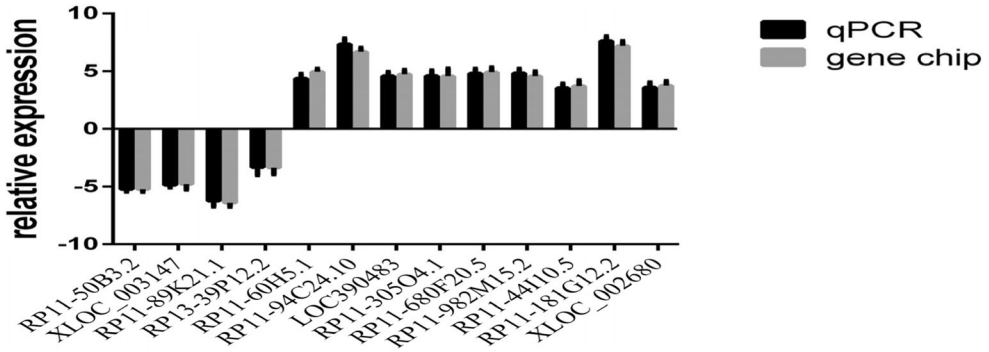

Fig. 4 Comparison between gene chip data and qPCR result. RP11-50B3.2, XLOC_ 003,147, RP11-89 K21.1, RP13-39P12.2, RP11-60H5.1, RP11-94C24.10, LOC390483, RP11-305O4.1, RP11-680F20.5, RP11-982 M15.2, RP11-44I10.5, RP11-181G12.2, XLOC_ 002680 determined to be obviouslly expressed in LAD samples compared with NT samples in six patients by microarray were validated by qPCR. The validation results of the 13 lncRNAs indicated that the microarray data correlated well with the qPCR results

lncRNAs from 30 LAD and normal lung tissue. According to Fig. 5, RP11-181G12.2 expression of LAD was significantly higher than the adjacent tissues (Mann-Whitney U = 189.87, P = 0.005), while RP11-89 K21.1 expression of LAD was significantly lower than the adjacent tissues (Mann-Whitney U = 193.12, P = 0.0013).

Discussion

Some studies confirmed lncRNAs can regulate the expression of key molecules of Wnt signaling pathway in liver cancer [11], bladder cancer [14], and so on. These lncRNAs played an important role in these cancers. While there is no reports about Wnt pathway related lncRNAs in LAD. Our study found that 232 lncRNAs and 100 mRNAs of Wnt pathway by microassay and were obviously expressed in lung adenocarcinoma samples and NT, hinting that many Wnt pathway related lncRNAs were aberrantly upregulated or downregulated in lung adenocarcinoma. So these lncRNAs might regulate the expression of key molecules of Wnt signaling pathway and play an important role in development of LAD.

We also found that 13 candidate Wnt pathway related lncRNAs were aberrantly expressed in lung adenocarcinoma compared with matched histologically normal lung tissues by qPCR and these lncRNAs are key molecules to regulate members of Wnt pathway. Among these lncRNAs, RP11-181G12.2 was the most significantly upregulated and RP11-89 K21.1 was the most significantly downregulated. This result suggests that RP11-181G12.2 and RP11-89 K21.1 might contribute to the development of lung adenocarcinoma; further study of the biological function of RP11-181G12.2 and RP11-89 K21.1 will be required to confirm this notion.

To summarize, our study revealed a set of Wnt pathway related lncRNAs in lung adenocarcinoma by microarray. The results revealed that many Wnt pathway related lncRNAs were differentially expressed in lung adenocarcinoma tissues and NT, suggesting that they may play a key role in tumor development. Moreover, we found that RP11-181G12.2 and RP11-89 K21.1 might contribute to the development of lung adenocarcinoma; further study of the biological function of the two lncRNAs will be required to confirm this notion.

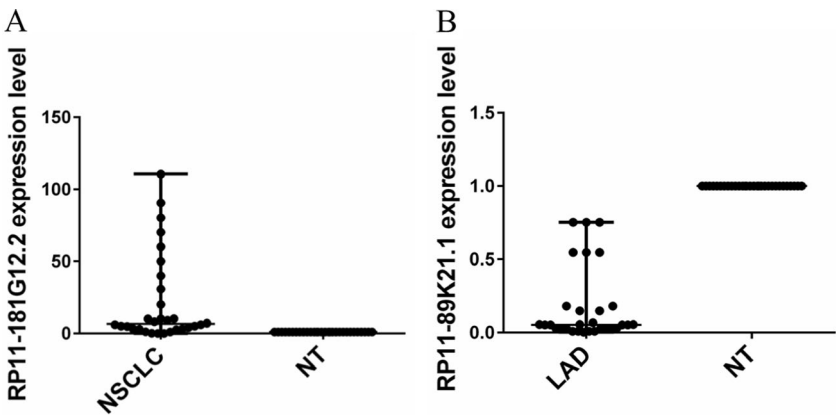

Fig. 5 The expression level of RP11-181G12.2 and RP11-89 K21.1 from LAD samples compared with NT

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Compliance with Ethical Standards

Conflict of Interest These conflicts did not interfere with the conduct of this study. All other authors have no other conflict of interest to declare.

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