Original Article
MicroRNA expression profile in stage IA lung adenocarcinoma and miR-940 target prediction

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Received April 13, 2016; Accepted December 10, 2018; Epub February 15, 2019; Published February 28, 2019

Abstract: Objectives: Non-small cell lung cancer (NSCLC) is the most common histologic type of lung cancer. Recent clinical studies have indicated that TNM staging does not adequately explain a significant stratification phenomenon in the prognosis of patients with stage IA lung adenocarcinoma. This study analyzed levels of microRNA expression in tumor tissues from patients with stage IA lung adenocarcinoma. A different prognostic stratification was used to explore alteration in molecular characteristics and the close relationship with lymph node micro-metastases.

Methods: Twenty-eight specimens from patients with stage IA lung adenocarcinoma, undergoing surgery in the Zhejiang Rongjun Hospital (Jiaxing, China) and Shaoxing People’s Hospital (Shaoxing, China), between May 2004 and October 2015, were selected (3 males and 25 females; age range, 53-71 years; and median age, 63 years). Six specimens from the high-risk group (more than 48 weeks) and 6 specimens from the low-risk group (less than 48 weeks) were used for analysis of miRNA expression profiles via TaqMan low density array analysis. Eight specimens from the high-risk group and 8 specimens from the low-risk group were used for reverse transcription PCR (RT-PCR) validation of miRNA. Results: TaqMan low-density array analysis showed that levels of expression of 21 kinds of miRNAs (let-7b-5p, miR-197-3p, miR-513a-5p, miR-940, miR-3620-3p, miR-3679-3p, miR-4484, miR-4510, miR-4635, miR-4640-3p, miR-4653-3p, miR-4695-3p, miR-5195-5p, miR-6511b-3p, miR-6784-3p, miR-6792-3p, miR-6793-3p, miR-6798-3p, miR-6803-3p, miR-6805-3p, and miR-6806-3p) were upregulated and levels of expression of 3 kinds of miRNAs (miR-4293, miR-3915, and miR-4476) were downregulated (P < 0.05 and FDR < 0.05). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to further validate miR-940, finding that results were the same with the expression tendency of miRNA revealed by array analysis. Targetscan and Miranda software were used to carry out significant Gene Ontology (GO) analysis and signaling pathway analysis for miR-940. It was shown that there were 18 significant target genes, based on GO analysis, and 2 signaling pathways. Conclusions: Taken together, the present study shows that these 24 kinds of miRNAs were abnormally expressed in stage IA lung adenocarcinoma, with different prognostic stratifications. They may participate in lymph node micro-metastases of stage IA lung adenocarcinoma. Of note, miR-940 was involved in a variety of targets and Wnt signaling pathways.

Keywords: MiRNA, lung adenocarcinoma, IA stage, lymph node micro-metastases, computational biology

Introduction

Lung cancer is a serious disease, posing a great threat to human health. With a worldwide range, epidemiologic reports of malignant tumors [1-3] have indicated that morbidity and mortality associated with lung cancer rank first among cancers. Non-small cell lung cancer (NSCLC) is the most common histologic type of lung cancer, accounting for 80%-85% of lung cancer in the patient population. Of these, the majority are lung adenocarcinomas [4]. TNM staging is carried out according to the size of the primary tumor, lymph node metastasis status, and whether there is distant metastases. TNM staging is the most commonly used staging method, worldwide. It is also the basis for establishing guidelines for diagnosis and treatment of lung cancer. TNM staging has played a huge role in the treatment of lung cancer [5]. However, results of recent clinical studies have indicated that TNM staging is insufficient to
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explain a significant stratification phenomenon in the prognosis of patients with stage IA lung adenocarcinoma [6, 7]. MicroRNAs are a small single-stranded non-coding RNA with a length of 18-25 nucleotides. They have a high degree of genetic stability. They degrade mRNA or inhibit the translation of mRNA mainly by combining with the 3’ untranslated regions (3’UTR) of the target gene mRNA, thus resulting in post-transcriptional regulation of gene expression [8]. As of August 2012, a total of 2,024 kinds of human miRNAs have been included in the Sanger miRBase (version 19.0). It is expected that miRNAs are involved in the regulation of nearly two-thirds of human protein-coding genes [9]. Moreover, miRNAs play important roles in cell proliferation, differentiation, apoptosis, and individual development. In recent years, studies involving the relationships between miRNAs and occurrence, metastasis, and invasiveness of tumors have become an intense focus of research. It has been reported that miRNA abnormalities are closely associated with occurrence and development of NSCLC [10-14]. There is a need for a thorough understanding on the pathogenetic mechanisms, diagnosis, prognosis, and treatment of NSCLC. However, studies on the roles of miRNAs in stage IA lung adenocarcinoma, with different prognostic stratifications, have rarely been reported. To this end, the current study examined the characteristics of miRNA expression profiles in stage IA lung adenocarcinoma, with different prognostic stratifications, thus laying a foundation for diagnosis and treatment of stage IA lung adenocarcinoma and discussion of the underlying mechanisms.

Materials and methods

Detection of miRNA expression profile

Experimental materials: Twenty-eight specimens from patients with stage IA lung adenocarcinoma, undergoing surgery in the Zhejiang Rongjun Hospital (Jiaxing, China) and Shaqiao People’s Hospital (Shaoxing, China), between May 2004 and October 2015, were selected (3 males and 25 females; age range, 53-71 years; and median age, 63 years). Six specimens from the high-risk group (more than 48 weeks) and 6 specimens from the low-risk group (less than 48 weeks) were used for analysis of miRNA expression profiles. Eight specimens from the high-risk group and 8 specimens from the low-risk group were used for experimental validation of miRNA. All specimens were fixed in 4% neutral formalin and embedded in paraffin.

Immunohistochemistry: The MaxVision two-step method was used for immunohistochemical staining. The primary antibody (CAM5.2; Beijing Zhongshan Golden Bridge Company, Beijing, China) was a mouse/rabbit anti-human monoclonal antibody. Sections were repaired through high pressure before staining. The staining procedure was performed according to kit instructions. Sections were developed with DAB. Tumor tissue was designated as the positive control and PBS was used instead of the primary antibody as the negative control.

MicroRNA expression profile analysis: (1) Extraction of total RNA: Six specimens from the high-risk group and 6 specimens from the low-risk group were selected. Five pieces of 8 μm wax membranes were harvested. The RecoverAll™ Total Nucleic Acid Isolation Kit (AB Company, Via Svizzera, Italy) was used for extraction of total RNA; (2) TaqMan low density array analysis: miRNAs from total RNA were extracted for quality control tests. A Ct value of the internal reference (U6 < 25) was considered the standard. Test results indicated that all Ct values amplified by internal reference small nuclear RNA U6 (U6 snRNA) were < 25 and the extracted total RNA was in accord with quality requirements of miRNA array analysis. Total RNA was reverse-transcribed into cDNA using MegaplexTM RT Primers, then cDNA preamplification was carried out using MegaplexTM PreAmp Primers. The solution was added with a low-density array containing 2,578 kinds of miRNAs (TaqMan Human MicroRNA Array A and B; AB company). Centrifugation was performed. The samples were evenly distributed to each reaction point. Finally, levels of miRNA expression were analyzed on an ABI-7900HT real-time quantitative polymerase chain reaction (qRT-PCR) instrument.

QRT-PCR test: Results of array validation: miR-940 was selected as the object. Expression levels in 8 patients of high-risk group and 8 patients of low-risk group were analyzed, respectively. Total RNA was extracted using miRNeasy FFPE kit (Qiagen Company), according to kit instructions. Reverse transcription: The primer
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of miR-940 (Megaplex III RT Primers, AB Company) and reverse transcription kit (TaqMan MicroRNA Reverse Transcription Kit, AB Company) took U6 snRNA as the internal reference. Three duplicate wells were made for each reaction. Amplification was performed using the qPCR instrument (ABI7500). The fold change of each tumor sample, relative to the normal sample, was calculated using 2-ΔΔCt \[ ΔΔCt = ΔCt \text{ experimental group} - ΔCt \text{ control group} = (Ct \text{ experimental group miRNA-Ct \text{ experimental group U6}}) - (Ct \text{ control group miRNA-Ct \text{ control group U6}}) \]. Fold changes I ≥ 2 indicated that expression levels of miRNA were increased, while fold changes I < 0.5 indicated that expression levels of miRNA were decreased. If the fold change ranged between 0.5 and 2.0, there were no changes in expression levels of miRNA.

Statistical analysis: Test results of TaqMan MicroRNA Array (A + B) were analyzed by t-test using the random variance model (RVM) based on a small sample [15]. P < 0.05 and FDR < 0.05 indicate statistically significant differences. Differences in miRNA expression profiles obtained from qRT-PCR were analyzed using
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Table 1. miRNA expression profiling of differences expression miRNA between the high-risk group and the low-risk group

<table>
<thead>
<tr>
<th>miRNA types</th>
<th>The average Ct value</th>
<th>Differences multiples</th>
<th>Log₂ (differences multiples)</th>
<th>P value</th>
<th>FDR value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>The high-risk group</td>
<td>The low-risk group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MiR-6798-3p</td>
<td>10.25253</td>
<td>9.401346</td>
<td>1.834516</td>
<td>-0.8754</td>
<td>2.13E-05</td>
</tr>
<tr>
<td>MiR-6793-5p</td>
<td>10.13036</td>
<td>9.420062</td>
<td>1.900571</td>
<td>-0.9263</td>
<td>0.000796</td>
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<tr>
<td>MiR-940</td>
<td>9.990828</td>
<td>9.248712</td>
<td>1.626923</td>
<td>-0.7021</td>
<td>0.000248</td>
</tr>
<tr>
<td>MiR-6805-3p</td>
<td>9.739469</td>
<td>9.085869</td>
<td>1.513253</td>
<td>-0.5976</td>
<td>3.44E-05</td>
</tr>
<tr>
<td>MiR-6792-3p</td>
<td>9.806777</td>
<td>9.262519</td>
<td>1.532499</td>
<td>-0.6158</td>
<td>0.008598</td>
</tr>
<tr>
<td>MiR-6862-3p</td>
<td>9.704607</td>
<td>9.152036</td>
<td>1.605594</td>
<td>-0.6831</td>
<td>0.006929</td>
</tr>
<tr>
<td>MiR-6803-3p</td>
<td>9.576438</td>
<td>9.060785</td>
<td>1.560961</td>
<td>-0.6424</td>
<td>0.038788</td>
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<tr>
<td>MiR-4635</td>
<td>9.918548</td>
<td>9.350358</td>
<td>1.57685</td>
<td>-0.6570</td>
<td>0.049333</td>
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<tr>
<td>MiR-4653-3p</td>
<td>10.08577</td>
<td>9.700849</td>
<td>1.625636</td>
<td>-0.701</td>
<td>0.011333</td>
</tr>
<tr>
<td>MiR-5195-5p</td>
<td>10.03154</td>
<td>9.662821</td>
<td>1.550274</td>
<td>-0.6325</td>
<td>0.041403</td>
</tr>
<tr>
<td>MiR-4510</td>
<td>9.735673</td>
<td>9.401018</td>
<td>1.5127</td>
<td>-0.5971</td>
<td>0.034722</td>
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<tr>
<td>Let-7b-5p</td>
<td>10.21238</td>
<td>9.652605</td>
<td>1.591627</td>
<td>-0.6705</td>
<td>0.039921</td>
</tr>
<tr>
<td>MiR-3679-3p</td>
<td>9.421976</td>
<td>8.699961</td>
<td>1.55665</td>
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<td>MiR-4640-3p</td>
<td>9.219585</td>
<td>8.632691</td>
<td>1.512135</td>
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<td>1.58E-05</td>
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<tr>
<td>MiR-3915</td>
<td>8.369949</td>
<td>8.826005</td>
<td>1.561028</td>
<td>0.642496315</td>
<td>0.018572</td>
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<td>MiR-4293</td>
<td>8.346554</td>
<td>8.698131</td>
<td>1.505972</td>
<td>0.59069524</td>
<td>0.005315</td>
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<td>MiR-3620-3p</td>
<td>10.43693</td>
<td>9.644077</td>
<td>1.644421</td>
<td>-0.7175</td>
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<td>MiR-197-3p</td>
<td>10.23322</td>
<td>9.465233</td>
<td>1.684515</td>
<td>-0.7523</td>
<td>0.00893</td>
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<tr>
<td>MiR-4695-3p</td>
<td>10.55888</td>
<td>9.911137</td>
<td>1.784779</td>
<td>-0.8357</td>
<td>0.049528</td>
</tr>
<tr>
<td>MiR-513a-5p</td>
<td>10.56582</td>
<td>9.951335</td>
<td>1.730559</td>
<td>-0.7912</td>
<td>0.002723</td>
</tr>
<tr>
<td>MiR-6784-3p</td>
<td>11.28824</td>
<td>10.44518</td>
<td>2.053846</td>
<td>-1.0383</td>
<td>0.010642</td>
</tr>
<tr>
<td>MiR-6511b-3p</td>
<td>11.23339</td>
<td>10.1246</td>
<td>2.224695</td>
<td>-1.1536</td>
<td>0.002143</td>
</tr>
<tr>
<td>MiR-4484</td>
<td>12.57223</td>
<td>12.14947</td>
<td>1.555869</td>
<td>-0.6377</td>
<td>0.021641</td>
</tr>
<tr>
<td>MiR-4476</td>
<td>10.84087</td>
<td>11.40172</td>
<td>1.635578</td>
<td>0.709800757</td>
<td>0.005315</td>
</tr>
</tbody>
</table>

SPSS19.0 software and two independent samples t-test. P <0.05 indicates statistically significant differences.

Cluster analysis: Methods of Euclidean distance function and average linking clustering were applied. Cluster-view 3.0 software was used to carry out clustering analysis for Ct values of 24 miRNAs, with significant expression differences in 6 patients from the high-risk group and 6 patients from the low-risk group.

Bioinformatics analysis

Target prediction and functional analysis: Target prediction software (miRanda [MicroRNA. Org/microrna/homedo] [16] and TargetScan [http://www.Targetscan.Org/] [17]) was used to carry out target prediction and functional analysis, respectively. For miR-940,miRNAs, there was a significant expression difference in 6 patients from the high-risk group and 6 patients from the low-risk group with stage IA lung adenocarcinoma.

Significant gene ontology analysis (GO-Analysis) of target: Two intersected targets predicated by target prediction software were taken to study the distribution status in the GO database. The functional embodiments of targets of miR-940 were clarified [18, 19]. Fisher’s exact test and χ² test were mainly used to obtain P and FDR values, respectively, (false positive rate and re-estimation of P value precision). Based on a P < 0.05 and FDR < 0.05, significant functions of targets were selected.

Significance analysis of signal transduction pathways (Pathway-Analysis): Pathway classification of miR-940 was carried out according to KEGG public databases. Significance analysis of targets based on the discrete distribution was carried out to obtain pathway classification of significance [20, 21]. Fisher’s exact test and
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Results

Immunohistochemistry

Of the 28 patients with stage IA lung adenocarcinoma, 14 patients in the high-risk group had lymph node micro-metastases (Figure 1), while 14 patients in the low-risk group had no lymph node micro-metastases.

Survival curves

Survival curves of 14 patients in the high-risk group and 14 patients in the low-risk group showed that the progression-free survival (PFS) was 25 weeks in the high-risk group and 135 weeks in the low risk group. (Figure 2A). Overall survival (OS) in the high-risk group was 25 weeks, while the follow-up endpoint in the low-risk group was not reached (P < 0.001; Figure 2B).

Cluster analysis

Clustering analyses of 24 kinds of miRNAs, with a significant expression difference from the patients in the high-and low-risk groups, were carried out. Results are shown in Figure 4 (A1, A2, A3, A4, A5, and A6 are representative of the high-risk group; B1, B2, B3, B4, B5, and B6 are representative of the low-risk group). As shown in Figure 4, the horizontal axis is the main miRNA cluster. miR-4293, miR-3915, and miR-4476 were downregulated in the high-risk group and 3 miRNA clusters were more apparent, while let-7b-5p, miR-197-3p, miR-513a-5p, miR-940, miR-3620-3p, miR-3679-3p, miR-4484, miR-4510, miR-4635, miR-4640-3p, miR-4653-3p, miR-4695-3p, miR-5195-5p, miR-6511b-3p, hsa-miR-6784-3p, miR-6792-3p, miR-6793-3p, miR-6798-3p, miR-6803-3p, miR-6805-3p, and miR-6862-3p were upregulated in the high-risk group. A total of 21 miRNA clusters were more obvious (Figure 4).

qRT-PCR validation

Compared with 8 patients from the low-risk group, miR-940 was upregulated in 8 patients from the high-risk group. The log2 differences multiples are significant differences of expression among 24 types of miRNA in lung adenocarcinoma miRNA expression profiling (1: miR-4293, 2: miR-3915, 3: miR-4476, 4: let-7b-5p, 5: miR-197-3p, 6: miR-513a-5p, 7: miR-940, 8: miR-3620-3p, 9: miR-3679-3p, 10: miR-4484, 11: miR-4510, 12: miR-4635, 13: miR-4640-3p, 14: miR-4653-3p, 15: miR-4695-3p, 16: miR-5195-5p, 17: miR-6511b-3p, 18: miR-6784-3p, 19: miR-6792-3p, 20: miR-6793-3p, 21: miR-6798-3p, 22: miR-6803-3p, 23: miR-6805-3p, 24: miR-6862-3p).
from the high-risk group, consistent with the results of array analysis (Table 2; Figure 5). Differences were statistically significant (P < 0.05).
Bioinformatics analysis of miR-940

In this study, miRanda and TargetScan were used to predict the targets of miR-940 and carry out GO and pathway analyses. GO analysis of the miR-940 target assembly showed that the assembly functions of targets regulated by miR-940 were mainly enriched in biological processes, such as protein transport, establishment of protein localization, and sodium ion transport ($P < 0.05$). Prediction results of pathway analysis of the miR-940 target assembly showed that signal transduction pathways of the miR-940 target assembly were enriched in the Axon guidance and Wnt signaling pathways ($P < 0.05$) (Figure 6).

Discussion

Goodgame et al. [6] studied the prognosis of 715 patients with NSCLC that underwent surgery. They reported that the 5-year survival rate of stage IA patients was 66%, but 19% of patients still had tumor metastases occurring within 5 years. Detterbeck et al. [7] published a very comprehensive review and analysis regarding the modification and recommendation of the 7th TNM staging system for NSCLC developed by the International Association for the Study of Lung Cancer (IASLC). Detterbeck et al. [7] reported that 73% of patients with stage IA lung cancer had a satisfactory prognosis. The tumor-free survival period was >5 years in the low-risk group. A total of 27% of patients still had a poor prognosis and most of the patients died due to post-operative tumor recurrence or metastasis in the high-risk group. Some studies have shown that one of the causes of early mortality after lung cancer surgery is the presence of lymph node micro-metastases [22-25]. Hanagiri et al. [26] found that among study samples from 131 stage IB and IIA patients, lymph node micro-metastases existed in the FOXP3 high-expression group. Prognosis was significantly worse than the FOXP3 low-expression group. Li et al. [27] studied samples from 44 stage IB and IIA patients, finding that 15 patients had lymph node micro-metastases. The tumor-free survival period was significantly lower than the group without lymph node micro-metastases. Dai et al. [28] examined 269 lymph node samples from 49 stage I-IIB patients, finding that 16 patients had lymph node micro-metastases. The disease-free survival period and 5-year survival rates were substantially reduced. These results indicate that the presence of lymph node micro-metastases in patients with stage IA lung cancer is a key factor affecting prognosis. It is an important reason explaining prognostic stratification in patients with stage IA lung adenocarcinoma. This study found that, for patients with stage IA lung cancer that should not have lymph

Table 2. qRT-PCR detection miR-940 of differences multiples between the the high-risk of 8 cases and the low-risk group of 8 cases

<table>
<thead>
<tr>
<th>MiRNA type</th>
<th>ΔCt mean</th>
<th>Differences multiples ($2^{-\Delta\Delta C_t}$)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>The low-risk group (n=8)</td>
<td>The high-risk group (n=8)</td>
<td></td>
</tr>
<tr>
<td>MiR-940</td>
<td>0.3</td>
<td>1.67</td>
<td>3.46</td>
</tr>
</tbody>
</table>

Figure 5. Relative expression of qRT-PCR detection miR-940 of differences multiples between the the high-risk of 8 cases and the low-risk group of 8 cases.
node metastases, even with the same histologic characteristics and undergoing surgical treatment using the same method in the same hospital by the same surgeon, there were significant differences in the therapeutic response rates when using the same chemotherapy regimen. Median survival time, PFS, and OS times among different groups of patients with lung cancer differed. In the current study, immunohistochemistry showed that all 14 patients with stage IA lung adenocarcinoma in the high-risk group had lymph node micro-metastases. None of the 14 patients with stage IA lung adenocarcinoma in the low-risk group had lymph node micro-metastases. Survival curves of 14 patients in the high-risk group and 14 patients in the low-risk group showed that the PFS was 25 weeks in the high-risk group and 135 weeks in the low-risk group (P = 0.01). The OS was 25 weeks in the high-risk group, while the follow-up endpoint in the low-risk group was not reached (P < 0.001).

In a study involving epigenetic factors in lymph node metastases of lung adenocarcinoma, Huang et al. [29] performed forward genetic screening using a miRNA expression library. They found that overexpression of miR-373 and miR-520c inhibited expression of CD44 proteins. Thus, tumor cells have a stronger metastatic potential, enhancing tumor metastasis. A study involving miR-21 showed that miR-21 can inhibit the function of cancer suppressor genes, such as PTEN, leading to high expression of downstream metastasis-associated proteins, such as MMP2 and MMP9 [29]. Degradation of the basement membrane promotes the epithelial-mesenchymal transition of cells, eventually mediating the occurrence of lymph node metastasis. Tavazoie et al. [30] reported that miR-335 and miR-126 expression is always downregulated in metastatic cells in an animal model. If expression of these miRNAs is restored in a xenograft mouse model, then the probability of emergence of metastases will be decreased. Watanabe et al. [31] reported that the methylation of miR-34 is significantly correlated with lymph node metastasis in non-small cell lung cancer and can be taken as the molecular marker of an aggressive phenotype in NSCLC. These results suggest that abnormal expression of miRNA in lung cancer cells also has a close relationship with lymph node micro-metastases. In this study, microarray detection revealed that 21 kinds of miRNAs (let-7b-5p, miR-197-3p, miR-513a-5p, miR-940, miR-3620-3p, miR-3679-3p, miR-4484, miR-4510, miR-4635, miR-4640-3p, miR-4653-3p, miR-4695-3p, miR-5195-5p, miR-6511b-3p, miR-6784-3p, miR-6792-3p, miR-6793-3p, miR-6798-3p, miR-6803-3p, miR-6805-3p, and miR-6862-3p) were upregulated. Three kinds of miRNAs (miR-4293, miR-3915, and miR-4476) in the high-risk group were downregulated (P < 0.05). Using qRT-PCR detection, this study validated that the fold change in miR-940 expression was 3.46 (P < 0.05), illustrating that miR-940 has a close relationship with lymph node micro-metastases.

This study predicted specifically-expressed miR-940 in stratified samples of stage IA pulmonary adenocarcinoma using target predic-

Figure 6. GO Analysis and Pathway Analysis of targets. A. GO analysis: X axis represents LgP value which negatively corresponds to P value and Y represents the upregulated genes with significant GO (a total of 18); B. Pathway analysis: X axis represents LgP value which negatively corresponds to P value, and Y represents the upregulated genes in indicated pathways (a total of 2).
tion software (miRanda and TargetScan). Due to different algorithms used in the software, an auxiliary method only can be used in the study of miRNA targets. Pathway analysis showed that miRNA participates in Wnt signaling pathways. Previous studies have shown that sustained activation of Wnt signaling pathways is often involved in stage IA pulmonary adenocarcinoma [32, 33], suggesting that miRNAs may participate in the regulation of the Wnt signal transduction pathways, leading to prognostic stratification of patients with stage IA lung adenocarcinoma.

Disclosure of conflict of interest

None.

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