Characterization of microRNA transcriptome in tumor, adjacent, and normal tissues of lung squamous cell carcinoma

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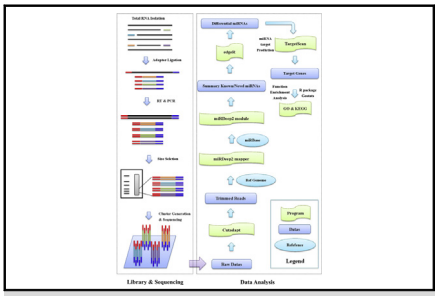
ABSTRACT

Objectives: MicroRNAs are a class of regulatory molecules involved in a wide variety of biological processes, including growth, development, and apoptosis. Given the widespread roles of microRNAs in biological processes, understanding their different expression profiles in normal, adjacent, and tumor tissues will provide insights into the consequences of aberrant expression.

Methods: With the use of next-generation deep sequencing technology, microRNA profiles in 3 pooled samples from normal, adjacent, and tumor tissues of 19 patients with lung squamous cell carcinoma were characterized comprehensively. Quantitative polymerase chain reaction was used to verify the primary findings in another 38 lung squamous cell carcinoma tumor samples. In situ hybridization also was performed for validation.

Results: A total of 368, 306, and 231 known microRNAs were identified from tumor, adjacent, and normal pooled samples, respectively, of which 40, 44, and 26 microRNAs displayed dysregulation with 2-fold or greater change in 3 compared groups of tumor versus normal, tumor versus adjacent, and adjacent versus normal, respectively. Sequencing data also showed that some coexpressed microRNAs displayed a pattern of progressive dysregulation. Some of the microRNAs exhibited consistent changes; among them, miR-425-5p and miR-218-5p were confirmed by quantitative polymerase chain reaction and in situ hybridization, and proved that the microRNA expression levels were closely related to tumor stages and sizes. It is suggested that some microRNAs, such as miR-425 and miR-183, might be a driver for tumor formation, growth, and progression to higher staging, whereas others, such as miR-218, might behave as a tumor suppressor in lung cancer. Functional annotation analysis indicated that the proteoglycan pathway in cancer and mitogen-activated protein kinase, Wnt, PI3K-Akt, and transforming growth factor-beta signaling pathways might be involved in the pathogenesis of lung squamous cell carcinoma.

Conclusions: This study describes the use of deep sequencing for comprehensive profiling of microRNAs in lung squamous cell carcinoma. The identified microRNA signatures may provide biomarkers for early detection, subclassification, and potential therapeutic targets of lung squamous cell carcinoma. This study also provides some insights into the molecular mechanism underlying the development and progression of lung squamous cell carcinoma, which may prove helpful for early diagnosis and treatment of the disease. (J Thorac Cardiovasc Surg 2015;149:1404-14)



Flow chart of library preparation and data analysis of the lung cancer tissue miRNA profiling by deep sequencing.

Central Message

MicroRNA profiles and signatures of lung squamous cell cancer identified by next generation deep-sequencing method may provide potential biomarkers for early detection, subclassification, and potential therapeutic targets of lung SCC. These will provide some insight into the molecular mechanism underlying the development and progression of lung SCC.

Perspective

With the increasing incidence of lung cancer, it is clinically imperative to develop new biomarkers that can provide targets for diagnosis and treatment. We obtained miRNA profiles of lung SCC and found some coexpressed miRNAs with a pattern of progressive dysregulation. Further analysis first revealed that some miRNAs (eg, miR-425, miR-183, and miR-218) might be drivers for tumor formation, growth, and progression, or behave as a tumor suppressor with similar mechanisms reported in previous studies of other cancers. The current study can provide important basic data for studying the pathogenesis of lung cancer and has laid the foundation for recognizing candidate biomarkers.

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Abbreviations and Acronyms

miRNA = microRNA
NSCLC = non–small-cell lung cancer
nt = nucleotide
qPCR = quantitative polymerase chain reaction
qRT-PCR = quantitative reverse transcription polymerase chain reaction
SCC = squamous cell carcinoma

Supplemental material is available online.

Lung cancer is the leading cause of cancer-related mortality worldwide, accounting for 29% of cancer deaths in male patients and 26% of cancer deaths in female patients.[1] Genome molecular assays recently have been used in the diagnosis of lung cancer. Despite recent advances in diagnosis and treatment strategies, the prognosis of lung cancer remains poor, with a 5-year overall survival of 16%.[2,3] The development of new therapeutic targets for early diagnosis and treatment may reduce the mortality of lung cancer.[3] Furthermore, the ability to identify lung cancer with lymph node metastasis or recurrence after surgical resection would provide a case of guiding significance in adjuvant therapies, while sparing patients without metastasis from unnecessary cytotoxic chemotherapy and reducing the death rate.[4]

Non–small-cell lung cancer (NSCLC), the most common type of lung cancer, includes 2 major histologic subtypes: squamous cell carcinoma (SCC) and adenocarcinoma.[5] Differentiation between SCC and adenocarcinoma has become an increasingly important issue in the management of lung cancer, because different treatments have diverse therapeutic or adverse effects depending on the histologic type.[6] Altogether, it is clinically imperative to develop new biomarkers that can improve early detection and subclassification, predict recurrence of NSCLC, and identify novel molecular targets for treatment.

MicroRNAs (miRNAs) are endogenous noncoding small RNAs of approximately 18 to 25 nucleotides (nt).[7] Mature miRNAs are highly conserved RNA molecules that can regulate the expression of genes by hybridizing to complementary sequences in the 3′ untranslated region of target miRNAs.[8] Many studies have demonstrated that miRNAs participate in various biological processes, including cell proliferation, differentiation, and apoptosis.[9] In addition, studies have shown that miRNAs play roles in the development and progression of lung cancer by promoting the expression of oncogenes or by inhibiting tumor suppressor genes.[10-14]

Although quantitative reverse transcription polymerase chain reaction (qRT-PCR) and microarrays have been used to identify lung cancer–associated miRNA aberrations,[15] these technologies only measure relative abundance of known miRNAs. Recent cancer profiling studies have focused on next-generation sequencing,[16] knowing that high throughput sequencing of miRNAs can avoid the limitations of microarrays and allow for massive parallel sequencing of millions of sequences on chips, thus generating a greater number of transcript sequences even if finding many novel miRNAs that is impossible by microarray analysis.[17]

Illumina (Illumina Inc, San Diego, Calif) and SOLiD (Applied Biosystems, Waltham, Mass) have been used to profile serum miRNAs of patients with lung cancer.[18,19] Several reports showed that miRNAs were differentially expressed in tumor tissues. However, there is no study reporting the use of next-generation deep sequencing for systematic characterization of miRNA transcriptome of tumor, adjacent, and normal tissues in patients with lung cancer. Deep sequencing-based miRNA profiling directly from clinically defined and histologically confirmed surgical tumor tissues is important because it is disease-specific. Characterization of miRNA transcriptome in lung tumor, adjacent, and normal tissues by next-generation deep sequencing may help develop strategies to improve early diagnosis and treatment of NSCLC.

The aim of the present study was to systematically characterize alterations of miRNAs in surgically resected lung tumor and adjacent tissues by using next-generation sequencing in an attempt to (1) comprehensively profile the expression pattern of miRNAs in SCC; (2) verify the findings obtained from the pairwise pooled SCC samples in other tumor tissues using quantitative polymerase chain reaction (qPCR); and (3) evaluate whether miRNAs could provide potential diagnosis and therapeutic targets for SCC by functionally analyzing the roles of the differentially expressed miRNAs.

MATERIAL AND METHODS

Sample Preparation and RNA Isolation

We collected 19 groups of clinical samples. Every group included cancerous tissues, tumor-adjacent tissues, and normal control tissues of SCCs. We also collected another 38 cancerous tissues from SCC for qPCR validation. All samples mentioned were obtained from Jiangsu Province People's Hospital with informed consent from all patients concerned. This study was approved by the Ethics Committee of Jiangsu Province People's Hospital. miRNAs were isolated from approximately 50 mg of tissue from each sample using a mirVana miRNA isolation Kit (Ambion, Austin, Tex), with some modifications according to the manufacturer's instructions. The quantity and quality of obtained miRNAs were measured with a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY) according to the manufacturer's protocol.

Deep Sequencing

For high-throughput small RNAs sequencing, 3 pooled samples (SCC tumor, adjacent, and normal tissues) were prepared by equally mixing the 19 corresponding samples. Small RNA libraries were established for deep sequencing as described in previous reports[17,20-24] according to the

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manufacturer's protocols. Briefly, sample RNAs were adapter-ligated using T4 RNA ligase and subsequently reverse-transcribed into cDNA by SuperScript II Reverse Transcriptase (Life Technologies). The cDNA fragments were polymerase chain reaction amplified for subsequent sequencing. Illumina HiSeq 2500 (Illumina Inc) was used for sequencing of the RNA libraries. The process of library preparation and sequencing data analysis is shown in Figure 1.

CAP-miRSeq was used for data analysis according to the user guider with some modifications.[25] The mean length of Illumina sequence reads was 41 nt, greater than the mean size of miRNAs (19-25 nt). Because the reads contained part of the 3′-adaptor at the end of the sequences, we used Novoalign (V2.08.01 Novocraft 2010; www.novocraft.com) to cut all reads at the 3′-end to remove the adapter sequences. After adaptor trimming, reads less than 12 bases were discarded. Then, trimmed reads were input into miRDeep2 to quantify known miRNAs against miRBase and predict novel miRNAs (http://sourceforge.net/projects/mireap/). Expression values of miRNAs were selected for the differential expression analysis using tool edgeR from Bioconductor, as described previously[26] (Figure 1). The screening criteria for differential miRNAs was defined as normalization reads greater than 50, the absolute value of fold-change (Log 2 [case/control]) was greater than 2, and its P value was less than .05.

Quantitative Validation of MicroRNAs by Quantitative Real-Time Polymerase Chain Reaction and In Situ Hybridization

qRT-PCR was performed to validate the expression levels of miRNAs analyzed by sequencing according to the previous studies.[18] Dissociation curve was performed in each reaction to verify the effectiveness. U6 small nuclear RNA was used as an internal reference control. The $\Delta\Delta$Ct method was used for data analysis.[27]

Detection of miRNAs by in situ hybridization with riboprobes was essentially as described by Li and colleagues[28] and Silahtaroglu and colleagues.[29] Briefly, the tissues were deparaffinized, dehydrated, and subsequently immersed in 0.2 N HCl, and the tissues were then fixed in 10% neutral-buffered formalin. After digestion with proteinase K, the slides were then prehybridized at 37°C for 2 hours, followed by the hybridization with probe at 37°C for 24 hours. miR-425-5p and miR-218-5p were detected by in situ hybridization for validation in this study.

Prediction and Enrichment Analysis of Target Genes

Target genes of differentially expressed miRNAs were predicted using TargetScan software (http://www.targetscan.org/).[30] Gene Ontology[31] enrichment analysis of the target genes was performed to investigate the functional distribution of differentially expressed miRNAs from tumor, adjacent, and normal tissues. In addition, KEGG pathway analysis of the target genes was also performed as previously described.[22,32]

RESULTS

Analysis of Sequenced Small RNAs

A total of 3.3 million (tumor tissue), 3.1 million (adjacent tissue), and 2.2 million (normal tissue) raw reads were generated from the 3 libraries. As shown in Figure E1, reads with a length of 22 nt were the most abundant, followed by 23 and 21 nt reads, which corresponded to the mean length of miRNAs. Small RNAs of 20 to 24 nt in length accounted for 94.4% (tumor), 88.2% (adjacent), and 86.5% (normal) of the total number of small RNA reads. In this study, a total of 196 novel miRNA sequences were identified from RNA libraries, with 84, 63, and 49 novel miRNAs in tumor, adjacent, and normal pooled samples, respectively.

Differential Expression Analysis of MicroRNAs in Tumor, Adjacent, and Normal Tissues of Lung Squamous Cell Carcinoma

A total of 44 (25 upregulated and 19 downregulated), 40 (26 upregulated and 14 downregulated), and 26 (8 upregulated and 18 downregulated) known miRNAs were identified to be differentially expressed in tumor versus normal samples, adjacent versus normal samples, and tumor versus adjacent samples, respectively. As shown in Table E1 and Figure E2, hsa-miR-135a-5p was the most downregulated miRNA in the tumor tissue compared with the normal control, representing an approximately 4-fold change. In contrast, hsa-miR-205-5p and hsa-miR-31-5p were the most upregulated, representing an approximately 6-fold change. With stricter cutoff criteria (fold change >3 and $P < .05$), 11 miRNAs displayed considerable expression differences between the tumor and normal tissues (all $P < .001$); 9 were upregulated and 2 were downregulated (Figure 2, *A*). Fourteen miRNAs exhibited substantial differences between the tumor and adjacent tissues; 10 were upregulated and 4 were downregulated (Figure 2, *B*). Only 6 miRNAs exhibited extremely differences between the adjacent and normal tissues; 2 were upregulated and 4 were downregulated (Figure 2, *C*). In addition, 5 miRNAs (hsa-miR-135a-5p, 205-5p, 31-5p, 196b-5p, and 1246) displayed altered expressions in both adjacent and normal tissues compared with the tumor tissue (Figure 2, *A*-*C*).

MicroRNA Expression Shows Gradual Changes From Normal to Tumor Tissues of Squamous Cell Carcinoma

In this study, we considered those miRNAs were gradually changed, which upregulated or downregulated significantly with fold-change greater than 2 and P value less than .05, from normal tissues to tumor tissues. We obtained 12 miRNAs as shown in Figure 3, *A*, of which 5 miRNAs (let-7a-3p, miR-140-5p, 34c-5p, 218-5p, and 449a) were gradually downregulated, and 7 miRNAs (miR-409-3p, 424-5p, 19a-3p, 454-3p, 708-5p, 425-5p, and 183-5p) were gradually upregulated. Among them, 2 of the gradually changed miRNAs (miR-425-5p, miR-218-5p) were confirmed by qPCR (Figure 3, *B*).

Quantitative Real-Time Polymerase Chain Reaction and In Situ Hybridization Validation of the Differential Expression of MicroRNAs

To confirm the deep sequencing results, qRT-PCR was used to assess the expression of 10 miRNAs (mir-205-5p, 31-5p, 196b-5p, 425-5p, 337-5p, 183-5p, 135a-5p, 218-5p, 625-3p, and let-7a-3p) in the same samples of SCC as those used in deep sequencing (19 tumor tissues, 19 adjacent tissues, and 19 normal tissues). These 10 miRNAs were selected to represent highly expressed miRNAs and low expressed miRNAs, but all showed significant dysregulation among the pooled samples of lung SCC. Of them, 2

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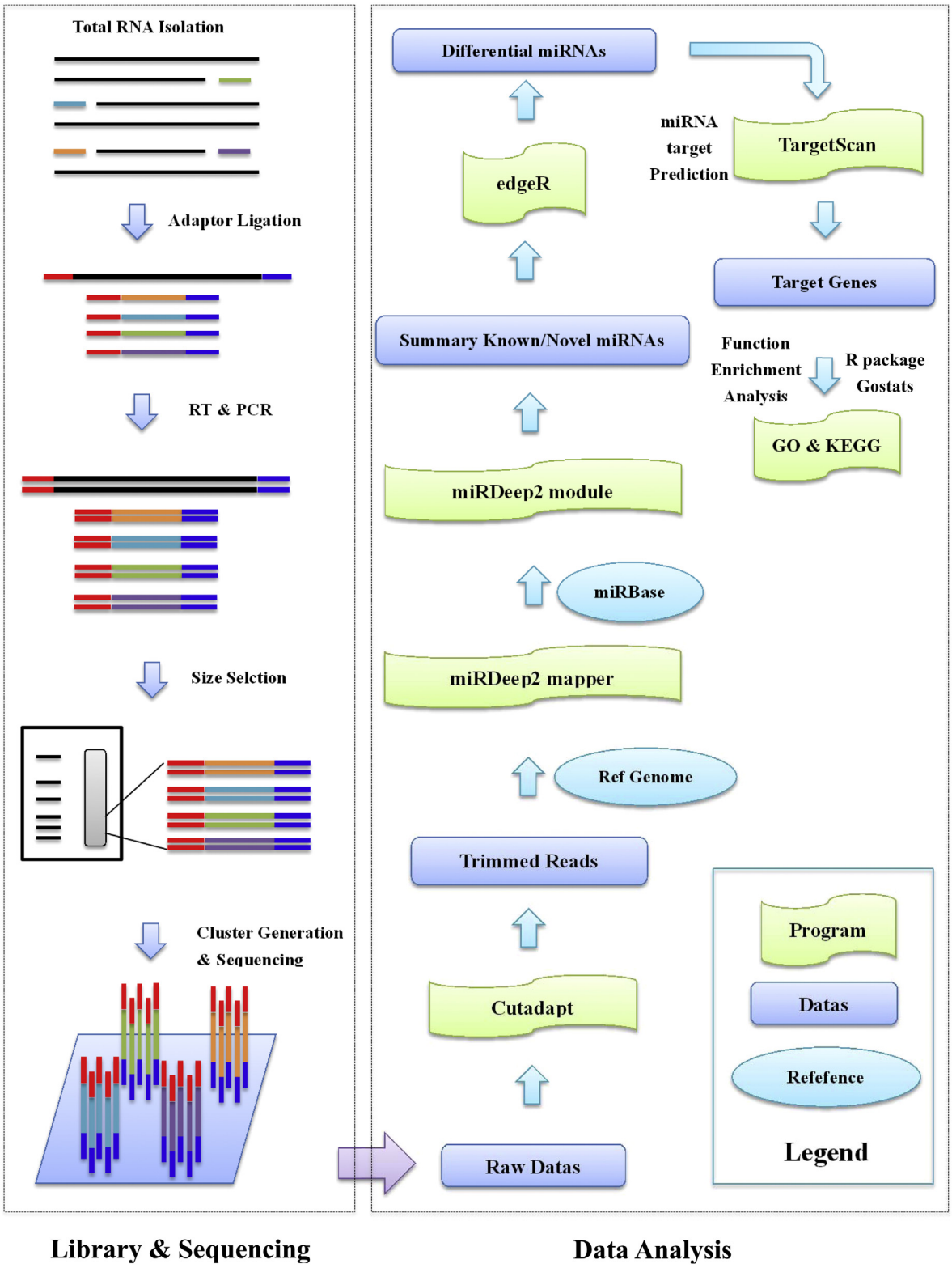

FIGURE 1. Flow chart of library preparation and data analysis of the lung cancer tissue miRNA profiling by deep sequencing. *GO*, Gene Ontology; *miRNA*, microRNA; *RT & PCR*, reverse transcription polymerase chain reaction; *KEGG*, Kyoto Encyclopedia of Genes and Genomes.

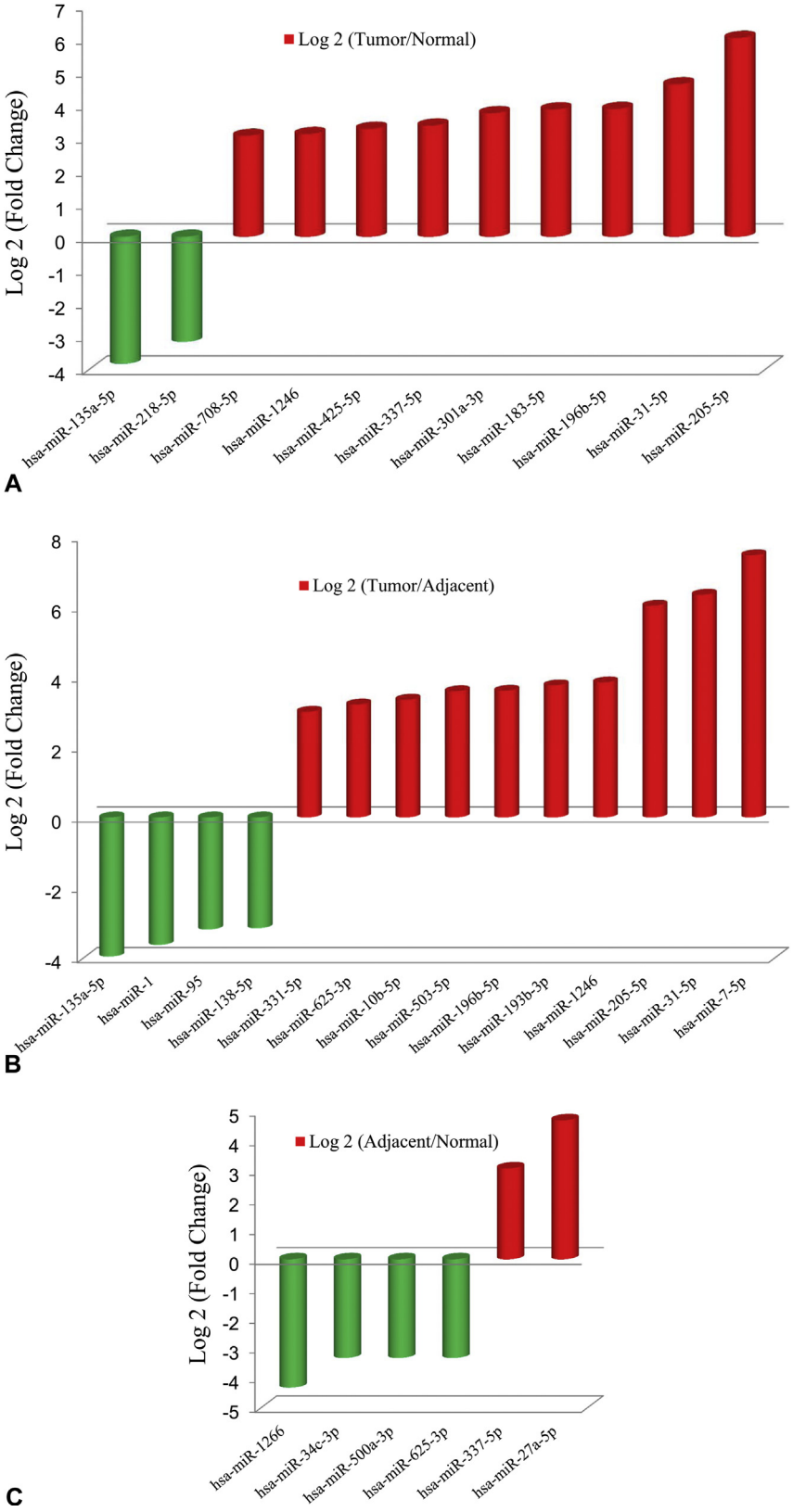

FIGURE 2. Significantly differentially expressed miRNAs in tumor, adjacent, and normal tissues. A, Upregulated and downregulated miRNAs in tumor versus normal tissues as defined by deep sequencing. B, Upregulated and downregulated miRNAs in tumor versus adjacent tissues as defined by deep sequencing. C, Upregulated and downregulated miRNAs in adjacent versus normal tissues as defined by deep sequencing.

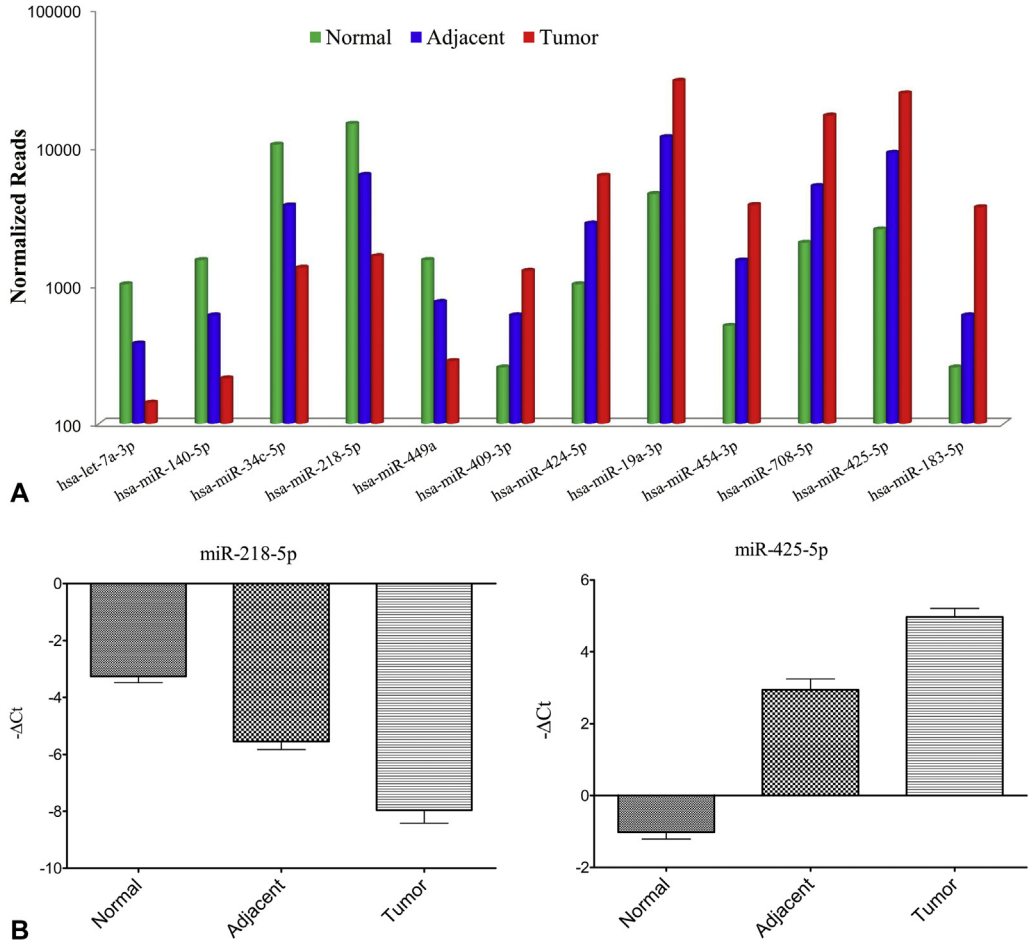

FIGURE 3. miRNA expression level shows a gradual significant change from normal tissue to tumor tissue. A, Some of them are upregulated successively, and others are downregulated successively. B, qPCR validation of the 2 related miRNAs shows gradual changes from normal tissue to tumor tissue. The expression level of miR-218-5p shows decreasing changes, and miR-425-5p shows increasing changes.

(mir-625-3p, let-7a-3p) had a Ct value greater than 35, suggesting that the 2 miRNAs were not reliably measured by qPCR and thus excluded from further analysis. The other 8 miRNAs exhibited significantly different levels in the SCC tumor tissue compared with the normal or adjacent tissue ($P < .05$) (Table E2). In addition, all of these changes detected by qRT-PCR were in the same direction as those detected by deep sequencing analysis, although the magnitude of changes differed between the 2 methods. The validation results obtained from a large set of tissues using qPCR suggested that the differential miRNAs could be signatures of SCC. As shown in Figure 4, the gradually changed expression of miR-425 and miR-218 from normal to tumor tissue of lung SCC was also confirmed by miRNA in situ hybridization.

Associations Between MicroRNA Expression and Histopathologic Parameters of Squamous Cell Carcinoma

To further substantiate the findings, expressions of 5 miRNAs (miR-425-5p, 31-5p, 337-5p, 183-5p, and 218-5p) selected from the results (Table E2) were assessed using another different 38 tumor tissues of SCCs with different stages and different tumor sizes. In line with the expected findings, all 5 miRNAs showed dysregulation in different tumor tissues of different stages. As shown in Figure 5, 4 miRNAs (miR-425-5p, 183-5p, 337-5p, and 218-5p) displayed distinctive expression levels in the tumor tissues of different stages (Figure 5, *A*) ($P < .05$), and 2 miRNAs (miR-183-5p and 425-5p) showed a close correlation with tumor size (Figure 5, *B*) ($P < .05$). However, there was no significant correlation between changes in miRNAs and age, gender, and race of the patients with SCC (all $P > .05$).

MicroRNA Target Prediction and Functional Annotation

A total of 5949 target genes of all differentially expressed miRNAs were predicted. All enriched Gene Ontology terms and the count of genes annotated are presented in Figure 6, *A-C*. We found a significant over-representation of the cellular process, single-organism process, and metabolic

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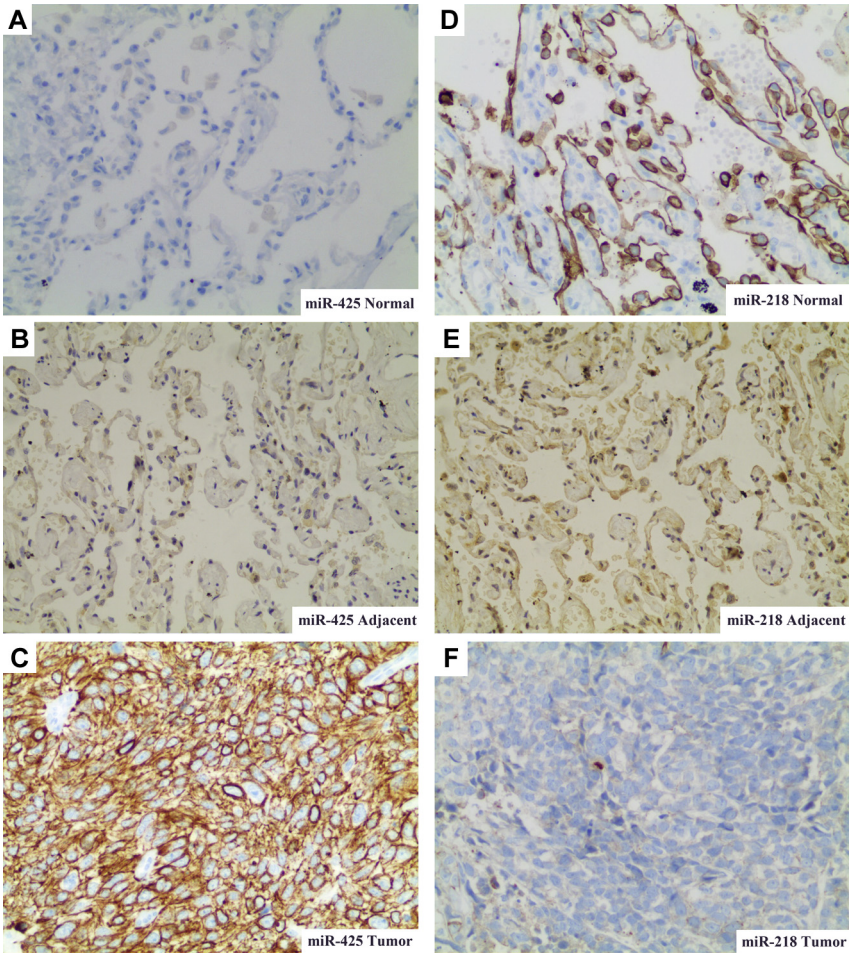

FIGURE 4. In situ hybridization assays for miR-425-5p and miR-218-5p were performed on tumor, adjacent, and normal lung tissues from patient with SCC. Representative results are shown. Gradually upregulated miR-425-5p (A-C) and gradually downregulated miR-218 (D-F) were detected in tumor, adjacent, and normal lung tissues.

processes for the biological process and binding, catalytic activity, and molecular transducer activity for the molecular function. As shown in Table 1, 20 significant pathways with a *P* value less than .01 were identified. Of note, the top canonical pathways of differentially expressed miRNAs targets included pathways in cancer, endocytosis, protein processing in endoplasmic reticulum, proteoglycans in cancer, MAPK signaling pathway, and transforming growth factor-beta signaling pathway.

DISCUSSION

In this study, we defined differential expression of miRNAs in the tumor, adjacent, and normal tissues from patients with lung SCC by deep sequencing technology and found successive dysregulated miRNAs in those differential miRNAs coexpressed in the tumor, adjacent, and normal tissues. Some of these significantly differentially expressed miRNAs were confirmed by qRT-PCR, showing that the expression level of miR-425-5p, 183-5p, 337-5p, and 218-5p increased or decreased in line with the stage and size of lung SCC tumors. These miRNAs were also defined as successive dysregulated miRNAs from normal to tumor tissues of lung SCC, implying that they might be acting as oncogene or tumor suppressor and associated with the development and progression of lung cancer. Further in-depth study on the molecular mechanism underlying the development of lung SCC is warranted.

Among the differential miRNAs found in this study, many of them were already discovered and their functions were validated by the previous studies.[12,33] The downregulated miRNAs in our study, such as let-7 family, has been verified to be inversely correlated to RAS protein expression[34] (Figure E2); the function of miR-126 that downregulated was also demonstrated to inhibit non–small cell lung cancer cell proliferation by targeting EGFL7 and SLC7A5 in the previous studies.[35,36] On the contrary, the upregulated miRNAs in lung cancer were also detected in our study, such as miR-21, miR-221, and miR-17-92

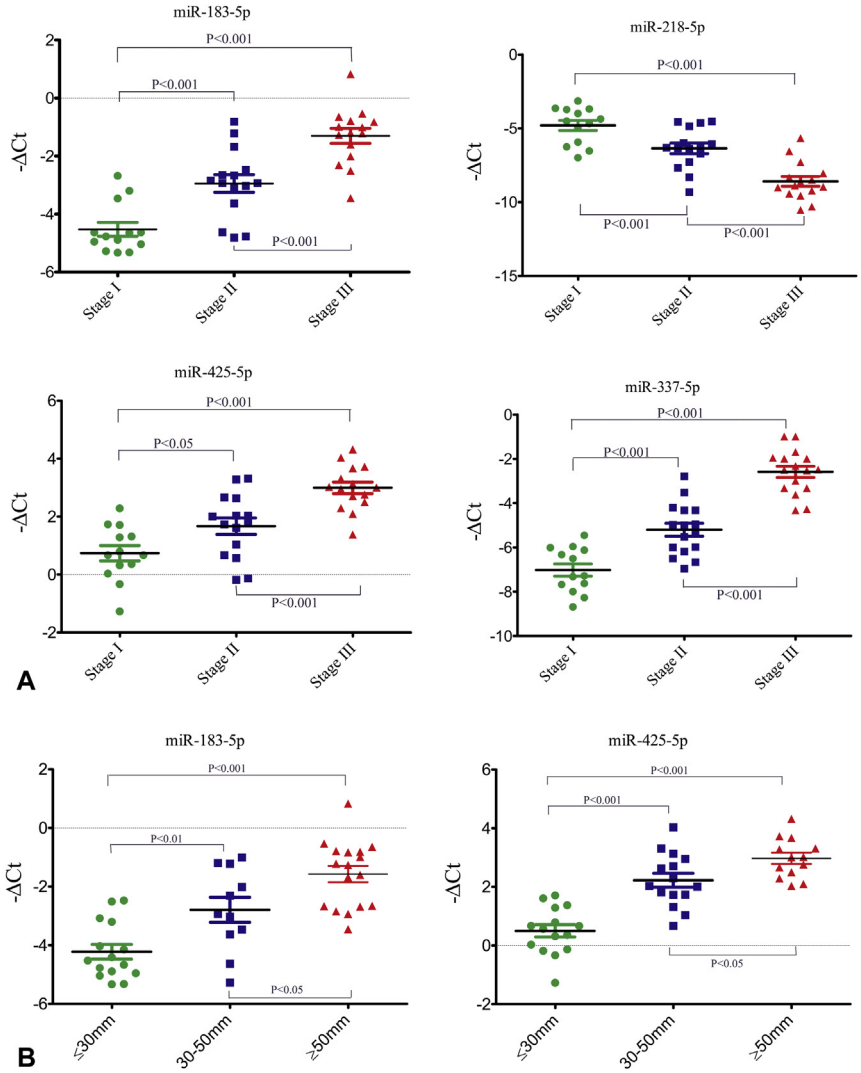

FIGURE 5. Associations between miRNA expression and different stages or tumor sizes of SCC. A, Expression levels of 4 miRNAs (miR-425-5p, 183-5p, 337-5p, and 218-5p) were significantly different and successively dysregulated from stage I to III of SCC as shown by qPCR. miR-218-5p showed downregulation, and other miRNAs showed upregulation. B, Expression levels of miR-183-5p and miR-425-5p increased with tumor size as shown by qPCR.

cluster, which has been defined as oncomir and denoted as epidermal growth factor receptor–regulated antiapoptotic factors in lung cancer.[37-39] In this study, except those miRNAs that have been demonstrated to be related to lung cancer, some of the differential miRNAs were rarely reported in the previous studies of lung cancer, such as miR-425, which may be a driver for tumor formation, growth, and progression to higher staging in studies on gastric cancer and breast cancer,[40,41] also upregulated successively from normal tissue to tumor tissues with further confirmation by qPCR and miRNA in situ hybridization, and the downregulated miR-218, which might be a tumor suppressor showing contrary tendency to miR-425, was also confirmed in our study[42] (Figure 3, *B*, and Figure 4). All of these results imply the roles of these miRNAs in tumor genesis and development in a view of combining clinical process and biological explanation.

Our study also demonstrated that the miRNA signatures of SCC may be involved in pathways of cancer, particularly NSCLC. Of the predicted targets of these miRNAs, numerous vital genes are believed to participate in several important signaling pathways, including MAPK, transforming growth factor-beta, Wnt, Hippo, Neurotrophin, PI3K-Akt, Insulin, and ErbB. Dysregulation of these miRNAs might function as oncogenes or tumor suppressors, thereby contributing to tumor initiation and progression. Modulation of miRNA expression holds great hope for potential cancer therapy.[43,44] Therefore, our prediction that the differentially expressed miRNAs have the capability to target multiple components in these critical pathways makes them promising molecular targets for the treatment of SCC.

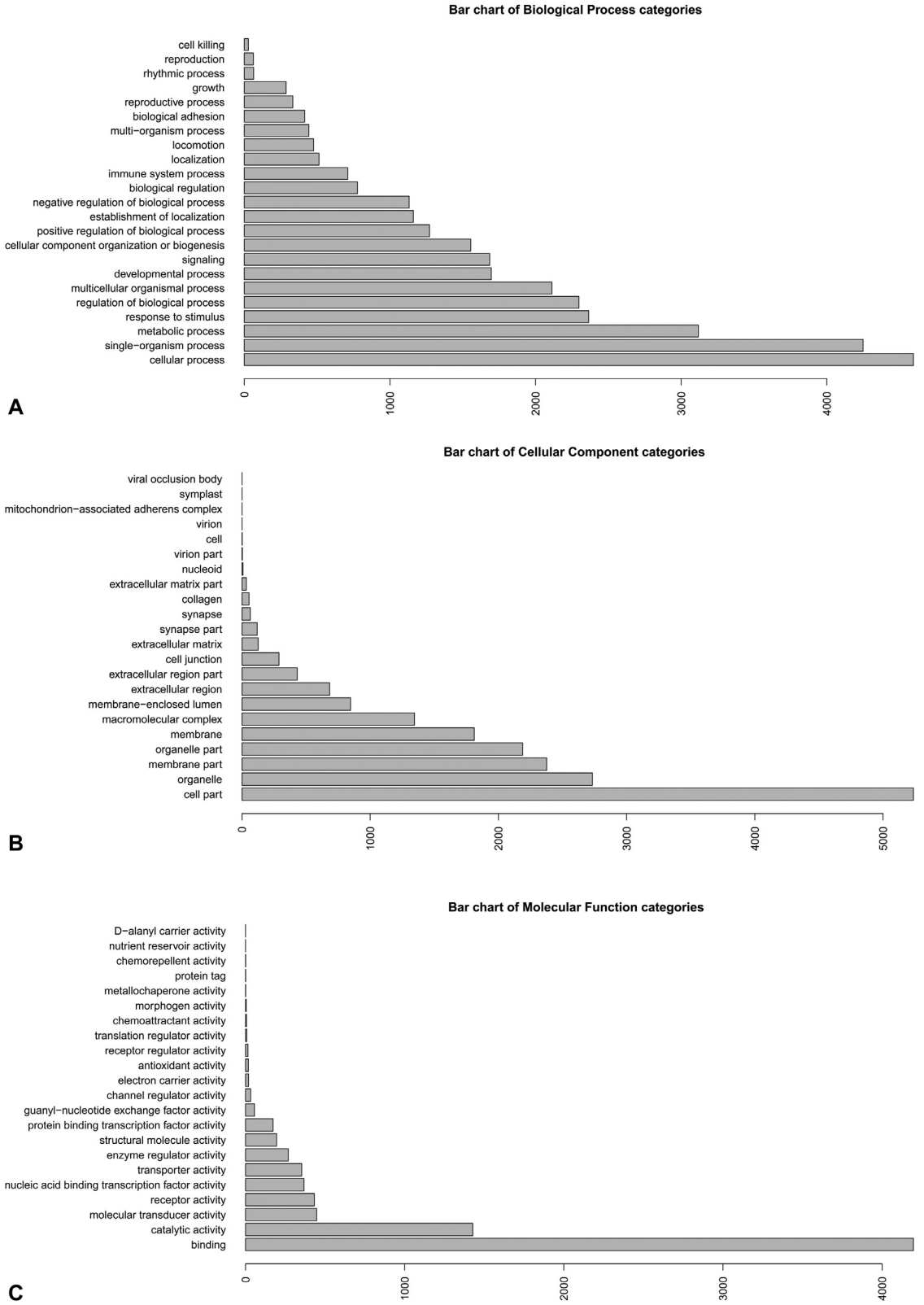

FIGURE 6. Categories and distribution of the Gene Ontology in terms of predicted miRNA targets. A, Biological process terms are enriched in cellular, metabolic, and single-organism processes. B, Cellular component terms are enriched in cell part, organelle, and membrane. C, Molecular function terms are enriched in binding, catalytic activity, and molecular transducer activity.

TABLE 1. Enriched KEGG pathway, number of differentially expressed genes, and adjusted *P* value and false discovery rate

KEGG pathway	Gene no.	Bonferroni *P* value	False discovery rate
Pathways in cancer	181	1.06E-18	1.06E-18
Endocytosis	122	2.57E-16	1.29E-16
Protein processing in endoplasmic reticulum	101	3.72E-14	1.24E-14
Proteoglycans in cancer	125	3.71E-13	9.28E-14
MAPK signaling pathway	135	2.68E-11	5.35E-12
TGF-beta signaling pathway	57	3.88E-11	6.46E-12
Prostate cancer	60	1.79E-10	2.55E-11
Renal cell carcinoma	48	4.54E-10	5.68E-11
Wnt signaling pathway	83	1.74E-09	1.94E-10
Ubiquitin mediated proteolysis	80	2.71E-09	2.71E-10
Regulation of actin cytoskeleton	112	3.03E-09	2.76E-10
HTLV-I infection	132	5.03E-09	4.20E-10
Focal adhesion	107	1.55E-08	1.19E-09
Hippo signaling pathway	85	3.60E-08	2.57E-09
Neurotrophin signaling pathway	69	6.42E-08	4.28E-09
PI3K-Akt signaling pathway	158	7.64E-08	4.77E-09
Insulin signaling pathway	76	1.55E-07	9.10E-09
Axon guidance	73	3.97E-07	2.20E-08
ErbB signaling pathway	53	1.01E-06	5.05E-08
Small cell lung cancer	52	2.04E-06	9.73E-08

HTLV-1, Human T-cell lymphotropic virus; *TGF*, transforming growth factor; *KEGG*, Kyoto Encyclopedia of Genes and Genomes; *MAPK*, mitogen-activated protein kinases; *ErbB*, V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog.

One of the advantages of the next-generation deep sequencing technology over conventional procedures is that it allows for the discovery of miRNAs that have not been related with malignancies by the traditional techniques. With the use of deep sequencing, we can identify a set of differentially expressed miRNAs whose dysregulation has not well been documented in lung tumorigenesis. Two of the 10 selected miRNAs that displayed dysregulations in SCC discovered by deep sequencing did not exhibit significant changes as assessed by qRT-PCR. The inconsistencies between the 2 approaches for assessing miRNA expression are not unusual, as demonstrated by previous studies of miRNAs using microarray, qRT-PCR, and high-throughput sequencing.[20,45,46] One of the reasons underlying the observation might be that qRT-PCR has lower sensitivity than deep sequencing, and thus is not able to measure the difference of some miRNAs between normal and tumor tissues. In addition, the different proportions of the tumors in the specimens tested by deep sequencing than in the specimens assessed by qRT-PCR could lead to the inconsistent results between the 2 techniques.

CONCLUSIONS

This study described the use of deep sequencing for comprehensive profiling of miRNAs in tumor, adjacent, and normal tissues of lung SCC. Our comprehensive survey of differentially expressed miRNAs not only confirms some existing findings but also discovers dysregulated miRNAs that had not been found in lung carcinogenesis, thereby providing new signatures of SCCs. In addition, by using qRT-PCR and in situ hybridization, we validated miRNA signatures that could distinguish tumor tissues from normal tissues and identified a small panel of miRNAs that can differentiate different stages of SCC by expression levels. The identified miRNAs may provide promising biomarkers to improve early detection, subclassification, and monitoring of SCC in the future.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: lung squamous cell carcinoma, microRNA, sequencing

ET/BS

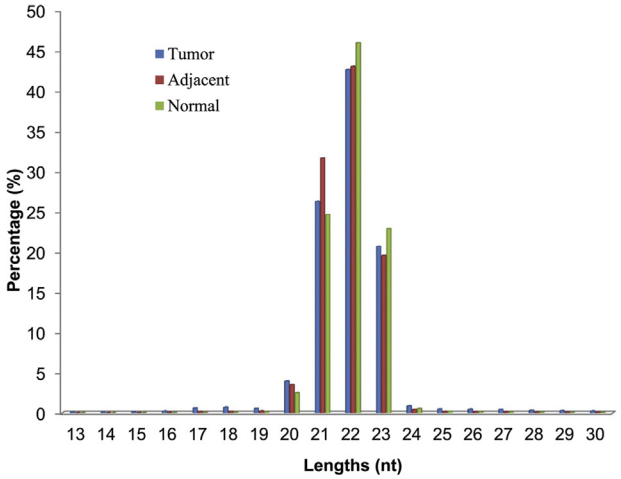

FIGURE E1. Length distribution and abundance of the small RNAs from ST, SA, and SN samples. Small RNA reads with a length of 22 nt were the most abundant, which accounted for approximately half of the total reads, followed by 21 and 21 nt reads.

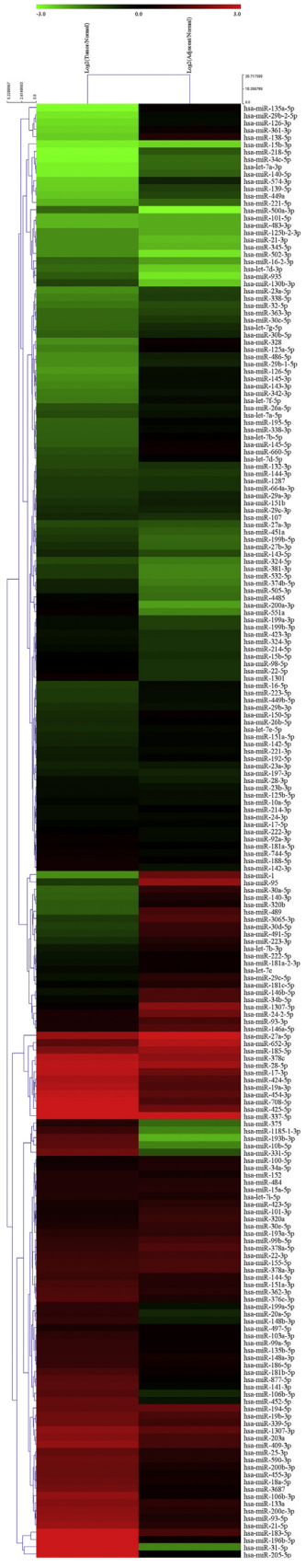

FIGURE E2. Hierarchical clustering analysis of miRNA expression in different tissues of lung SCC. Differential expression of miRNA in tumor and adjacent tissue compared with the normal tissue control. The data removed those low copy number miRNAs (<10). The column shows each group to the control, and the *line* shows the fold change of each miRNA in lung cancer tissue. The color of each pattern represents the fold change as log2, from high (*red*) to low (*green*).

TABLE E1. Upregulated and downregulated microRNAs among tumor, adjacent, and normal tissues as defined by deep sequencing

miRNAs	Log2(tumor/normal)	miRNAs	Log2(adjacent/normal)	miRNAs	Log2(tumor/adjacent)
hsa-miR-135a-5p	−3.85	hsa-miR-1266	−4.33	hsa-miR-135a-5p	−3.96
hsa-miR-218-5p	−3.18	hsa-miR-34c-3p	−3.33	hsa-miR-1	−3.62
hsa-miR-34c-5p	−2.96	hsa-miR-500a-3p	−3.33	hsa-miR-95	−3.19
hsa-let-7a-3p	−2.85	hsa-miR-625-3p	−3.33	hsa-miR-138-5p	−3.15
hsa-miR-15b-3p	−2.85	hsa-miR-502-3p	−2.75	hsa-miR-361-3p	−2.84
hsa-miR-140-5p	−2.85	hsa-miR-7-5p	−2.75	hsa-miR-29b-2-5p	−2.69
hsa-miR-654-5p	−2.85	hsa-miR-935	−2.75	hsa-miR-489	−2.52
hsa-miR-760	−2.85	hsa-miR-15b-3p	−2.58	hsa-miR-126-3p	−2.50
hsa-miR-29b-2-5p	−2.77	hsa-let-7d-3p	−2.51	hsa-miR-27a-5p	−2.28
hsa-miR-126-3p	−2.65	hsa-miR-130b-3p	−2.43	hsa-miR-3065-3p	−2.27
hsa-miR-361-3p	−2.58	hsa-miR-21-3p	−2.33	hsa-miR-30a-5p	−2.26
hsa-miR-574-3p	−2.54	hsa-miR-345-5p	−2.26	hsa-miR-328	−2.10
hsa-miR-139-5p	−2.52	hsa-miR-193b-3p	−2.23	hsa-miR-1307-5p	−2.09
hsa-miR-138-5p	−2.50	hsa-miR-101-5p	−2.16	hsa-miR-574-3p	−2.01
hsa-miR-625-5p	−2.43	hsa-miR-125b-2-3p	−2.16	hsa-miR-106b-5p	2.05
hsa-miR-449a	−2.43	hsa-miR-483-3p	−2.16	hsa-miR-200a-3p	2.07
hsa-miR-101-5p	−2.26	hsa-miR-16-2-3p	−2.06	hsa-miR-106b-3p	2.07
hsa-miR-221-5p	−2.26	hsa-miR-200a-3p	−2.03	hsa-miR-378d	2.07
hsa-miR-4766-3p	−2.26	hsa-miR-1307-5p	2.19	hsa-miR-210	2.12
hsa-miR-185-5p	2.05	hsa-miR-378c	2.25	hsa-miR-375	2.17
hsa-miR-133a	2.15	hsa-miR-95	2.34	hsa-miR-339-3p	2.22
hsa-miR-203a	2.16	hsa-miR-185-5p	2.43	hsa-miR-193a-3p	2.31
hsa-miR-1307-3p	2.24	hsa-miR-28-5p	2.50	hsa-miR-335-3p	2.48
hsa-miR-200c-3p	2.27	hsa-miR-652-3p	2.74	hsa-miR-183-5p	2.60
hsa-miR-93-5p	2.32	hsa-miR-337-5p	3.06	hsa-miR-136-5p	2.71
hsa-miR-106b-3p	2.32	hsa-miR-27a-5p	4.68	hsa-miR-361-5p	2.71
hsa-miR-409-3p	2.32			hsa-miR-224-5p	2.71
hsa-miR-27a-5p	2.40			hsa-miR-1180	2.81
hsa-miR-21-5p	2.44			hsa-miR-182-5p	2.82
hsa-miR-424-5p	2.61			hsa-miR-200b-5p	2.90
hsa-miR-19a-3p	2.72			hsa-miR-331-5p	3.01
hsa-miR-378c	2.74			hsa-miR-625-3p	3.22
hsa-miR-17-3p	2.80			hsa-miR-10b-5p	3.36
hsa-miR-28-5p	2.85			hsa-miR-503-5p	3.60
hsa-miR-454-3p	2.91			hsa-miR-196b-5p	3.61
hsa-miR-708-5p	3.05			hsa-miR-193b-3p	3.77
hsa-miR-1246	3.11			hsa-miR-1246	3.85
hsa-miR-425-5p	3.26			hsa-miR-205-5p	6.04
hsa-miR-337-5p	3.36			hsa-miR-31-5p	6.36
hsa-miR-301a-3p	3.74			hsa-miR-7-5p	7.48
hsa-miR-183-5p	3.85				
hsa-miR-196b-5p	3.87				
hsa-miR-31-5p	4.61				
hsa-miR-205-5p	6.01				

miRNA, MicroRNA.

TABLE E2. Expression levels of 8 microRNAs in 19 groups of tumor, adjacent, and normal tissues of squamous cell carcinoma as determined by quantitative reverse transcription polymerase chain reaction

miRNAs	Mean ± SD in tumor tissues	Mean ± SD in adjacent tissues	Mean ± SD in normal tissues	*P* value (tumor vs normal)	*P* value (tumor vs adjacent)	*P* value (adjacent vs normal)
miR-205-5p	−5.35 ± 0.23	1.95 ± 0.03	2.02 ± 0.13	<.001	<.001	>.05
miR-31-5p	−4.61 ± 0.13	3.84 ± 0.19	1.45 ± 0.05	<.001	<.001	<.001
miR-196b-5p	0.35 ± 0.09	4.21 ± 0.25	4.28 ± 0.08	<.001	<.001	>.05
miR-425-5p	−1.64 ± 0.09	0.42 ± 0.10	1.85 ± 0.08	<.001	<.001	<.001
miR-337-5p	1.87 ± 0.10	2.22 ± 0.11	4.86 ± 0.25	<.001	<.001	<.001
miR-183-5p	0.27 ± 0.02	3.11 ± 0.20	5.23 ± 0.19	<.001	<.001	<.001
miR-135a-5p	4.44 ± 0.20	0.86 ± 0.04	0.91 ± 0.04	<.001	<.001	>.05
miR-218-5p	4.36 ± 0.09	0.23 ± 0.10	−0.88 ± 0.01	<.001	<.001	<.001

miRNA, MicroRNA; *SD*, standard deviation.