Construction and validation of a seven-microRNA signature as a prognostic tool for lung squamous cell carcinoma

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Objective: The aim of this study was to construct and validate a microRNA (miR)-based signature as a prognostic tool for lung squamous cell carcinoma (LUSC).

Materials and methods: With the use of mature miR expression profiles downloaded from The Cancer Genome Atlas database, we identified differentially expressed miRs between LUSC and matched healthy lung tissue. Thereafter, we carried out an evaluation of the association of differentially expressed miRs with overall survival (OS) with the use of univariate and multivariate Cox regression analysis. This analysis was eventually employed for the construction of a miR-based signature, which effectively predicted the prognosis. The functional enrichment analysis of the miRs included in the signature was used to explore their potential molecular mechanism in LUSC.

Results: A total of 316 miRs were differentially expressed between LUSC and matched healthy lung tissues in the training set. Following the univariate and multivariate Cox regression analysis, we found that seven miRs were independent prognostic factors. Each patient received a signature index ranging from 0 to 7. Patients with LUSC were divided into high-risk, intermediate-risk, and low-risk groups in accordance with their signature index and the OS in the three groups was significantly different. This finding remains consistent in the validation set. Besides that, this seven-miR signature remained an independent prognostic factor in comparison with routine clinicopathologic features. The seven-miR signature is a promising biomarker for predicting the 5-year survival rate of LUSC with an area under the receiver operating characteristic curve of 0.712 in the training set and 0.688 in the validation set, respectively. The target genes of seven miRs may be involved in various pathways associated with lung cancer, for instance the mitogen-activated protein kinase signaling pathway and the Wnt signaling pathway.

Conclusion: Using this signature, patients with LUSC can be divided into high-risk, intermediate-risk, and low-risk groups for more personalized management.

Keywords: lung squamous cell carcinoma, microRNA-based signature, prognosis

Introduction

Lung cancer remains the leading cause of cancer incidence and mortality across the globe,1 and approximately 80% of lung cancers are classified histopathologically as nonsmall cell lung cancer (NSCLC). NSCLC can be segregated into two major classes, which include lung nonsquamous cell carcinoma and lung squamous cell carcinoma (LUSC). In spite of the curative surgery for patients with early stage disease, approximately 40% of patients will relapse within a period of 5 years,2 with the 5-year overall survival (OS) rate amounting to 50–60%.3,4 This suggests
that there are still some high-risk individuals among patients who have early stage disease. A reliable prognostic prediction model for the identification of these high-risk individuals is obviously valuable. While advanced lung nonsquamous cell cancer has greatly benefited from the detection and targeting of oncogenic alterations, for instance ALK rearrangement and EGFR mutations, LUSC has been challenging in the identification and targeting of driving mutations. Thus, such a prognostic prediction model is urgently needed for LUSC.

MicroRNAs (miRs) are short noncoding RNA molecules, playing crucial roles in transcriptional regulation of gene expression through several mechanisms. The deregulation of miRs has been shown to be associated with various cancers, including NSCLC. Although a few miR signatures have been proposed for predicting the outcome of NSCLC, including LUSC, the results of these studies are significantly inconsistent and lack validation. This may have resulted from the small sample sizes as well as the discovery of new miRs.

In this work, we propose a new miR-based signature to predict the prognosis of LUSC. With the use of this signature, LUSC can be effectively divided into high-risk, intermediate-risk, and low-risk groups. Furthermore, these results will be validated in an independent data set.

Materials and methods

Data processing

The preprocessed LUSC mature miR expression profiles in The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/) database, displayed as log₂ converted reads per million (log₂(RPM+1)), were downloaded from University of California Santa Cruz Xena (https://xenabrowser.net/datapages/, version 09-08-2017). The corresponding clinical information was downloaded from TCGA database (download date 09-26-2018). These contain two mature miR expression data, which are based on two different platforms, including 380 samples (336 LUSC tissues and 44 matched healthy lung tissues) based on the IlluminaHiSeq_miRNASeq platform (Illumina Inc., San Diego, CA, USA) and 131 LUSC tissues based on the IlluminaGA_miRNASeq platform. The samples based on the IlluminaHiSeq_miRNASeq platform were used as the training set to identify differentially expressed miRs and construct a miR-based signature for predicting prognosis, and the samples based on the IlluminaGA_miRNASeq platform were used as the validation set to verify the signature.

Screening of differentially expressed miRs

In the training set, miRs that did not express over 10% of samples were removed. The differentially expressed miRs between LUSCs and matched healthy lung tissues were analyzed using the “limma” package in R language. The fold changes (FCs) in the expression of individual miRs were calculated, and differentially expressed miRs with $|\log_{2}FC| > 0.585$ and $p < 0.05$ (adjusted by the false discovery rate) were considered significant. We applied bidirectional hierarchical clustering to differentially express miRs based on the Euclidean distance and displayed the results as a heatmap.

Construction and validation of the miR-based prognostic signature for LUSC

In the training set, patients with a survival time of less than 30 days were removed for survival analysis. The remaining patients (N=318) were separated into high and low-level groups based on the median value of the differentially expressed miRs, followed by univariate and multivariate Cox proportional hazards analyses. We found that seven miRs were independent factors of survival. We assigned each patient 1 point for the high-risk expression level of these seven miRs. Accordingly, each patient received a score ranging from 0 to 7, which we called the signature index. We considered patients having a signature index of 6 or 7 to be high risk, those with an index of 3–5 to be intermediate risk, and those with an index <3 to be low risk. The survival time was compared in the three groups using Kaplan–Meier analysis with the log-rank test. In the validation set, two patients without a survival time were removed for survival analysis. The validation set was used to confirm the robustness of the miR-based prognostic signature. Furthermore, to compare the relative prognostic value of this miR-based signature with that of routine clinicopathologic features, we carried out the univariate and multivariate Cox proportional hazards analyses in the training and validation sets. The time-dependent receiver operating characteristic (ROC) curve was used to assess the miR-based signature’s predictive value for the 5-year survival rate of LUSC and was performed using the “survivalROC” package in R.

Target gene prediction and functional enrichment analysis

Target gene prediction of the seven miRs was performed using the miRDB online tool (http://mirdb.org/). Target genes of a miR provided by miRDB are ranked by the target score. The top 50 target genes with the highest target score
for each miR were extracted to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses using the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.abcc.ncifcrf.gov/home.jsp). p<0.05 was set as the cutoff criterion. These seven miRs and their target genes would be displayed as the miR-target network using cytoscape software.

Statistical analysis

The \( \chi^2 \)-test was used for the categorical data and an unpaired \( t \)-test was used to screen differentially expressed miRs. Univariate/multivariate Cox proportional hazards analyses and Kaplan–Meier survival analysis were used to compare survival between the two groups of patients. The \( \chi^2 \)-test and survival analysis were performed using IBM SPSS statistics software program version 22.0 (IBM Corporation, Armonk, NY, USA). All tests were two-sided and \( p<0.05 \) was considered statistically significant.

**Results**

**Differentially expressed miRs between LUSC and matched healthy lung tissues**

The LUSC patients’ detailed clinical characteristics including gender, age at diagnosis, and TNM stage are listed in Table 1. The training set contained more patients with early stage (I/II) disease (83.33% vs 74.42%, \( \chi^2 \)-test \( p=0.033 \)) and patients with Mx (22.96% vs 3.10%, \( \chi^2 \)-test \( p=0.000 \)) than the validation set. In accordance with the cutoff criteria (\( p<0.05 \) and \( |\log_{2}FC|>0.585 \)), 316 miRs were differentially expressed between LUSC and matched healthy lung tissues in the training set. These included 223 miRs that were upregulated and 93 miRs that were downregulated in LUSC tissues. The result of the expression analysis was presented as a heatmap (Figure 1), and the result of hierarchical clustering showed that these differentially expressed miR expression patterns could basically distinguish LUSC tissues and healthy lung tissues. miR-6499-5p, miR-4746-5p, miR-1293, and miR-4664-3p were upregulated whereas miR-326, miR-30d-3p, and miR-30e-3p were downregulated in LUSC tissues (Figure 2).

**Construction of miR-based signature with differentially expressed miRs in the training set**

For each of the 316 differentially expressed miRs, we used the median expression level as a cutoff point to stratify the 318 patients into a high-level group and a low-level group. The univariate Cox proportional hazards regression analysis revealed that a total of 10 miRs had prognostic value. We then applied a multivariate Cox proportional hazards regression analysis to identify seven miRs (Table 2) – miR-326, miR-6499-5p, miR-30d-3p, miR-4746-5p, miR-1293, miR-4664-3p, and miR-30e-3p – as independent prognostic factors (Figure 3A–G). We scored the seven-miR signature by the value assigned for each miR. Each patient was assigned one score for low expression of miR-6499-5p, miR-1293, and miR-4664-3p, respectively, and one score for high expression of miR-326, miR-30d-3p, and miR-30e-3p, respectively. Then, we summed the scores for each patient, resulting in a signature index ranging from 0 to 7. Moreover, we considered patients with a signature index of 6 or 7 to be high risk, those with an index of 3–5 to be intermediate risk, and those with an index <3 to be low risk. The comparison of the survival time was carried out in the three groups using Kaplan–Meier analysis with the log-rank test. The OS in the low-risk (N=70), intermediate-risk (N=229), and high-risk (N=19) patients was 3838 days (95%
CI 3236–4439 days), 1655 days (95% CI 1223–2086 days), and 408 days (95% CI 233–582 days), respectively. The OS in the three groups was significant (log-rank \( p=0.000 \), Figure 3H). This indicated that the seven-miR signature created was associated with survival in LUSC.

Validation of this seven-miR signature in the validation set

Just as in the training set, patients in the validation set were divided into the low-risk, intermediate-risk, and
high-risk groups according to this seven-miR signature index and Kaplan–Meier analysis was used to compare OS. The OS in the low-risk (N=30), intermediate-risk (N=91), and high-risk (N=8) patients was 3149 days (95% CI 2825–3472 days), 1470 days (95% CI 521–2418 days), and 345 days (95% CI 0–1104 days), respectively. The OS in the three groups was significantly different (log-rank $p$=0.014, Figure 4A).

Prognostic value of this seven-miR signature
The time-dependent ROC curve was used to assess this seven-miR signature’s predictive value of the 5-year survival rate for LUSC in both the training set and the validation set. The seven-miR signature index is a promising biomarker for predicting the 5-year-survival rate of LUSC with an area under the ROC curve (AUC) of 0.712 ($p$=0.034) in the training set and 0.688 in the validation set ($p$=0.034), respectively. Furthermore, this seven-miR signature index remained an independent prognostic factor in comparison with routine clinicopathologic features in both the training set (Table 3) and the validation set (Table 4).

Target gene prediction and functional enrichment analysis of these seven miRs
Target gene prediction of these seven miRs was performed using the miRDB online tool. These seven miRs and their respective top 50 target genes (if targets $\geq$50) were displayed as the miR-target network (Figure 5). Moreover, biological functions of these seven miRs were explored by KEGG pathway enrichment analyses for their target genes using the DAVID. The target genes were involved in various pathways associated with lung cancer, for instance the mitogen-activated protein kinase signaling pathway and the Wnt signaling pathway (Figure 6).

Discussion
LUSC accounts for 20–30% of lung cancer cases, and the ratio is larger in the countries and regions where tobacco is not controlled, such as the People's Republic of China. The management of LUSC remains dependent on the stage of disease of individual patients and without suggested personalized biomarkers. miR expression profiles are highly specific to individual types of cells, tissues, and organs and may serve as potential biomarkers of clinical relevance. In our present study, we proposed a new seven-miR signature to predict the prognosis of LUSC. In the same manner as that of several other studies, patients with a survival time of less than 30 days were removed for survival analysis in order to rule out acute death from nontumor causes. Patients with LUSC could be divided into high-risk, intermediate-risk, and low-risk groups using this seven-miR signature. The OS in the three
groups was significantly different in both the training set and the validation set. Furthermore, this seven-miR signature remained an independent prognostic factor in comparison with routine clinicopathologic features. The seven-miR signature is a promising biomarker for predicting the 5-year-survival rate of LUSC with an AUC of 0.712 in the training set and 0.688 in the validation set, respectively. In addition, it is worth noting that the training set contained more patients with early stage (I/II) disease and patients with Mx than the validation set, and the miR expression profiles of the training set and validation set were based on different platforms. Therefore, this might indicate that this seven-miR signature is still robust in different populations and suitable for different platforms. Based on the

Table 3 Univariate and multivariate analyses of clinicopathological features of the seven-microRNA signature in the training set

| Factor                              | Univariate Cox analysis |       | Multivariate Cox analysis |
|-------------------------------------|-------------------------|-------|---------------------------|
|                                     | \( p \)-value          | HR (95% CI) | \( p \)-value | HR (95% CI) |
| Gender (male/female)                | 0.791                   | 0.622–1.436 | 0.611                   | 0.661–2.021 |
| Age (>65 years/≤65 years)           | 0.692                   | 0.744–1.562 | 0.166                   | 0.641–13.300 |
| T stage (T3–4/T1–2)                 | 0.032\*                | 1.040–2.351 | 0.214                   | 0.807–2.601 |
| Lymph node stage (N1–3/N0)          | 0.462                   | 0.798–1.644 | 0.661                   | 0.661–2.021 |
| Metastasis (M1/M0)                  | 0.009\*                | 1.600–27.030 | 1.000\*       | 1.492–4.439 |
| Pathological stage (III–IV/I–II)    | 0.032\*                | 1.040–2.427 | 0.166                   | 0.807–2.601 |
| PI (≥4/≤3 scores)                   | 0.000\*                | 2.146–4.550 | 1.985                   | 4.699       |

Note: *\( p \)<0.05.

Table 4 Univariate and multivariate analyses of clinicopathological features of the seven-microRNA signature in the validation set

| Factor                              | Univariate Cox analysis |       | Multivariate Cox analysis |
|-------------------------------------|-------------------------|-------|---------------------------|
|                                     | \( p \)-value          | HR (95% CI) | \( p \)-value | HR (95% CI) |
| Gender (male/female)                | 0.221                   | 0.370–1.258 | 0.135                   | 0.823–4.286 |
| Age (>65 years/≤65 years)           | 0.191                   | 0.823–2.647 | 0.135                   | 0.823–4.286 |
| T stage (T3–4/T1–2)                 | 0.004\*                | 1.340–4.724 | 0.486                   | 0.613–2.794 |
| Lymph node stage (N1–3/N0)          | 0.247                   | 0.798–2.396 | 0.486                   | 0.613–2.794 |
| Metastasis (M1/M0)                  | 0.449                   | 0.418–7.203 | 0.486                   | 0.613–2.794 |
| Pathological stage (III–IV/I–II)    | 0.025\*                | 1.087–3.429 | 0.486                   | 0.613–2.794 |
| PI (≥4/≤3 scores)                   | 0.001\*                | 1.492–4.439 | 0.001\*       | 1.411–4.227 |

Note: *\( p \)<0.05.

Figure 4 Kaplan–Meier curves with the log–rank test of the seven-miR signature prognosis index in the validation set (A). ROC curves of seven-miR signature prognosis index in the training set (B) and the validation set (C).

Abbreviations: AUC, area under the ROC curve; miR, microRNA; ROC, receiver operating characteristic.
predictive seven-miR signature, high-risk patients could be followed up more frequently and accept more active management than low-risk patients.

Tan et al\textsuperscript{24} proposed a five-miR (miR-210, miR-182, miR-486-5p, miR-30a, and miR-140-3p) signature for LUSC diagnosis and miR-31 for prognosis based on LUSCs from the People's Republic of China in 2011. Interestingly, these six miRs are not included in our miR signature, suggesting that the tumor heterogeneity of LUSC in races may also be reflected in the expression patterns of miRs. Huang et al\textsuperscript{25} proposed that miR-140-3p was a positive prognostic biomarker for LUSC. Filipiska et al\textsuperscript{26} proposed that miR-192 and miR-662 enhance chemoresistance and invasiveness of LUSC. Luo et al\textsuperscript{27} found that miR-223-3p functions as a tumor suppressor in LUSC by the miR-223-3p-mutant p53 regulatory feedback loop. However, these miRs have not been involved in our miR signature after univariate and multivariate analyses. Taking into account the sample size of these studies, this may suggest that these miRs may not be a reliable prognostic marker, although they have crucial biological functions. Gao et al\textsuperscript{28} proposed a seven-miR (miR-101-2, miR-139, miR-182, miR-190, miR-326, and miR-944) signature using miR stem loop expression profiles in TCGA database to divide patients into high-risk and low-risk groups, and the AUC of their signature model for the training set (N=224) and the test set (N=223) was only 0.604 and 0.610, respectively. Beer et al\textsuperscript{11} found that miR-146b alone may be a biomarker for predicting prognosis in LUSC in 2009. Wang et al\textsuperscript{29} suggested a seven-miR (miR-101-2, miR-139, miR-182, miR-190, miR-326, and miR-944) signature using miR stem loop expression profiles in TCGA database to divide patients into high-risk and low-risk groups, and the AUC of their signature model for the training set (N=224) and the test set (N=223) was only 0.604 and 0.610, respectively. Beer et al\textsuperscript{11} found that miR-146b alone may be a biomarker for predicting prognosis in LUSC in 2009.

Figure 5 The network of the seven microRNAs and their target genes.

Figure 6 Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses of target genes of the seven microRNAs.

Abbreviation: MAPK, mitogen-activated protein kinase.
into high-risk, intermediate-risk, and low-risk groups for more personalized management.

Among our seven miRs, miR-326 was identified as a tumor suppressor miR in various cancers and was downregulated in LUSC, but high expression of miR-326 was a high risk factor of LUSC in our study. This indicates that the molecular mechanism of miR-326 is complex, and its function in different tumors may be inconsistent. miR-30d was found to be significantly downregulated in LUSC compared with normal lung tissues in a previous study, which is consistent with the results of our study. Another study reported that exosomal miR-30e-3p was lung adenocarcinoma specific, rather than LUSC specific. However, our results indicated that miR-30e-3p in tumor tissue was a prognostic factor for LUSC. This is an indication of the fact that the expression of miR-30e-3p in LUSC tissues and exosomes may be different. The functions of the other four miRs are still rarely reported. To explore the potential biological functions of the seven miRs, their target genes were predicted respectively using miRDB online analysis tools. The target genes were involved in various pathways associated with lung cancer, for instance the mitogen-activated protein kinase signaling pathway and the Wnt signaling pathway. Given their prognostic value in LUSC, further exploration of the molecular function of these seven miRs is encouraged.

There exist some limitations to this work. There were more LUSC tissues than healthy lung tissues. Despite the fact that a preliminary exploration was carried out, the molecular function of these seven miRs in LUSC was unknown and further experimental validation was lacking. Therefore, it is not clear whether these seven miRs are causal or merely markers for predicting prognosis in LUSC. It may be essential to validate or even improve this seven-miRNA signature in a larger independent cohort.

Conclusion
We propose the first seven-miR signature to predict the prognosis of LUSC. With the use of this signature, patients with LUSC can be divided into high-risk, intermediate-risk, and low-risk groups for personalized management.

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Author contributions
All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure
The authors report no conflicts of interest in this work.

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