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MiR-219-5p decrease the risk of cancer-related mortality in patients with small cell lung cancer

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MiR-219-5p decrease the risk of cancer-related mortality in patients with small cell lung cancer

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Running title: MiR-219-5p decrease the risk of SCLC patients

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ABSTRACT

Objectives Small cell lung cancer (SCLC) is a lethal human malignancy, and previous studies support the contribution of microRNA (miRNA) to cancer progression. The prognostic value of miR-219-5p in SCLC patients remains unclear. This study evaluated the risk factors for SCLC and created a prediction model for them.

Design Retrospective observational cohort study.

Setting The programme has yielded a database of all patients with SCLC in 2 defined geographical regions of China.

Participants We did a real-world study, including data from 133 patients with SCLC between Mar 1, 2010 and June 1, 2015. We collected 86 NSCLC patients in the external validation step.

Primary and secondary outcome measures MiR-219-5p was recorded during the admission. Cox proportional hazard model was applied for survival analyses and for analyzing risk factors for cancer-related mortality and to create a nomogram for prediction. The accuracy of the model was evaluated by C-index and calibration curve. An external data of 86 SCLC patients from Sichuan Cancer Hospital and the First affiliated hospital of Soochow University was conducted.

Results In our data, the mortality in group with high miR-219-5p level (≥ 1.50) was 74.6%. Based on univariate analysis, we put factors (P <0.05) into a multivariate regression model, patients with high miR-219-5p level (P <0.001, HR=0.36), immunotherapy (P <0.001, HR=0.44), PNI score >47.9 (P =0.01, HR=0.45) remained statistically factors for better OS and regarded as independent protective factors. These independently associated risk factors were used to establish an OS estimation nomogram. Nomogram revealed good accuracy in estimating the risk, with a bootstrap-corrected C index of 0.691. External validation displayed an AUC of 0.749 (0.709-0.788).

Conclusions MiR-219-5p decreased the risk of cancer-related mortality in patients with SCLC. Nomogram based on multivariate analysis demonstrated good accuracy in estimating the risk of overall mortality.

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Keywords: small cell lung cancer, miR-219-5p, overall survival, nomogram, prediction model

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BACKGROUND

Lung cancer is the leading cause of cancer deaths worldwide, with millions of new cases diagnosed each year[1]. Small cell lung cancer (SCLC) is a kind of neuroendocrine malignant tumor with poor prognosis, accounting for about 15% of lung cancer patients[2]. SCLC is generally divided into limited disease (LD-SCLC) and extensive disease (ED-SCLC). Platinum combined with etoposide is the first-line therapeutic strategy of SCLC, and most patients are easy to receive initial chemotherapy[3]. However, the 5-year survival rates of LD-SCLC and ED-SCLC are only 15% and 3%, respectively[4]. Therefore, improvement in early diagnosis and prognostic prediction of SCLC is vital.

MicroRNAs (miRNAs) are endogenous non-coding RNAs (~ 22 nt), which regulate mRNA activity by hybridization with 3 '- untranslated region (UTR) of specific genes[5]. Many studies have shown that miRNAs could participate in a variety of cell biological processes, including cell growth, differentiation and apoptosis[6, 7]. In addition, researches have demonstrated that miRNAs are frequently dysregulated in cancers[8, 9], and some miRNAs can serve as diagnostic and prognostic biomarkers for caners[10]. Recently, several miRNAs have been proved to participant in the occurrence and development of SCLC, but few of them are likely to be a biomarker or therapeutic target for SCLC.

Recently, miR-219-5p has been found to be abnormally expressed and play a significant role in different cancers. Ma et al. found that the expression of miR-219-5p was significantly decreased in esophageal squamous cell carcinoma (ESCC) tissues compared with normal tissues[11]. A study of Gong et al. revealed a tumor suppressive role for miR-219-5p by targeting glypican-3 in hepatocellular carcinoma (HCC)[12]. On the contrary, Yang et al. indicated that miR-219-5p could promote cell growth and metastasis of HCC and serve as a prognostic marker for HCC patients[13]. A research investigated by Wei et al. suggested that miR-219-5p could inhibit proliferation, migration and invasion of epithelial ovarian cancer through downregulation of the Wnt signaling pathway, and it could serve as a diagnostic

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biomarker and therapeutic target for epithelial ovarian cancer[14]. However, the biological functions of miR-219-5p and its potential prognostic role for biomarker in SCLC are still unknown.

In this study, we aimed to examine the variation in the expression levels of miR-219-5p in patients with SCLC and explored the potential prognostic role of miR-219-5p for SCLC. We also displayed a nomogram that could provide individualized, evidence-based, highly accurate risk estimates. Nomograms were easy to performed and could facilitate management-related decision making.

METHODS

Study Design and Patient Characteristics

We did a real-world study, including data obtained from 133 patients with SCLC between Mar 2010 and June 2015, in the Suzhou Xiangcheng People's Hospital. Those participants who lacked information on complement components data, withdrew from treatment or lacked follow-up information were excluded. Clinical information of patients, including gender, age, BMI, neutrophils count, lymphocytes count, serum CEA level, CRP level, albumin level, hemoglobin level, stage of SCLC, platelet count, PNI score, KPS score, NLR, pathologic type, immunotherapy, therapy of radiation, application of platinum, application of VEGF inhibitor, target therapy, application of TKI, smoking, ACS, diabetes, heart failure and hyperlipemia, were recorded. Diagnosis of SCLC was confirmed by histopathological examination. The median length of follow-up was 23.6 months. The definition and details of all the variables above were provided in Supplemental Materials Part I. Data from 86 patients with NSCLC at Sichuan Cancer Hospital and the First affiliated hospital of

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Soochow University were applied for external validation. Inform, and consent was obtained from all patients or their immediate family members. All protocols were in line with the guidelines with the ethic committee of Suzhou Xiangcheng People's Hospital, Sichuan Cancer Hospital, the First affiliated hospital of Soochow University and following the Declaration of Helsinki.

Assays for Detection of MiR-219-5p Levels

The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was conducted for the detection of miR-219-5p expression levels.

Total RNA from tissues was isolated and extracted using miRcute Extraction and Separation of miRNAs kit (Tiangen Biotech Co., Ltd., Beijing, China), and then reversely transcribed into cDNA by PrimeScript™ Ⅱ 1st strand cDNA synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co., Ltd.) was used for qRT-PCR. The thermocycling conditions were as follows: one cycle at 95˚C for 3 min (initial denaturation), 40 cycles at 95˚C for 15 s and 60˚C for 30 s. U6 small nuclear RNA (U6) served as the respective internal control. The relative expression of miR-219-5p was quantified by the $2^{-\Delta\Delta Ct}$ methods, and normalized to the U6. The following primers were used: miR-219-5p forward, 5'-ACACTCCAGCTGGGTGATTGTCCAAACGCAAT-3' and reverse, 5'-CTCAACTGGTGTCGTGGA-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The experiments were repeated at least 3 times.

Statistical Analysis

Sample size assessment was performed using NCSS-PASS software version 11.0

(https://www.ncss.com/software/pass/). Power was set as 0.99, and alpha was 0.5. The mortalities of both miR-219-5p high-level group and miR-219-5p low-level group in our previous data (2008-2009) (0.750 and 0.950) were entered into the PASS. The Actual Hazard Ratio was set as 0.50. Then the sample size was calculated using PASS, and the minimum sample size was 103 (control = 51, experiment = 43). Our sample size was 133 (66 and 67 for each group), which was suitable. The report of sample size assessment was displayed in Supplemental Material Part II. The missing data (<5.0%) were estimated by random forest algorithm using the mice package in RStudio (R version 3.6.1). Categorical variates were presented as percentages and compared via the κ^2 test. Continuous variates with skewed and normal distributions were presented as median with interquartile ranges and mean ± standard deviation. The Mann-Whitney U test and the unpaired t-test were applied for comparison between Groups. Cumulative mortality was showed by the Kaplan-Meier curve and analyzed by the log-rank test. Univariate and multivariate survival analyses for OS were conducted using the Cox regression model. The forest plots were used to visualize the significance of covariates to the prognosis. The restricted cubic spline analyses were performed with Harrell's Regression Modelling Strategies (rms) package.

To create a prognostic risk model, the Lasso regression was conducted to identify risk factors correlated with prognosis. The contribution of each covariate was quantified and visualized in a prognostic nomogram with internal validation via 1000-times bootstrapping. The consistency of the resulting model was assessed by the calibration assay. Decision curve analyses were performed to evaluate net clinical benefits of the model compared with conventional prognostic scores. The scatter plots

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were applied for visualization of the consistency of each model. A 1000-time bootstrapping was employed as indicated. The association between miR-219-5p class and survival endpoints was evaluated by Kaplan-Meier curves and log-rank test. Statistical analysis was performed using the RStudio (R version 3.6.1) with the following packages: 'ggplot2', 'rms', 'PredictABLE', 'risk regression', and 'survminer'.

Patient and public involvement

This study was conducted without patient involvement. Patients were not invited to comment on the study design and were not consulted to develop patient-relevant outcomes or interpret the results. Moreover, patients were not allowed to contribute to the writing or editing of this document for readability or accuracy.

RESULTS

Baseline Characteristics

A total of 133 SCLC patients who were diagnosed between Mar 2010 and June 2015 were included in this study. A flow chart of the screening process was shown in figure 1. The median age of these patients was 64 years old (58-70), and it contained 106 (80.0%) males. Median serum CEA and CRP level was 3.43 ng/ml and 7.83 μmol/L, respectively. For the stage of SCLC, 51 (38.0%) patients were diagnosed with limited disease, 82 (61.0%) patients were extensive disease. 25 (19.0%) patients accepted the immunotherapy therapy. 54 (41.0%) patients got the therapy of radiation. In addition, platinum was applied for 131 (98.0%) patients, and TKI was used for 15 (12.0%) patients. KPS score of these patients was examined, and the results revealed that 107 (81.0%) patients got 80 or higher points. The distribution of basic diseases was also

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assessed in our data. Diabetes was found in 17 (13.0%) patients, while hyperlipemia was 13 (10.0%). Cardiovascular diseases such as heart failure and ACS were found in 3 (2.0%) and 4 (3.0%) patients, respectively. 13 (10.0%) patients suffered from hypertension. In addition, 82 (62.0%) patients had a habit of smoking among all the patients. The baseline characteristics of these patients were listed in table 1.

Among all the 133 patients, the overall mortality was 87.2%. The mortality in high miR-219-5p level group was 74.6%. Moreover, in the high miR-219-5p level group, patients with extensive disease were 35 (52.0%), while the low group was 47 (71.0%) (table 1).

Table 1. Study Participant Characteristics at Enrollment

Variation	Total (n=133)	Cohort, median (IQR)		p.value
		mir-219-5p<1.50, (n=66)	mir-219-5p≥1.50, (n=67)	
Age, (year)	64(58,70)	65(59,70)	63(56.5,68)	0.276
BMI, (kg/m2)	23.12±3.09	22.99±3.22	23.26±2.98	0.619
Serum CEA level, (ng/ml)	3.43(1.96,9.26)	3.34(1.92,10.05)	3.43(1.99,8.11)	0.87
Serum CRP level, (μmol/L)	7.83(1.68,12.78)	10.68(2.78,13.15)	5.64(1.2,12.16)	0.107
Albumin level, (g/L)	39.46±5.2	38.86±4.68	40.05±5.65	0.188
Neutrophils count, (10^9/L)	4.55(3.59,5.88)	4.54(3.77,5.75)	4.55(3.48,5.92)	0.975
Lymphocytes count, (10^9/L)	1.63(1.31,1.95)	1.5(1.21,1.78)	1.73(1.38,2.08)	0.031*
Hemoglobin level, (g/L)	133(125,145)	134(124,145)	132(125,143.5)	0.564
Platelet count, (10^9/L)	233(184,288)	244.5(180,293.75)	226(184.5,273.5)	0.306
PNI score	47.9(43.95,51.85)	45.98(42.51,50.38)	48.7(44.92,54)	0.026*
NLR	2.66(1.99,4.19)	2.75(2.11,4.57)	2.59(1.92,3.7)	0.232
Gender, (n%)				0.211
Female	27(20)	10(15)	17(25)	
Male	106(80)	56(85)	50(75)	
Metastasis, n(%)				0.299
No	45(34)	19(29)	26(39)	
Yes	88(66)	47(71)	41(61)	
Stage of SCLC				0.038*
Limited disease	51(38)	19(29)	32(48)	
Extensive disease	82(62)	47(71)	35(52)	
Immunotherapy, (n%)				0.197
No	108(81)	57(86)	51(76)	
Yes	25(19)	9(14)	16(24)	
Therapy of radiation, (n%)				0.417
No	79(59)	42(64)	37(55)	

Yes	54(41)	24(36)	30(45)	
Application of platinum, (n%)				0.244
No	2(2)	2(3)	0(0)	
Yes	131(98)	64(97)	67(100)	
Chemotherapy				0.45
AP	28(21)	12(18)	16(24)	
DP	15(11)	6(9)	9(13)	
EP	71(53)	35(53)	36(54)	
GP	3(2)	2(3)	1(1)	
Others	16(12)	11(17)	5(7)	
Target therapy, (n%)				0.627
No	116(87)	59(89)	57(85)	
Yes	17(13)	7(11)	10(15)	
Application of TKI, (n%)				0.449
No	118(89)	60(91)	58(87)	
TKI I	9(7)	4(6)	5(7)	
TKI II	1(1)	1(2)	0(0)	
TKI III	5(4)	1(2)	4(6)	
Application of VEGF inhibitor, n(%)				0.645
No	114(86)	58(88)	56(84)	
Yes	19(14)	8(12)	11(16)	
KPS score, n(%)				0.678
40	2(2)	0(0)	2(3)	
50	5(4)	3(5)	2(3)	
60	7(5)	3(5)	4(6)	
70	12(9)	8(12)	4(6)	
80	29(22)	14(21)	15(22)	
90	56(42)	29(44)	27(40)	
100	22(17)	9(14)	13(19)	
Smoking, n(%)				0.255
No	51(38)	29(44)	22(33)	
Yes	82(62)	37(56)	45(67)	
Hypertension, n(%)				1
No	80(60)	40(61)	40(60)	
Yes	53(40)	26(39)	27(40)	
Diabetes, n(%)				1
No	116(87)	58(88)	58(87)	
Yes	17(13)	8(12)	9(13)	
Hyperlipemia, n(%)				0.579
No	120(90)	61(92)	59(88)	
Yes	13(10)	5(8)	8(12)	
Heart failure, n(%)				1
No	130(98)	65(98)	65(97)	
Yes	3(2)	1(2)	2(3)	

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ACS, n(%)				0.619
No	129(97)	65(98)	64(96)	
Yes	4(3)	1(2)	3(4)	

Abbreviation: IQR, interquartile range; CRP, C-reactive protein; PNI, neutrophil lymphocyte ratio; NLR, neutrophil lymphocyte ratio; SCLC, small-cell lung cancer; TKI, Tyrosine Kinase Inhibitor; VEGF, vascular endothelial growth factor; KPS, Karnofsky Performance Status; ACS, acute coronary syndrome. ***p<0.001, **p<0.01, *p<0.05.

MiR-219-5p Expression Level, and Clinical Risk Factors Predict the Development of SCLC Patients

According to the univariate analysis, high miR-219-5p levels (≥ 1.50) was a strong protective predictor of cancer-related mortality (HR 0.36, 95% CI 0.25-0.53, *P* <0.001) (table 2). Kaplan Meier curve showed that patients in the high miR-219-5p group had a decreased cumulative rate of death than those in the low miR-219-5p group (log-rank *P* <0.001) (figure 2A). Meanwhile, patients who accepted immunotherapy also showed a low morality compared to those patients without accepting immunotherapy in the survival curve (HR 0.28, 95% CI 0.15-0.52, *P* <0.001) (figure 2B).

In addition, gender, age, serum CRP level, albumin level, lymphocytes count, PNI score, immunotherapy, heart failure, and KPS score were also correlated with overall mortality (table 2). When adjusted by age and gender, patients in the miR-219-5p high-level group also displayed a low cumulative rate death compared to those in the low-level group.

Table 2. Cox Regression Analysis of Hazard Ratio on SCLC patients

Variation	Non-adjustment		Model 1	
	Hazard Ratio (95% CI)	p.value	Hazard Ratio (95% CI)	p.value
Gender, male vs. female	1.66 [1.02, 2.69]	0.041*	-	-
Age (year), ≥60 vs. < 60	1.52 [1.03, 2.26]	0.036*	-	-
BMI, ≥23.12 kg/m2 vs. < 22.86kg/m2	0.99 [0.69, 1.43]	0.97	0.95 [0.66, 1.38]	0.806
Serum CEA level, >3.43 ng/ml vs. ≤3.43 ng/ml	1.01 [0.70, 1.46]	0.954	1.00 [0.69, 1.45]	0.999
Serum CRP level, >7.83 μmol/L vs. ≤7.83 μmol/L	1.91 [1.31, 2.78]	0.001**	1.92 [1.32, 2.79]	0.001**
Albumin level, >39.46 g/L vs. ≤39.46 g/L	1.66 [1.15, 2.40]	0.007**	1.60 [1.10, 2.31]	0.013*
Neutrophils count, >4.55x10^9/L vs. ≤ 4.55x10^9/L	1.18 [0.82, 1.71]	0.367	1.15 [0.79, 1.66]	0.464
Lymphocytes count, >1.63x10^9/L vs. ≤ 1.63x10^9/L	0.59 [0.40, 0.85]	0.005**	0.61 [0.42, 0.89]	0.01*

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Hemoglobin level, >133 g/L vs. ≤ 133 g/L	0.80 [0.56, 1.16]	0.244	0.72 [0.49, 1.05]	0.089
Platelet count, >233x10^9/L vs. ≤ 233x10^9/L	1.23 [0.85, 1.78]	0.275	1.23 [0.85, 1.78]	0.275
PNI score, >47.9 vs. ≤47.9	0.54 [0.37, 0.78]	0.001**	0.53 [0.37, 0.77]	0.001**
NLR, >2.66 vs. ≤2.66	1.18 [0.82, 1.71]	0.367	1.20 [0.83, 1.74]	0.341
Metastasis, Yes vs. No	1.05 [0.70, 1.57]	0.81	1.10 [0.73, 1.64]	0.654
Stage of NSCLC, IV, III vs. II and I	0.94 [0.50, 1.75]	0.838	1.05 [0.56, 1.98]	0.868
Stage of SCLC, Yes vs. No	1.33 [0.90, 1.96]	0.154	1.43 [0.97, 2.12]	0.074
Immunotherapy, Yes vs. No	0.28 [0.15, 0.52]	<0.001***	0.30 [0.16, 0.55]	<0.001***
Therapy of radiation, Yes vs. No	0.88 [0.60, 1.27]	0.488	0.79 [0.54, 1.16]	0.235
Application of platinum, Yes vs. No	0.61 [0.15, 2.47]	0.483	0.66 [0.16, 2.70]	0.562
Target therapy, Yes vs. No	0.75 [0.42, 1.33]	0.323	0.81 [0.45, 1.45]	0.481
Application of TKI, Yes vs. No	0.77 [0.41, 1.44]	0.415	0.82 [0.44, 1.53]	0.534
Application of VEGF inhibitor, Yes vs. No	0.83 [0.47, 1.45]	0.518	0.90 [0.51, 1.59]	0.723
Chemotherapy, AP vs. others	0.61 [0.37, 1.00]	0.052	0.66 [0.40, 1.09]	0.104
Smoking, Yes vs. No	1.23 [0.84, 1.79]	0.292	0.90 [0.56, 1.43]	0.645
Hypertension, Yes vs. No	1.13 [0.78, 1.64]	0.531	1.10 [0.75, 1.60]	0.622
Diabetes, Yes vs. No	0.90 [0.51, 1.61]	0.726	0.97 [0.54, 1.75]	0.927
Hyperlipemia, Yes vs. No	0.79 [0.42, 1.48]	0.461	0.77 [0.40, 1.46]	0.421
Heart failure, Yes vs. No	5.61 [1.71, 18.42]	0.004**	6.43 [1.94, 21.26]	0.002**
ACS, Yes vs. No	0.74 [0.23, 2.35]	0.612	0.69 [0.21, 2.21]	0.53
KPS score, >80 vs. ≤ 80	0.52 [0.35, 0.75]	0.001**	0.51 [0.35, 0.74]	<0.001***
miR-219-5p ≥ 1.50 vs. < 1.50	0.36 [0.25, 0.53]	<0.001***	0.37 [0.25, 0.55]	<0.001***

Abbreviation: HR, hazard risk; BMI, Body Mass Index; CRP, C-reactive protein; PNI, neutrophil lymphocyte ratio; NLR, neutrophil lymphocyte ratio; SCLC, small-cell lung cancer; TKI, Tyrosine Kinase Inhibitor; VEGF, vascular endothelial growth factor; KPS, Karnofsky Performance Status; ACS, acute coronary syndrome. ***p<0.001, **p<0.01, *p<0.05.

Model 1: Adjusted by age and gender

Independent Prognostic Factors for OS of Patients With SCLC

After multivariate adjustment, the miR-219-5p high level (HR 0.39, 95% CI 0.26-0.59, $P < 0.001$) was also associated with a low increase in the risk of death (figure 3). Meanwhile, gender, PNI score, immunotherapy and heart failure were also the independent risk factors for OS.

Development and Validation of an OS-predicting Nomogram

The independently related risk factors derived from the multivariate analysis were used to create an OS estimation nomogram (figure 4). The prognostic model was internally validated according to the bootstrap validation method. With an unadjusted

C index of 0.691 and a bootstrap-corrected C index of 0.691, the nomogram displayed excellent accuracy in estimating the risk of OS. In the validation cohort, the nomogram showed a C index of 0.691 for the estimation of OS. A suitable calibration curve for risk estimation was also displayed (R^2=0.455, LR chi2=80.55) (figure 4B). We collected 86 NSCLC patients from Sichuan Cancer Hospital in the external validation step. The ROC curve showed an AUC of 0.783 (0.743-0.822) for predicting 5-year overall survival, compared with an AUC of 0.749 (0.709-0.788) for the external validation data (figure 5).

Discussion

In this study, we detected the expression of miR-219-5p in a large cohort of SCLC patients at a single institution, between Mar 2010 and June 2015. The results suggested that reduced expression of miR-219-5p was significantly correlated with unfavorable clinical features. Moreover, patients in high miR-219-5p expression group displayed better OS compared with those in low miR-219-5p expression group. The multivariate analysis demonstrated miR-219-5p an independent prognostic factor for OS. In addition, to propose, and retrospectively verify in an independent cohort of patients, these independent risk factors were applied to establish a nomogram for OS estimation. The nomogram revealed good accuracy in estimating the risk of OS.

Carcinogenesis involves multiple biological processes which are related to many key genes[15, 16]. The characteristics of cancer occurrence represent properties that a cell acquire a certain ability to become and maintain itself as a cancer cell[17]. The key genes guide the cellular signaling pathways related to occurrence and progression of cancers[18, 19]. Using miRNA expression to predict the clinical diagnosis and prognosis of cancer has more advantages than mRNAs, as miRNAs are proved to be the vital posttranscriptional regulators of gene expression[20, 21]. In comparison with mRNAs, these vital gene regulators are highly conserved among species[22].

It has been reported that miRNAs were related to the initiation and progression of various cancers, and many miRNAs have been identified as a promising biomarker

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for prognostic prediction of cancer[10, 23]. Recently, some miRNAs have been proved to be a novel prognostic biomarker for SCLC[24, 25]. A study of Yu et al. indicated that miR-92a-2 was significantly higher in SCLC patients group compared to healthy control, and detection of miR-92a-2 levels could be a potential biomarker for patients with SCLC[26]. As a promising biomarker, miR-219-5p has been identified as a prognostic factor for different cancers. Long et al. found that miR-219-5p expression level was distinctly decreased in melanoma tissues and cell lines, and the modulation of miR-219-5p expression could be a prognostic biomarker and treatment strategy in melanoma[27]. A study from Huang et al. suggested a role of miR-219-5p for prognostic prediction and therapeutic strategy in colorectal cancer[28]. However, there is no studies exploring the role of miR-219-5p for biomarker in patients with SCLC. To the best of our knowledge, this study was the first attempt ever made to comprehensively evaluate the role for prognostic prediction based on miR-219-5p expression in patients with SCLC. In the current study, we initially examined the expression levels of miR-219-5p in SCLC patients. We, for the first time, demonstrated a correlation of the altered miR-219-5p expression with available clinical parameters. We found that miR-219-5p was significantly associated with lymphocytes count, PNI score and stage of SCLC. The univariate analysis indicated that increased miR-219-5p expression was a protective predictor for mortality. Kaplan-Meier curve displayed that patients with elevated miR-219-5p expression levels or accepted immunotherapy had low cumulative incidence of death compared to those with reduced miR-219-5p expression or unaccepted

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immunotherapy, respectively. In addition, gender, age, serum CRP level, albumin level, lymphocytes count, PNI score, immunotherapy, heart failure, KPS score and miR-219-5p level were associated with overall mortality. The multivariate analysis showed that miR-219-5p, gender, PNI score, immunotherapy and heart failure could predict OS as the independent risk factors.

Nomograms are applied for visualization of statistical models, graphical evaluation of variable significance and examination of predicted values[29, 30]. They have been widely performed to predict cancer risks and therapeutic outcomes[31, 32]. Most recently, several studies have successfully established a prognostic nomogram that combined a miRNA with clinical-related variables for OS estimation in different cancers[33-35]. Although nomograms are becoming increasingly popular, no studies have built prognostic models using combination of miR-219-5p and clinical risk factors in SCLC patients. In this study, based on the combination of miR-219-5p and independent clinicopathological variables, we created a nomogram model that could provide an individual prognostic prediction for OS estimation in SCLC patients. The results indicated excellent accuracy in estimating the risk of OS. There was a suitable calibration curve for risk estimation, indicating a well-performed nomogram, and good agreements between observation and prediction. To further verify the accuracy and efficiency of the model, an external date containing 86 patients from Sichuan Cancer Hospital was conducted. The results indicated that the prognositc model could accurately predict the prognosis of SCLC patients. Hence, this was the first prognostic nomogram for patients with SCLC that considered clinical parameters in addition to miR-219-5p. This nomogram could provide comprehensive information for patients, as well as a better guidance for clinical therapy. Based on the model, the potential high-risk patients with low survival rate could be more accurately selected for a specific therapeutic strategy.

There are some limitations in this article. Firstly, experimental research explaining the biological processes of miR-219-5p is needed. Thus, the molecular

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mechanism of miR-219-5p should be investigated in further research. Secondly, the prognostic nomogram needs to be further calculated by a prospective and large-scale multicenter study before it can be applied to clinical practice.

CONCLUSIONS

In conclusion, we found that the miR-219-5p expression levels were significantly correlated with clinical parameters of SCLC patients. Furthermore, miR-219-5p was proved to be an independent factor for prognostic prediction in patients with SCLC. Moreover, nomogram based on multivariate analysis showed excellent accuracy in estimating the risk of OS.

Acknowledgements The authors would like to thank the referees and the associate editor for their constructive advice.

Contributors ZHX and XMW designed the study. XHZ, JGZ and MQX collected and analysed the data. XHZ, JGZ and MQX drafted the initial manuscript. ZHX and XMW reviewed and edited the article. All authors read and approved the final manuscript.

Funding Not applicable.

Competing interests The authors declare that they have no competing interests.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not required.

Ethics approval

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The study was approved by ethics committee of Suzhou Xiangcheng People's Hospital. The reference number was 20140193. All procedures performed in the present study were in accordance with the principles outlined in the 1964 Helsinki Declaration and its later amendments.

Provenance and peer review Not commissioned; externally peer reviewed.

Data Availability Statement The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Figure Legends

Figure 1 A flow chart of the screening process.

Figure 2 Overall survival (OS) of SCLC patients in different levels of miR-219-5p and different treatments. (A) OS of SCLC patients with high or low level of miR-219-5p. (B) OS of SCLC patients with different treatments (immunotherapy vs non-immunotherapy).

Figure 3 Multivariate cox regression analysis of 5-year overall survival on data in the SCLC patients.

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Figure 4 Nomogram for overall survival (OS) risk estimation of SCLC patients and its predictive performance. (A) Nomogram to estimate the OS risk of SCLC patients in different variations. To build the nomogram, find the position of each variable on the corresponding axis, draw a line to the points axis for the number of points, add the points from all of the variables, and draw a line from the total points axis to determine the OS probabilities at the lower line of the nomogram. (B) Validity of the predictive performance of the nomogram in estimating the OS risk of SCLC patients.

Figure 5 External validation of the prognostic model.

Table 1 Study participant characteristics at enrollment.

Table 2 Univariate cox regression analysis of overall survival on SCLC patients.

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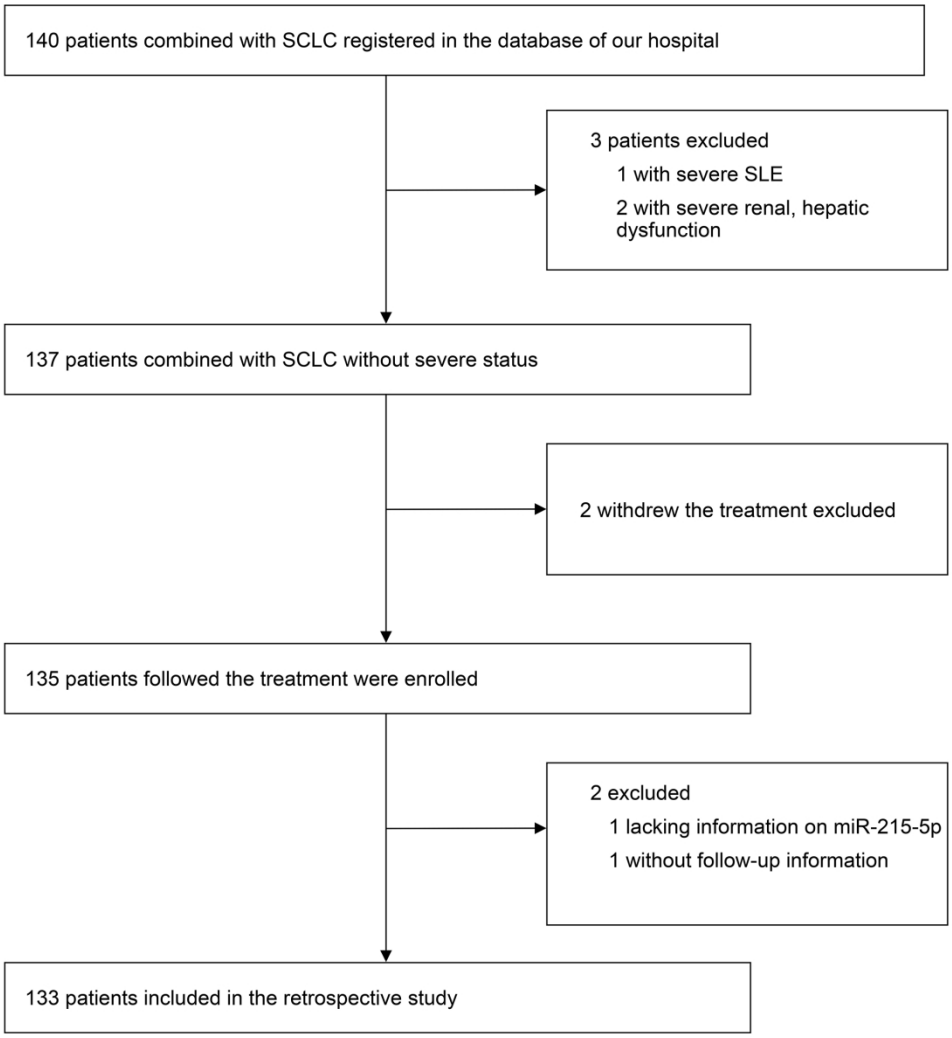

Figure 1 A flow chart of the screening process.

180x195mm (300 x 300 DPI)

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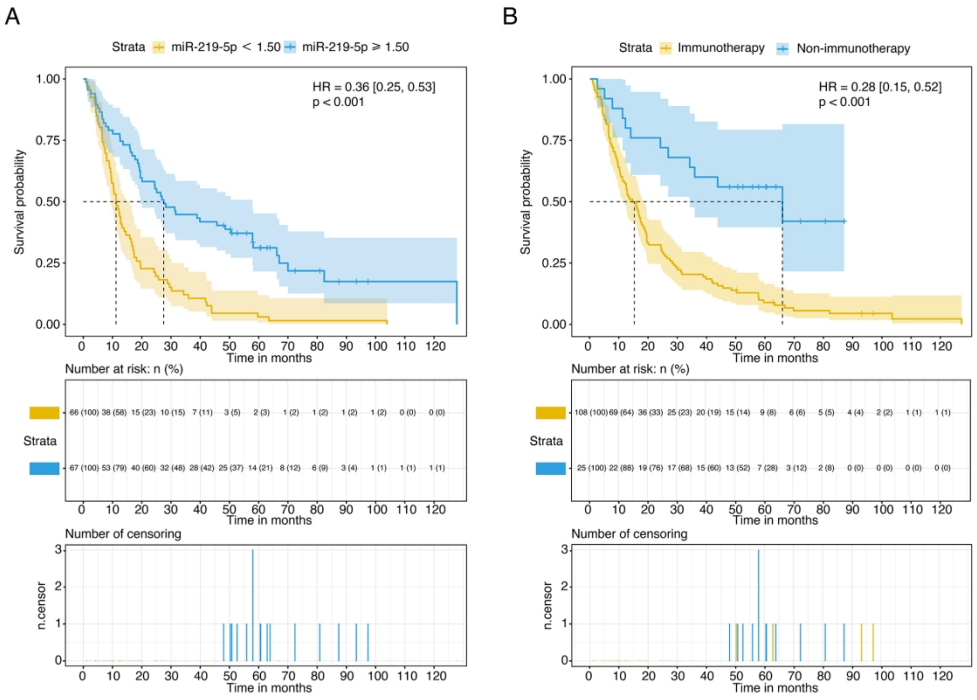

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180x126mm (300 x 300 DPI)

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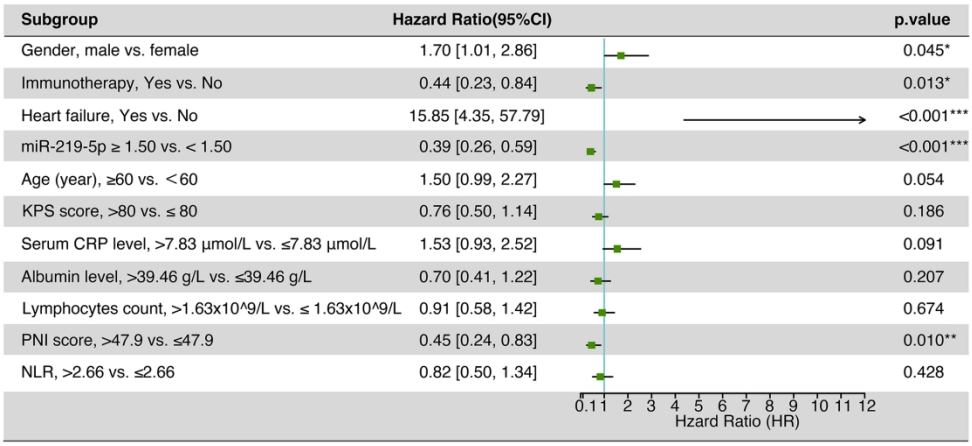

Figure 3 Multivariate cox regression analysis of 5-year overall survival on data in the SCLC patients.

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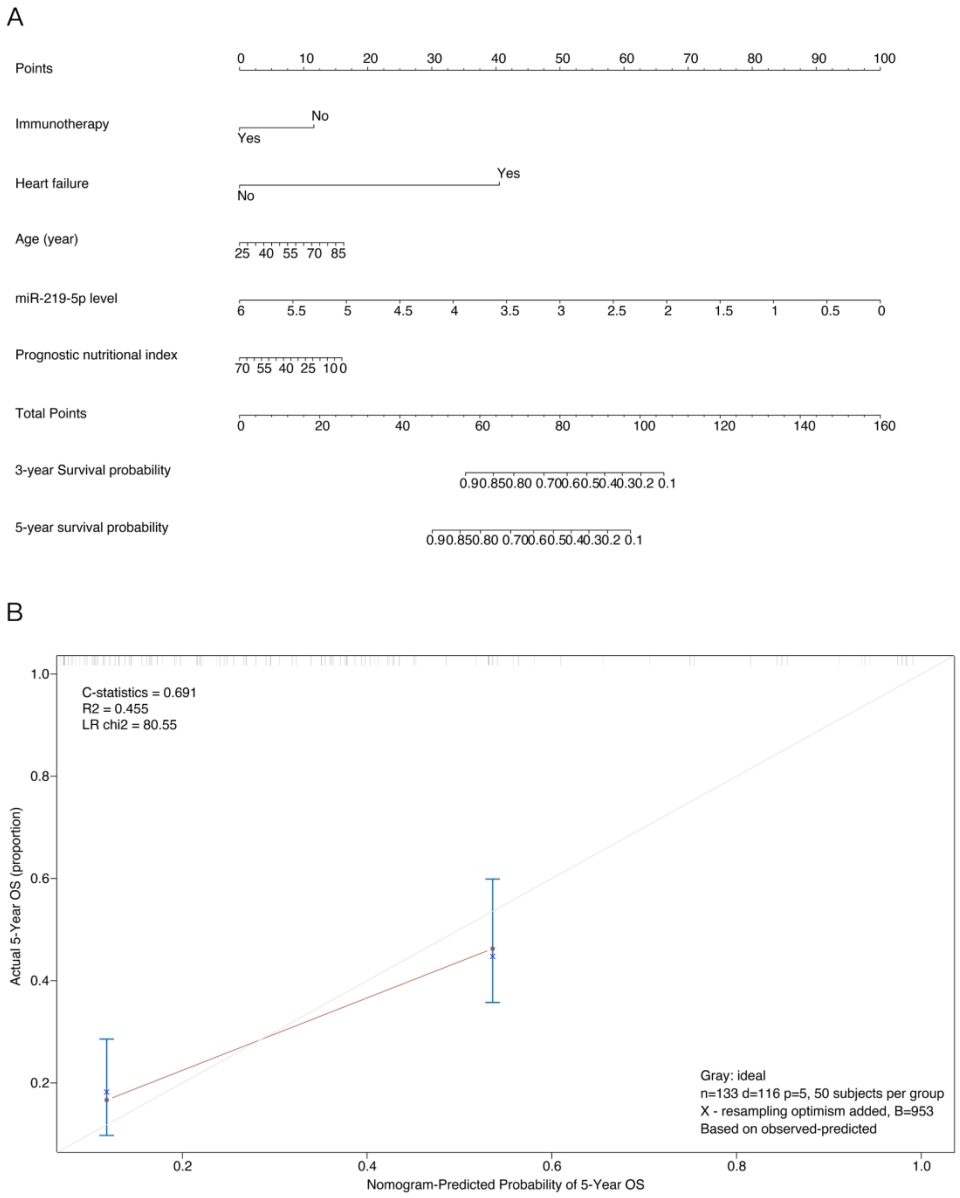

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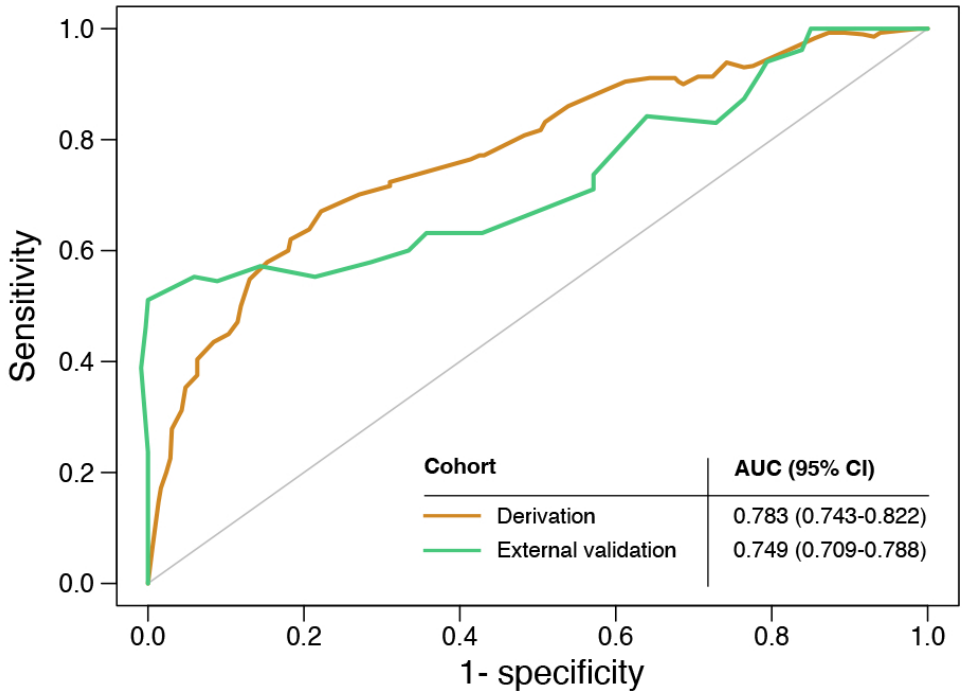
Figure 5



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Supplement Materials

Part I. Details of variants

Patients' age: The age of patients was based on the time of diagnosis of NSCLC.

Body mass index: The equation of body mass index (BMI) is calculated as follow: BMI (kg/m^2) = weight (kg) / height $(m)^2$. The data of BMI was recorded before clinical treatment.

Serum CEA level: The serum level of carcinoembryonic antigen (CEA) is one of tumor marks， the higher serum *CEA* level than that of healthy individuals led to its clinical application as a diagnostic biomarker for cancer.

Serum CRP level: C-reactive protein (CRP) is detected by olorimetric determination through fasting blood collection and CRP is one of the inflamation markers.

Albumin level: Serum albumin is detected by colorimetric determination through fasting blood collection. And serum albumin is one of the indicators of nutritional status.

Neutrophils count, lymphocytes count:, hemoglobin level, platelet count: All these indexes were extracted from blood routine examination to evaluate preoperative status of patients.

PNI score: Prognostic nutritional index (PNI) is an index to evaluate the nutritional status of surgical patients, to predict the risk of surgery and to help make prognostic judgment. The equation is listed as below: PNI = serum albumin (g/L) + 5 * lymphocytes ($*10^9$/L).

NLR: Neutrophil to Lymphocyte Ratio (NLR) is an index of inflammation markers to predict the prognosis of various cancer, including lung cancer.

Pathologic type: Non-small cell lung cancer can be classified as adenocarcinoma, squamous cell carcinoma, large cell lung cancer, or others, depending on surgical or tumor biopsy specimens

Stage of NSCLC: Non-small cell lung cancer was staged according to the seventh edition of international union against cancer (UICC) TNM was published in 2009

Surgery: For early and locally advanced NSCLC, which is generally acceptable, there is a history of surgical resection.

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Therapy of radiation: Radiation therapy is a medical procedure that uses the delivery of high-energy radiation to kill cancer cells and shrink tumors, including external beam radiation therapy, internal radiation therapy and stereotactic body radiotherapy (SBRT). It aims to cure small tumors, treat lung cancer both locally and nearby lymph nodes, treat metastases, relieve symptoms, and also at prevention.

Application of platinum: Platinum-based chemotherapy may be used as a first or second line therapy for advanced lung cancer, after surgery (adjuvant chemotherapy), or before surgery to reduce the size of a tumor (neoadjuvant therapy). The most commonly used platinum drugs are cisplatin, carboplatin and nedaplatin. Treatment of non-small cell lung cancer begins with either cisplatin or carboplatin combined with another medication, such as docetaxel, pemetrexed, paclitaxel, gemcitabine and gemcitabine.

Target therapy: Targeted therapies for lung cancer are drugs that are tailored to attack certain mutations of cancer cells. These mutations lead to the production of abnormal proteins which guide the growth and development of a cancer cell. Common driver mutations include EGFR mutations, ALK rearrangements, ROS1 rearrangements, MET amplifications, KRAS mutations, HER2 mutations, BRAF mutations and so on.

Application of TKI: Tyrosine kinase inhibitors (TKI) act as competitive inhibitors of ATP binding to tyrosine kinases, or as tyrosine analogs that block tyrosine kinase activity and inhibit cell proliferation. Epithelial growth factor receptor (EGFR) is a protein that is present on the surface of both normal cells and lung cancer cells. Mutations in EGFR can occur at different locations on exon 18 to 21. ALK-positive lung cancer refers to people who have lung cancer that tests positive for the EML4-ALK fusion mutation. Several FDA-approved medications available to treat EGFR-positive and ALK-positive lung adenocarcinoma, including:

- EFGR inhibitors - Tarceva (erlotinib), Gilotrif (afatinib), Iressa (gefitinib), Tagrisso (osimertinib), and Portrazza (necitumumab)
- ALK inhibitors - Xalkori (crizotinib), Zykadia (ceritinib), and Alecensa (alectinib)
- ROS1 inhibitor - Xalkori (crizotinib)

Application of VEGF inhibitor: VEGF (Vascular endothelial growth factor) can activate angiogenesis through different signaling pathways. VEGF inhibitors are drugs that block the ability of tumors to form new blood vessels, and hence, grow and spread. Some currently available medications include bevacizumab, ramucirumab, axitinib, endostar and anlotinib.

KPS score: A performance measure for rating the ability of a person to perform usual activities, evaluating a patient's progress after a therapeutic procedure, and determining a patient's suitability for therapy. It is used most commonly in the

prognosis of cancer therapy, usually after chemotherapy and customarily administered before and after therapy. It was named for Dr. David A. Karnofsky, an American specialist in cancer chemotherapy. Patients with more than 80 scores had better postoperative status and longer survival time. And patients with more than 70 scores can suffer from chemoradiotherapy.

Smoking: Smoker refers to continuous or cumulative smoking > 1 cigarette/day over a lifetime of more than 6 months. (1997, WHO)

Hypertension: Hypertension is defined as a repeatedly elevated blood pressure exceeding 140 over 90 mmHg.

Diabetes: Diabetes is a group of metabolic diseases characterized by hyperglycemia. And fasting blood glucose more than 7.0 mmol/l or blood glucose more than 11.1 mmol/l within 2hoursafter meal can be diagnosed diabetes.

Hyperlipemia: Hyperlipemia means the presence of excess fat or lipids in the blood. And total cholesterol ≥ 6.2 mmol/L, low density lipoprotein cholesterin ≥ 4.1 mmol/L, triglyceride ≥ 2.3 mmol/L, high density lipoprotein cholesterin < 1.0 mmol/L can be diagnosed hyperlipemia.

Heart failure: The information was recorded through history taking and verified after hospitalization. Heart failure means inability of the heart to keep up with the demands on it and, specifically, failure of the heart to pump blood with normal efficiency. Heart failure may be due to failure of the right or left or both ventricles. The signs and symptoms depend upon which side of the heart is failing. They can include shortness of breath (dyspnea), asthma due to the heart (cardiac asthma), pooling of blood (stasis) in the general body (systemic) circulation or in the liver's (portal) circulation, swelling (edema), blueness or duskiness (cyanosis), and enlargement (hypertrophy) of the heart.

ACS: Acute coronary syndrome is a term for a group of conditions that suddenly stop or severely reduce blood from flowing to the heart. When blood cannot flow to the heart, the heart muscle can become damaged. Heart attack and unstable angina are both acute coronary syndromes (ACS).

Withdraw treatment: Reasons for patients withdrew from treatment were listed below: 1) High cost of the treatment: In China, the cost of hormone therapy can be up to 3000 RMB a month if without health insurance. 2) Poor patient compliance: Some patients discontinue treatment because they do not comply with the treatment plan prescribed by their doctor.

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Part II. Report of sample size assessment

PASS 15.0.5

Tests for Two Survival Curves Using Cox's Proportional Hazards Model

Numeric Results with Ha: HR ≠ 1

Power	Total Sample Size N	Control Sample Size N1	Trtmnt Sample Size N2	Prop'n Control N1/N P1	Hazard Ratio h2/h1 HR	Control Prob Event Pev1	Trtmnt Prob Event Pev2	Control Events E1	Trtmnt Events E2	Alpha	Beta
0.9005	103	51	52	0.500	0.500	0.750	0.950	38.3	49.4	0.050	0.100

References

Chow, S.C., Shao, J., Wang, H. 2008. Sample Size Calculations in Clinical Research, 2nd Edition. Chapman & Hall/CRC.

Schoenfeld, David A. 1983. 'Sample Size Formula for the Proportional-Hazards Regression Model', Biometrics, Volume 39, Pages 499-503.

Report Definitions

Power is the probability of rejecting a false null hypothesis. Power should be close to one.
N is the total sample size.
N1 and N2 are the sample sizes of the control and treatment groups.
P1 is the proportion of the total sample that is in the control group, group 1.
HR is the hazard ratio: h2/h1.
Pev1 and Pev2 are the probabilities of an event in the control and the treatment groups.
E1 and E2 are the number of events required in the control and the treatment groups.
Alpha is the probability of a type one error: rejecting a true null hypothesis.
Beta is the probability of a type two error: failing to reject a false null hypothesis.

Summary Statements

A two-sided test of whether the hazard ratio is one with an overall sample size of 103 subjects (of which 51 are in the control group and 52 are in the treatment group) achieves 90% power at a 0.050 significance level when the hazard ratio is actually 0.500. The number of events required to achieve this power is 87.7. It is anticipated that proportions of subjects having the event during the study is 0.750 for the control group and 0.950 for the treatment group. These results assume that the hazard ratio is constant throughout the study and that Cox proportional hazards regression is used to analyze the data.

Procedure Input Settings

Autosaved Template File

\\Mac\Home\Documents\PASS 15\Procedure Templates\Autosave\Tests for Two Survival Curves Using Cox's

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Proportional Hazards Model - Autosaved 2020_1_24-9_57_39.t92

Design Tab

Solve For:	Sample Size
Alternative Hypothesis:	Ha: HR ≠ 1
Power:	0.90
Alpha:	0.05
Group Allocation:	Equal (N1 = N2)
Pev1 (Probability of a Control Event):	0.750
Pev2 (Probability of a Treatment Event):	0.950
HR (Actual Hazard Ratio = h2/h1):	0.5

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			**5**
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*6*
Bias	9	Describe any efforts to address potential sources of bias	7
Study size	10	Explain how the study size was arrived at	7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7
		(*b*) Describe any methods used to examine subgroups and interactions	7
		(*c*) Explain how missing data were addressed	7
		(*d*) If applicable, explain how loss to follow-up was addressed	7
		(*e*) Describe any sensitivity analyses	7
Results			

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8
		(b) Give reasons for non-participation at each stage	8
		(c) Consider use of a flow diagram	8
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9
		(b) Indicate number of participants with missing data for each variable of interest	9
		(c) Summarise follow-up time (eg, average and total amount)	9
Outcome data	15*	Report numbers of outcome events or summary measures over time	10
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10
		(*b*) Report category boundaries when continuous variables were categorized	11
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	11
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	12
Discussion			
Key results	18	Summarise key results with reference to study objectives	13-14
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15
Generalisability	21	Discuss the generalisability (external validity) of the study results	15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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MiR-219-5p decrease the risk of cancer-related mortality in patients with small cell lung cancer

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Running title: MiR-219-5p decrease the risk of SCLC patients

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ABSTRACT

Objectives Small cell lung cancer (SCLC) is a lethal human malignancy, and previous studies support the contribution of microRNA (miRNA) to cancer progression. The prognostic value of miR-219-5p in SCLC patients remains unclear. This study evaluated the risk factors for SCLC and created a prediction model for them.

Design Retrospective observational cohort study.

Setting The programme has yielded a database of all patients with SCLC in 2 defined geographical regions of China.

Participants We did a real-world study, including data from 133 patients with SCLC between Mar 1, 2010 and June 1, 2015. We collected 86 NSCLC patients in the external validation step.

Primary and secondary outcome measures MiR-219-5p was recorded during the admission. Cox proportional hazard model was applied for survival analyses and for analyzing risk factors for cancer-related mortality and to create a nomogram for prediction. The accuracy of the model was evaluated by C-index and calibration curve. An external data of 86 SCLC patients from Sichuan Cancer Hospital and the First affiliated hospital of Soochow University was conducted.

Results In our data, the mortality in group with high miR-219-5p level (≥ 1.50) was 74.6%. Based on univariate analysis, we put factors (P <0.05) into a multivariate regression model, patients with high miR-219-5p level (P <0.001, HR=0.36), immunotherapy (P <0.001, HR=0.44), PNI score >47.9 (P =0.01, HR=0.45) remained statistically factors for better overall survival (OS) and regarded as independent protective factors. These independently associated risk factors were used to establish an OS estimation nomogram. Nomogram revealed good accuracy in estimating the risk, with a bootstrap-corrected C index of 0.691. External validation displayed an AUC of 0.749 (0.709-0.788).

Conclusions MiR-219-5p decreased the risk of cancer-related mortality in patients with SCLC. Nomogram based on multivariate analysis demonstrated good accuracy in estimating the risk of overall mortality.

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Keywords: small cell lung cancer, miR-219-5p, overall survival, nomogram, prediction model

Strengths and limitations of this study

- ► The study established the first risk nomogram for predicting the 3-year and 5-year specific mortality probability for SCLC
- ► Based on the retrospective sample, the nomogram can improve the ability of clinicians in predicting survival probabilities in individual patients.
- ► The model is not comprehensive enough because the epidemiology and end results database does not include all prognostic factors for SCLC.
- ► The available data on treatment status are not detailed enough to distinguish the impact of various treatment plans.
- ► The model needs to be prospectively studied to confirm its reliability

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BACKGROUND

Lung cancer is the leading cause of cancer deaths worldwide, with millions of new cases diagnosed each year[1]. Small cell lung cancer (SCLC) is a kind of neuroendocrine malignant tumor with poor prognosis, accounting for about 15% of lung cancer patients[2]. SCLC is generally divided into limited disease (LD-SCLC) and extensive disease (ED-SCLC). Platinum combined with etoposide is the first-line therapeutic strategy of SCLC, and most patients are easy to receive initial chemotherapy[3]. However, the 5-year survival rates of LD-SCLC and ED-SCLC are only 15% and 3%, respectively[4]. Therefore, improvement in early diagnosis and prognostic prediction of SCLC is vital.

MicroRNAs (miRNAs) are endogenous non-coding RNAs (~ 22 nt), which regulate mRNA activity by hybridization with 3 '- untranslated region (UTR) of specific genes[5]. Many studies have shown that miRNAs could participate in a variety of cell biological processes, including cell growth, differentiation and apoptosis[6, 7]. In addition, researches have demonstrated that miRNAs are frequently dysregulated in cancers[8, 9], and some miRNAs can serve as diagnostic and prognostic biomarkers for caners[10]. Recently, several miRNAs have been proved to participant in the occurrence and development of SCLC, but few of them are likely to be a biomarker or therapeutic target for SCLC.

Recently, miR-219-5p has been found to be abnormally expressed and play a significant role in different cancers. Ma et al. found that the expression of miR-219-5p was significantly decreased in esophageal squamous cell carcinoma (ESCC) tissues compared with normal tissues[11]. A study of Gong et al. revealed a tumor suppressive role for miR-219-5p by targeting glypican-3 in hepatocellular carcinoma (HCC)[12]. On the contrary, Yang et al. indicated that miR-219-5p could promote cell growth and metastasis of HCC and serve as a prognostic marker for HCC patients[13]. A research investigated by Wei et al. suggested that miR-219-5p could inhibit proliferation, migration and invasion of epithelial ovarian cancer through downregulation of the Wnt signaling pathway, and it could serve as a diagnostic

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biomarker and therapeutic target for epithelial ovarian cancer[14]. However, the biological functions of miR-219-5p and its potential prognostic role for biomarker in SCLC are still unknown.

In this study, we aimed to examine the variation in the expression levels of miR-219-5p in patients with SCLC and explored the potential prognostic role of miR-219-5p for SCLC. We also displayed a nomogram that could provide individualized, evidence-based, highly accurate risk estimates. Nomograms were easy to performed and could facilitate management-related decision making.

METHODS

Study Design and Patient Characteristics

We did a real-world study, including data obtained from 133 patients with SCLC between Mar 2010 and June 2015, in the Suzhou Xiangcheng People's Hospital. Those participants who lacked information on complement components data, withdrew from treatment or lacked follow-up information were excluded. Clinical information of patients, including gender, age, BMI, neutrophils count, lymphocytes count, serum CEA level, CRP level, albumin level, hemoglobin level, stage of SCLC, platelet count, PNI score, KPS score, NLR, pathologic type, immunotherapy, therapy of radiation, application of platinum, application of VEGF inhibitor, target therapy, application of TKI, smoking, ACS, diabetes, heart failure and hyperlipemia, were recorded. Diagnosis of SCLC was confirmed by histopathological examination. The median length of follow-up was 23.6 months. Median was used as the cut-off value. The definition and details of all the variables above were provided in Supplemental Materials Part I. Data from 86 patients with NSCLC at Sichuan Cancer Hospital and

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the First affiliated hospital of Soochow University were applied for external validation. Inform, and consent was obtained from all patients or their immediate family members. All protocols were in line with the guidelines with the ethic committee of Suzhou Xiangcheng People's Hospital, Sichuan Cancer Hospital, the First affiliated hospital of Soochow University and following the Declaration of Helsinki.

Assays for Detection of MiR-219-5p Levels

The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was conducted for the detection of miR-219-5p expression levels.

Total RNA from tissues was isolated and extracted using miRcute Extraction and Separation of miRNAs kit (Tiangen Biotech Co., Ltd., Beijing, China), and then reversely transcribed into cDNA by PrimeScript™ Ⅱ 1st strand cDNA synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co., Ltd.) was used for qRT-PCR. The thermocycling conditions were as follows: one cycle at 95˚C for 3 min (initial denaturation), 40 cycles at 95˚C for 15 s and 60˚C for 30 s. U6 small nuclear RNA (U6) served as the respective internal control. The relative expression of miR-219-5p was quantified by the $2^{-\Delta\Delta Ct}$ methods, and normalized to the U6. The following primers were used: miR-219-5p forward, 5'-ACACTCCAGCTGGGTGATTGTCCAAACGCAAT-3' and reverse, 5'-CTCAACTGGTGTCGTGGA-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The experiments were repeated at least 3 times.

Statistical Analysis

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Sample size assessment was performed using NCSS-PASS software version 11.0 (https://www.ncss.com/software/pass/). Power was set as 0.99, and alpha was 0.5. The mortalities of both miR-219-5p high-level group and miR-219-5p low-level group in our previous data (2008-2009) (0.750 and 0.950) were entered into the PASS. The Actual Hazard Ratio was set as 0.50. Then the sample size was calculated using PASS, and the minimum sample size was 103 (control = 51, experiment = 43). Our sample size was 133 (66 and 67 for each group), which was suitable. The report of sample size assessment was displayed in Supplemental Material Part II. The missing data (<5.0%) were estimated by random forest algorithm using the mice package in RStudio (R version 3.6.1). Categorical variates were presented as percentages and compared via the κ^2 test. Continuous variates with skewed and normal distributions were presented as median with interquartile ranges and mean ± standard deviation. The Mann-Whitney U test and the unpaired t-test were applied for comparison between Groups. Cumulative mortality was showed by the Kaplan-Meier curve and analyzed by the log-rank test. Univariate and multivariate survival analyses for OS were conducted using the Cox regression model. The forest plots were used to visualize the significance of covariates to the prognosis. The restricted cubic spline analyses were performed with Harrell's Regression Modelling Strategies (rms) package.

We screened multifactor analysis for statistically significant indicators for inclusion in the prediction model. To build the nomogram, find the position of each variable on the corresponding axis, draw a line to the points axis for the number of points, add the points from all the variables, and draw a line from the total points axis

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to determine the OS probabilities at the lower line of the nomogram. The contribution of each covariate was quantified and visualized in a prognostic nomogram with internal validation via 1000-times bootstrapping. The consistency of the resulting model was assessed by the calibration assay. Decision curve analyses were performed to evaluate net clinical benefits of the model compared with conventional prognostic scores. The scatter plots were applied for visualization of the consistency of each model. A 1000-time bootstrapping was employed as indicated. The association between miR-219-5p class and survival endpoints was evaluated by Kaplan-Meier curves and log-rank test. Statistical analysis was performed using the RStudio (R version 3.6.1) with the following packages: 'ggplot2', 'rms', 'PredictABLE', 'risk regression', and 'survminer'.

Patient and public involvement

This study was conducted without patient involvement. Patients were not invited to comment on the study design and were not consulted to develop patient-relevant outcomes or interpret the results. Moreover, patients were not allowed to contribute to the writing or editing of this document for readability or accuracy.

RESULTS

Baseline Characteristics

A total of 133 SCLC patients who were diagnosed between Mar 2010 and June 2015 were included in this study. A flow chart of the screening process was shown in figure 1. The median age of these patients was 64 years old (58-70), and it contained 106 (80.0%) males. Median serum CEA and CRP level was 3.43 ng/ml and 7.83 μmol/L, respectively. For the stage of SCLC, 51 (38.0%) patients were diagnosed with limited disease, 82 (61.0%) patients were extensive disease. 25 (19.0%) patients accepted the immunotherapy therapy. 54 (41.0%) patients got the therapy of radiation. In addition,

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platinum was applied for 131 (98.0%) patients, and TKI was used for 15 (12.0%) patients. KPS score of these patients was examined, and the results revealed that 107 (81.0%) patients got 80 or higher points. The distribution of basic diseases was also assessed in our data. Diabetes was found in 17 (13.0%) patients, while hyperlipemia was 13 (10.0%). Cardiovascular diseases such as heart failure and ACS were found in 3 (2.0%) and 4 (3.0%) patients, respectively. 13 (10.0%) patients suffered from hypertension. In addition, 82 (62.0%) patients had a habit of smoking among all the patients. The baseline characteristics of these patients were listed in table 1.

Among all the 133 patients, the overall mortality was 87.2%. The mortality in high miR-219-5p level group was 74.6%. Moreover, in the high miR-219-5p level group, patients with extensive disease were 35 (52.0%), while the low group was 47 (71.0%) (table 1).

Table 1. Study Participant Characteristics at Enrollment

Variation	Total (n=133)	Cohort, median (IQR)		p.value
		mir-219-5p<1.50, (n=66)	mir-219-5p≥1.50, (n=67)	
Age, (year)	64(58,70)	65(59,70)	63(56.5,68)	0.276
BMI, (kg/m2)	23.12±3.09	22.99±3.22	23.26±2.98	0.619
Serum CEA level, (ng/ml)	3.43(1.96,9.26)	3.34(1.92,10.05)	3.43(1.99,8.11)	0.87
Serum CRP level, (μmol/L)	7.83(1.68,12.78)	10.68(2.78,13.15)	5.64(1.2,12.16)	0.107
Albumin level, (g/L)	39.46±5.2	38.86±4.68	40.05±5.65	0.188
Neutrophils count, (10^9/L)	4.55(3.59,5.88)	4.54(3.77,5.75)	4.55(3.48,5.92)	0.975
Lymphocytes count, (10^9/L)	1.63(1.31,1.95)	1.5(1.21,1.78)	1.73(1.38,2.08)	0.031*
Hemoglobin level, (g/L)	133(125,145)	134(124,145)	132(125,143.5)	0.564
Platelet count, (10^9/L)	233(184,288)	244.5(180,293.75)	226(184.5,273.5)	0.306
PNI score	47.9(43.95,51.85)	45.98(42.51,50.38)	48.7(44.92,54)	0.026*
NLR	2.66(1.99,4.19)	2.75(2.11,4.57)	2.59(1.92,3.7)	0.232
Gender, (n%)				0.211
Female	27(20)	10(15)	17(25)	
Male	106(80)	56(85)	50(75)	
Metastasis, n(%)				0.299
No	45(34)	19(29)	26(39)	
Yes	88(66)	47(71)	41(61)	
Stage of SCLC				0.038*
Limited disease	51(38)	19(29)	32(48)	

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Extensive disease	82(62)	47(71)	35(52)	
Immunotherapy, (n%)				0.197
No	108(81)	57(86)	51(76)	
Yes	25(19)	9(14)	16(24)	
Therapy of radiation, (n%)				0.417
No	79(59)	42(64)	37(55)	
Yes	54(41)	24(36)	30(45)	
Application of platinum, (n%)				0.244
No	2(2)	2(3)	0(0)	
Yes	131(98)	64(97)	67(100)	
Chemotherapy				0.45
AP	28(21)	12(18)	16(24)	
DP	15(11)	6(9)	9(13)	
EP	71(53)	35(53)	36(54)	
GP	3(2)	2(3)	1(1)	
Others	16(12)	11(17)	5(7)	
Target therapy, (n%)				0.627
No	116(87)	59(89)	57(85)	
Yes	17(13)	7(11)	10(15)	
Application of TKI, (n%)				0.449
No	118(89)	60(91)	58(87)	
TKI I	9(7)	4(6)	5(7)	
TKI II	1(1)	1(2)	0(0)	
TKI III	5(4)	1(2)	4(6)	
Application of VEGF inhibitor, n(%)				0.645
No	114(86)	58(88)	56(84)	
Yes	19(14)	8(12)	11(16)	
KPS score, n(%)				0.678
40	2(2)	0(0)	2(3)	
50	5(4)	3(5)	2(3)	
60	7(5)	3(5)	4(6)	
70	12(9)	8(12)	4(6)	
80	29(22)	14(21)	15(22)	
90	56(42)	29(44)	27(40)	
100	22(17)	9(14)	13(19)	
Smoking, n(%)				0.255
No	51(38)	29(44)	22(33)	
Yes	82(62)	37(56)	45(67)	
Hypertension, n(%)				1
No	80(60)	40(61)	40(60)	
Yes	53(40)	26(39)	27(40)	
Diabetes, n(%)				1
No	116(87)	58(88)	58(87)	
Yes	17(13)	8(12)	9(13)	

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Hyperlipemia, n(%)				0.579
No	120(90)	61(92)	59(88)	
Yes	13(10)	5(8)	8(12)	
Heart failure, n(%)				1
No	130(98)	65(98)	65(97)	
Yes	3(2)	1(2)	2(3)	
ACS, n(%)				0.619
No	129(97)	65(98)	64(96)	
Yes	4(3)	1(2)	3(4)	

Abbreviation: IQR, interquartile range; CRP, C-reactive protein; PNI, neutrophil lymphocyte ratio; NLR, neutrophil lymphocyte ratio; SCLC, small-cell lung cancer; TKI, Tyrosine Kinase Inhibitor; VEGF, vascular endothelial growth factor; KPS, Karnofsky Performance Status; ACS, acute coronary syndrome. ***p<0.001, **p<0.01, *p<0.05.

MiR-219-5p Expression Level, and Clinical Risk Factors Predict the Development of SCLC Patients

According to the univariate analysis, high miR-219-5p levels (≥ 1.50) was a strong protective predictor of cancer-related mortality (HR 0.36, 95% CI 0.25-0.53, *P* <0.001) (table 2). Kaplan Meier curve showed that patients in the high miR-219-5p group had a decreased cumulative rate of death than those in the low miR-219-5p group (log-rank *P* <0.001) (figure 2A). Meanwhile, patients who accepted immunotherapy also showed a low morality compared to those patients without accepting immunotherapy in the survival curve (HR 0.28, 95% CI 0.15-0.52, *P* <0.001) (figure 2B).

In addition, gender, age, serum CRP level, albumin level, lymphocytes count, PNI score, immunotherapy, heart failure, and KPS score were also correlated with overall mortality (table 2). When adjusted by age and gender, patients in the miR-219-5p high-level group also displayed a low cumulative rate death compared to those in the low-level group.

Table 2. Cox Regression Analysis of Hazard Ratio on SCLC patients

Variation	Non-adjustment		Model 1	
	Hazard Ratio (95% CI)	p.value	Hazard Ratio (95% CI)	p.value
Gender, male vs. female	1.66 [1.02, 2.69]	0.041*	-	-
Age (year), ≥60 vs. < 60	1.52 [1.03, 2.26]	0.036*	-	-

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BMI, ≥23.12 kg/m2 vs. < 22.86kg/m2	0.99 [0.69, 1.43]	0.97	0.95 [0.66, 1.38]	0.806
Serum CEA level, >3.43 ng/ml vs. ≤3.43 ng/ml	1.01 [0.70, 1.46]	0.954	1.00 [0.69, 1.45]	0.999
Serum CRP level, >7.83 μmol/L vs. ≤7.83 μmol/L	1.91 [1.31, 2.78]	0.001**	1.92 [1.32, 2.79]	0.001**
Albumin level, >39.46 g/L vs. ≤39.46 g/L	1.66 [1.15, 2.40]	0.007**	1.60 [1.10, 2.31]	0.013*
Neutrophils count, >4.55x10^9/L vs. ≤ 4.55x10^9/L	1.18 [0.82, 1.71]	0.367	1.15 [0.79, 1.66]	0.464
Lymphocytes count, >1.63x10^9/L vs. ≤ 1.63x10^9/L	0.59 [0.40, 0.85]	0.005**	0.61 [0.42, 0.89]	0.01*
Hemoglobin level, >133 g/L vs. ≤ 133 g/L	0.80 [0.56, 1.16]	0.244	0.72 [0.49, 1.05]	0.089
Platelet count, >233x10^9/L vs. ≤ 233x10^9/L	1.23 [0.85, 1.78]	0.275	1.23 [0.85, 1.78]	0.275
PNI score, >47.9 vs. ≤47.9	0.54 [0.37, 0.78]	0.001**	0.53 [0.37, 0.77]	0.001**
NLR, >2.66 vs. ≤2.66	1.18 [0.82, 1.71]	0.367	1.20 [0.83, 1.74]	0.341
Metastasis, Yes vs. No	1.05 [0.70, 1.57]	0.81	1.10 [0.73, 1.64]	0.654
Stage of NSCLC, IV, III vs. II and I	0.94 [0.50, 1.75]	0.838	1.05 [0.56, 1.98]	0.868
Stage of SCLC, Yes vs. No	1.33 [0.90, 1.96]	0.154	1.43 [0.97, 2.12]	0.074
Immunotherapy, Yes vs. No	0.28 [0.15, 0.52]	<0.001***	0.30 [0.16, 0.55]	<0.001***
Therapy of radiation, Yes vs. No	0.88 [0.60, 1.27]	0.488	0.79 [0.54, 1.16]	0.235
Application of platinum, Yes vs. No	0.61 [0.15, 2.47]	0.483	0.66 [0.16, 2.70]	0.562
Target therapy, Yes vs. No	0.75 [0.42, 1.33]	0.323	0.81 [0.45, 1.45]	0.481
Application of TKI, Yes vs. No	0.77 [0.41, 1.44]	0.415	0.82 [0.44, 1.53]	0.534
Application of VEGF inhibitor, Yes vs. No	0.83 [0.47, 1.45]	0.518	0.90 [0.51, 1.59]	0.723
Chemotherapy, AP vs. others	0.61 [0.37, 1.00]	0.052	0.66 [0.40, 1.09]	0.104
Smoking, Yes vs. No	1.23 [0.84, 1.79]	0.292	0.90 [0.56, 1.43]	0.645
Hypertension, Yes vs. No	1.13 [0.78, 1.64]	0.531	1.10 [0.75, 1.60]	0.622
Diabetes, Yes vs. No	0.90 [0.51, 1.61]	0.726	0.97 [0.54, 1.75]	0.927
Hyperlipemia, Yes vs. No	0.79 [0.42, 1.48]	0.461	0.77 [0.40, 1.46]	0.421
Heart failure, Yes vs. No	5.61 [1.71, 18.42]	0.004**	6.43 [1.94, 21.26]	0.002**
ACS, Yes vs. No	0.74 [0.23, 2.35]	0.612	0.69 [0.21, 2.21]	0.53
KPS score, >80 vs. ≤ 80	0.52 [0.35, 0.75]	0.001**	0.51 [0.35, 0.74]	<0.001***
miR-219-5p ≥ 1.50 vs. < 1.50	0.36 [0.25, 0.53]	<0.001***	0.37 [0.25, 0.55]	<0.001***

Abbreviation: HR, hazard risk; BMI, Body Mass Index; CRP, C-reactive protein; PNI, neutrophil lymphocyte ratio; NLR, neutrophil lymphocyte ratio; SCLC, small-cell lung cancer; TKI, Tyrosine Kinase Inhibitor; VEGF, vascular endothelial growth factor; KPS, Karnofsky Performance Status; ACS, acute coronary syndrome. ***p<0.001, **p<0.01, *p<0.05.

Model 1: Adjusted by age and gender

Independent Prognostic Factors for OS of Patients With SCLC

After multivariate adjustment, the miR-219-5p high level (HR 0.39, 95% CI 0.26-0.59, $P < 0.001$) was also associated with a low increase in the risk of death (figure 3). Meanwhile, gender, PNI score, immunotherapy and heart failure were also the independent risk factors for OS.

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Development and Validation of an OS-predicting Nomogram

The independently related risk factors derived from the multivariate analysis were used to create an OS estimation nomogram (figure 4). The prognostic model was internally validated according to the bootstrap validation method. With an unadjusted C index of 0.691 and a bootstrap-corrected C index of 0.691, the nomogram displayed excellent accuracy in estimating the risk of OS. In the validation cohort, the nomogram showed a C index of 0.691 for the estimation of OS. A suitable calibration curve for risk estimation was also displayed (R^2=0.455, LR chi2=80.55) (figure 4B). We collected 86 NSCLC patients from Sichuan Cancer Hospital in the external validation step. The ROC curve showed an AUC of 0.783 (0.743-0.822) for predicting 5-year overall survival, compared with an AUC of 0.749 (0.709-0.788) for the external validation data (figure 5). We calculated the total score using Nomogram for patients in the training and validation sets, respectively, and divided them into four groups according to 40-60,61-80,81-100,101-120, and performed Kaplan-Meier analysis and plotted survival curves, which were found to have good separation and were statistically significant (Figure S1a, S1b).

Discussion

In this study, we detected the expression of miR-219-5p in a large cohort of SCLC patients at a single institution, between Mar 2010 and June 2015. The results suggested that reduced expression of miR-219-5p was significantly correlated with unfavorable clinical features. Moreover, patients in high miR-219-5p expression group displayed better OS compared with those in low miR-219-5p expression group. The multivariate analysis demonstrated miR-219-5p an independent prognostic factor for OS. In addition, to propose, and retrospectively verify in an independent cohort of patients, these independent risk factors were applied to establish a nomogram for OS estimation. The nomogram revealed good accuracy in estimating the risk of OS.

Carcinogenesis involves multiple biological processes which are related to many key genes[15, 16]. The characteristics of cancer occurrence represent properties that a

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cell acquire a certain ability to become and maintain itself as a cancer cell[17]. The key genes guide the cellular signaling pathways related to occurrence and progression of cancers[18, 19]. Using miRNA expression to predict the clinical diagnosis and prognosis of cancer has more advantages than mRNAs, as miRNAs are proved to be the vital posttranscriptional regulators of gene expression[20, 21]. In comparison with mRNAs, these vital gene regulators are highly conserved among species[22].

It has been reported that miRNAs were related to the initiation and progression of various cancers, and many miRNAs have been identified as a promising biomarker for prognostic prediction of cancer[10, 23]. Recently, some miRNAs have been proved to be a novel prognostic biomarker for SCLC[24, 25]. A study of Yu et al. indicated that miR-92a-2 was significantly higher in SCLC patients group compared to healthy control, and detection of miR-92a-2 levels could be a potential biomarker for patients with SCLC[26]. As a promising biomarker, miR-219-5p has been identified as a prognostic factor for different cancers. Long et al. found that miR-219-5p expression level was distinctly decreased in melanoma tissues and cell lines, and the modulation of miR-219-5p expression could be a prognostic biomarker and treatment strategy in melanoma[27]. A study from Huang et al. suggested a role of miR-219-5p for prognostic prediction and therapeutic strategy in colorectal cancer[28]. However, there is no studies exploring the role of miR-219-5p for biomarker in patients with SCLC. To the best of our knowledge, this study was the first attempt ever made to comprehensively evaluate the role for prognostic prediction based on miR-219-5p expression in patients with SCLC. In the current study, we initially examined the expression levels of miR-219-5p in SCLC patients. We, for the first time, demonstrated a correlation of the altered miR-219-5p expression with

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available clinical parameters. We found that miR-219-5p was significantly associated with lymphocytes count, PNI score and stage of SCLC. The univariate analysis indicated that increased miR-219-5p expression was a protective predictor for mortality. Kaplan-Meier curve displayed that patients with elevated miR-219-5p expression levels or accepted immunotherapy had low cumulative incidence of death compared to those with reduced miR-219-5p expression or unaccepted immunotherapy, respectively. In addition, gender, age, serum CRP level, albumin level, lymphocytes count, PNI score, immunotherapy, heart failure, KPS score and miR-219-5p level were associated with overall mortality. The multivariate analysis showed that miR-219-5p, gender, PNI score, immunotherapy and heart failure could predict OS as the independent risk factors.

Nomograms are applied for visualization of statistical models, graphical evaluation of variable significance and examination of predicted values[29, 30]. They have been widely performed to predict cancer risks and therapeutic outcomes[31, 32]. Most recently, several studies have successfully established a prognostic nomogram that combined a miRNA with clinical-related variables for OS estimation in different cancers[33-35]. Although nomogram is becoming increasingly popular, no studies have built prognostic models using combination of miR-219-5p and clinical risk factors in SCLC patients. In this study, based on the combination of miR-219-5p and independent clinicopathological variables, we created a nomogram model that could provide an individual prognostic prediction for OS estimation in SCLC patients. The results indicated excellent accuracy in estimating the risk of OS. There was a suitable calibration curve for risk estimation, indicating a well-performed nomogram, and good agreements between observation and prediction. To further verify the accuracy and efficiency of the model, an external date containing 86 patients from Sichuan Cancer Hospital was conducted. The results indicated that the prognositc model could

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accurately predict the prognosis of SCLC patients. Hence, this was the first prognostic nomogram for patients with SCLC that considered clinical parameters in addition to miR-219-5p. This nomogram could provide comprehensive information for patients, as well as a better guidance for clinical therapy. Based on the model, the potential high-risk patients with low survival rate could be more accurately selected for a specific therapeutic strategy.

There are some limitations in this article. Firstly, experimental research explaining the biological processes of miR-219-5p is needed. Thus, the molecular mechanism of miR-219-5p should be investigated in further research. Secondly, the prognostic nomogram needs to be further calculated by a prospective and large-scale multicenter study before it can be applied to clinical practice.

CONCLUSIONS

In conclusion, we found that the miR-219-5p expression levels were significantly correlated with clinical parameters of SCLC patients. Furthermore, miR-219-5p was proved to be an independent factor for prognostic prediction in patients with SCLC. Moreover, nomogram based on multivariate analysis showed excellent accuracy in estimating the risk of OS.

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Contributors ZHX and XMW designed the study. ZJC, XHZ, JGZ and MQX collected and analysed the data. ZJC, JGZ and XHZ drafted the initial manuscript. ZHX and XMW reviewed and edited the article. All authors read and approved the final manuscript.

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Competing interests The authors declare that they have no competing interests.

Patient consent for publication Not required.

Ethics approval

The study was approved by ethics committee of Suzhou Xiangcheng People's Hospital. The reference number was 20140193. All procedures performed in the present study were in accordance with the principles outlined in the 1964 Helsinki Declaration and its later amendments.

Provenance and peer review Not commissioned; externally peer reviewed.

Data Availability Statement The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Figure Legends

Figure 1 A flow chart of the screening process.

Figure 2 Overall survival (OS) of SCLC patients in different levels of miR-219-5p and different treatments. (A) OS of SCLC patients with high or low level of

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miR-219-5p. (B) OS of SCLC patients with different treatments (immunotherapy vs non-immunotherapy).

Figure 3 Multivariate cox regression analysis of 5-year overall survival on data in the SCLC patients.

Figure 4 Nomogram for overall survival (OS) risk estimation of SCLC patients and its predictive performance. (A) Nomogram to estimate the OS risk of SCLC patients in different variations. (B) Validity of the predictive performance of the nomogram in estimating the OS risk of SCLC patients.

Figure 5 External validation of the prognostic model.

Table 1 Study participant characteristics at enrollment.

Table 2 Univariate cox regression analysis of overall survival on SCLC patients.

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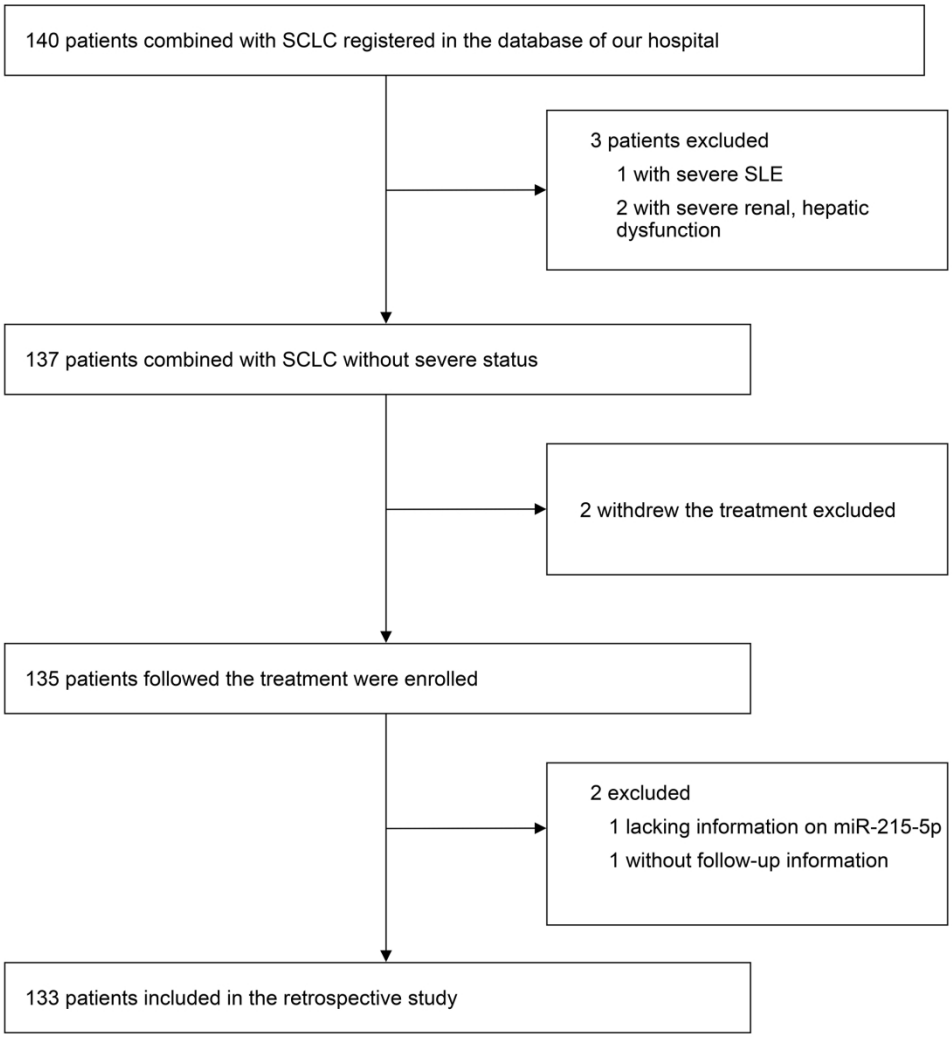

Figure 1 A flow chart of the screening process.

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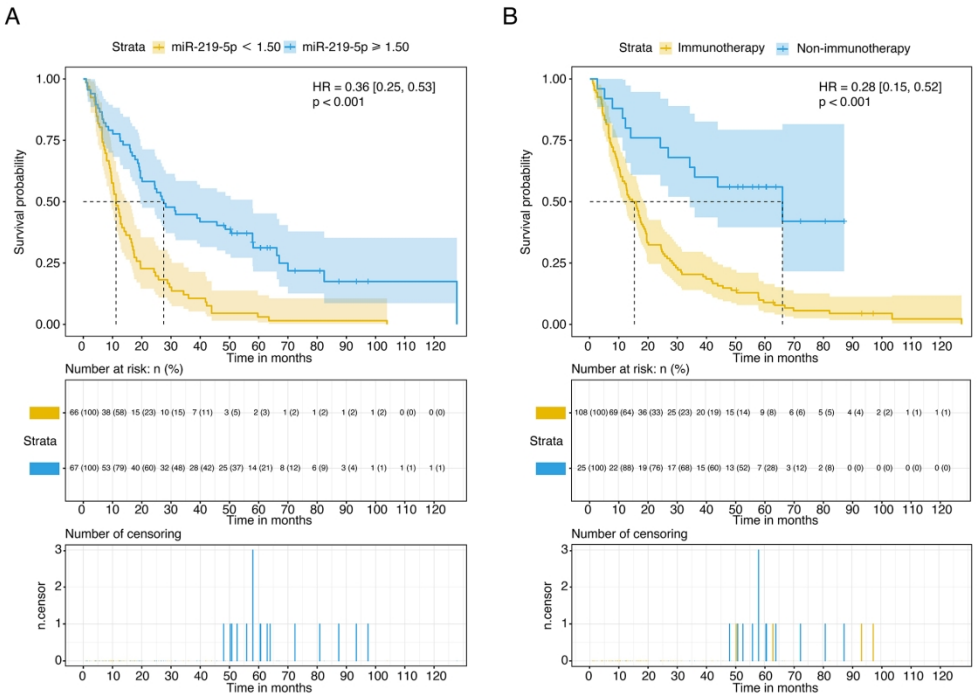

Figure 2 Overall survival (OS) of SCLC patients in different levels of miR-219-5p and different treatments. (A) OS of SCLC patients with high or low level of miR-219-5p. (B) OS of SCLC patients with different treatments (immunotherapy vs non-immunotherapy).

180x126mm (300 x 300 DPI)

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Figure 3

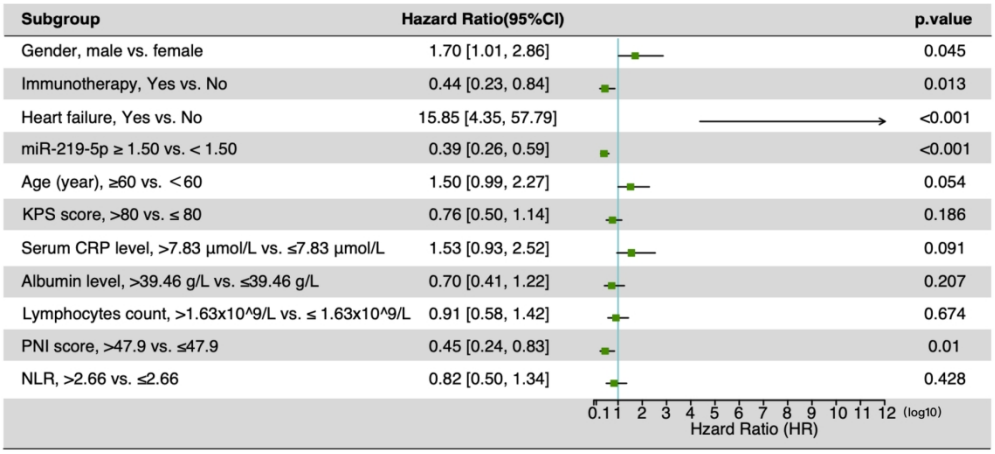

Figure 3 Multivariate cox regression analysis of 5-year overall survival on data in the SCLC patients.

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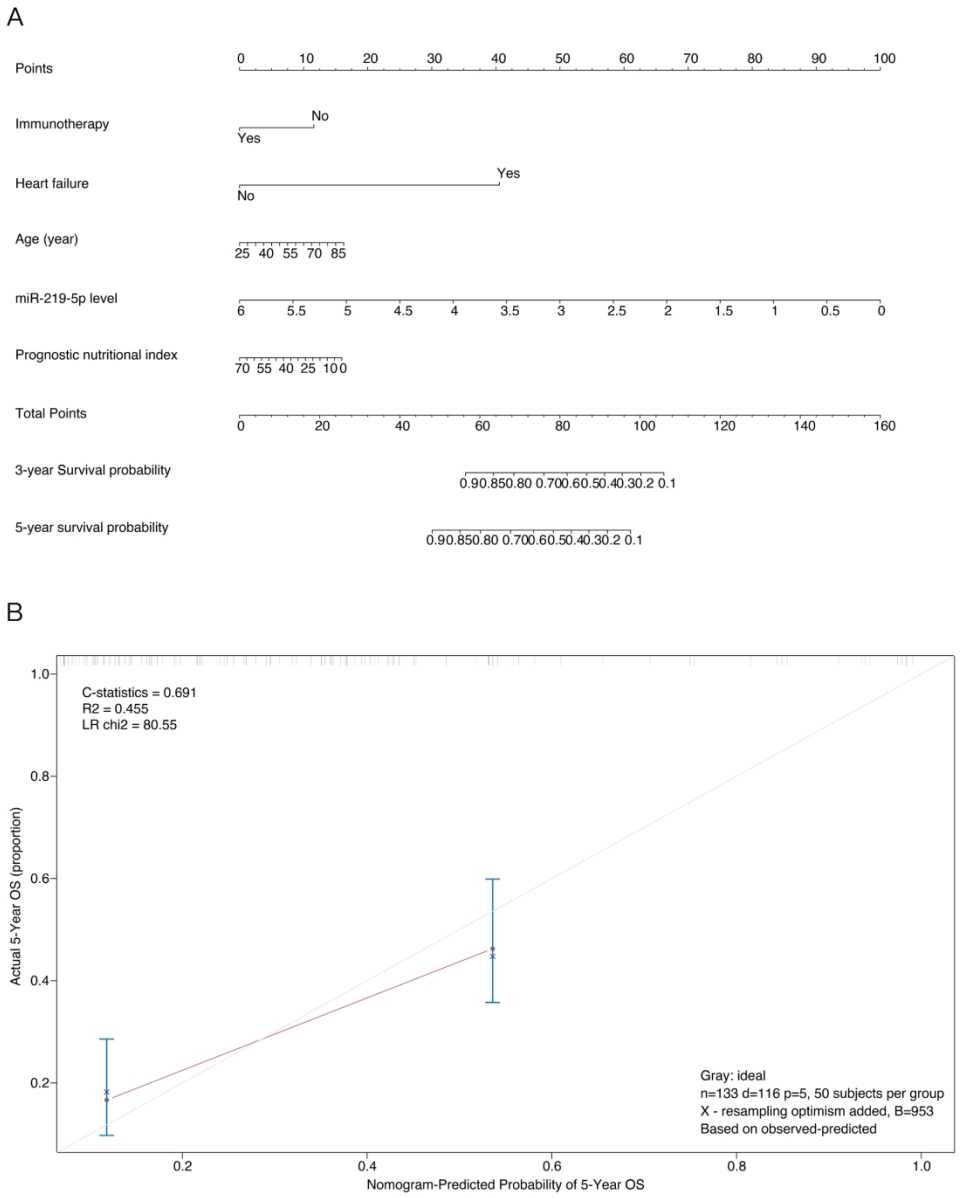

Figure 4 Nomogram for overall survival (OS) risk estimation of SCLC patients and its predictive performance. (A) Nomogram to estimate the OS risk of SCLC patients in different variations. To build the nomogram, find the position of each variable on the corresponding axis, draw a line to the points axis for the number of points, add the points from all of the variables, and draw a line from the total points axis to determine the OS probabilities at the lower line of the nomogram. (B) Validity of the predictive performance of the nomogram in estimating the OS risk of SCLC patients.

180x216mm (300 x 300 DPI)

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Figure 5

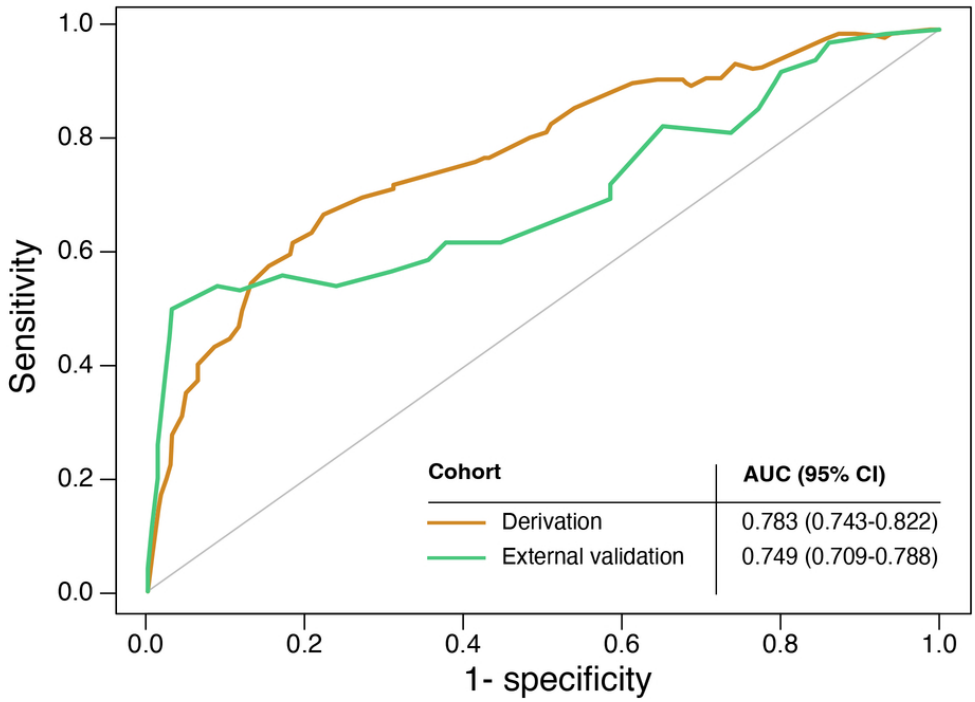

Figure 5 External validation of the prognostic model.

80x63mm (300 x 300 DPI)

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Supplement Materials

Part I. Details of variants

Patients' age: The age of patients was based on the time of diagnosis of NSCLC.

Body mass index: The equation of body mass index (BMI) is calculated as follow: BMI (kg/m^2) = weight (kg) / height $(m)^2$. The data of BMI was recorded before clinical treatment.

Serum CEA level: The serum level of carcinoembryonic antigen (CEA) is one of tumor marks， the higher serum *CEA* level than that of healthy individuals led to its clinical application as a diagnostic biomarker for cancer.

Serum CRP level: C-reactive protein (CRP) is detected by olorimetric determination through fasting blood collection and CRP is one of the inflamation markers.

Albumin level: Serum albumin is detected by colorimetric determination through fasting blood collection. And serum albumin is one of the indicators of nutritional status.

Neutrophils count, lymphocytes count:, hemoglobin level, platelet count: All these indexes were extracted from blood routine examination to evaluate preoperative status of patients.

PNI score: Prognostic nutritional index (PNI) is an index to evaluate the nutritional status of surgical patients, to predict the risk of surgery and to help make prognostic judgment. The equation is listed as below: PNI = serum albumin (g/L) + 5 * lymphocytes ($*10^9/L$).

NLR: Neutrophil to Lymphocyte Ratio (NLR) is an index of inflammation markers to predict the prognosis of various cancer, including lung cancer.

Pathologic type: Non-small cell lung cancer can be classified as adenocarcinoma, squamous cell carcinoma, large cell lung cancer, or others, depending on surgical or tumor biopsy specimens

Stage of NSCLC: Non-small cell lung cancer was staged according to the seventh edition of international union against cancer (UICC) TNM was published in 2009

Surgery: For early and locally advanced NSCLC, which is generally acceptable, there is a history of surgical resection.

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Therapy of radiation: Radiation therapy is a medical procedure that uses the delivery of high-energy radiation to kill cancer cells and shrink tumors, including external beam radiation therapy, internal radiation therapy and stereotactic body radiotherapy (SBRT). It aims to cure small tumors, treat lung cancer both locally and nearby lymph nodes, treat metastases, relieve symptoms, and also at prevention.

Application of platinum: Platinum-based chemotherapy may be used as a first or second line therapy for advanced lung cancer, after surgery (adjuvant chemotherapy), or before surgery to reduce the size of a tumor (neoadjuvant therapy). The most commonly used platinum drugs are cisplatin, carboplatin and nedaplatin. Treatment of non-small cell lung cancer begins with either cisplatin or carboplatin combined with another medication, such as docetaxel, pemetrexed, paclitaxel, gemcitabine and gemcitabine.

Target therapy: Targeted therapies for lung cancer are drugs that are tailored to attack certain mutations of cancer cells. These mutations lead to the production of abnormal proteins which guide the growth and development of a cancer cell. Common driver mutations include EGFR mutations, ALK rearrangements, ROS1 rearrangements, MET amplifications, KRAS mutations, HER2 mutations, BRAF mutations and so on.

Application of TKI: Tyrosine kinase inhibitors (TKI) act as competitive inhibitors of ATP binding to tyrosine kinases, or as tyrosine analogs that block tyrosine kinase activity and inhibit cell proliferation. Epithelial growth factor receptor (EGFR) is a protein that is present on the surface of both normal cells and lung cancer cells. Mutations in EGFR can occur at different locations on exon 18 to 21. ALK-positive lung cancer refers to people who have lung cancer that tests positive for the EML4-ALK fusion mutation. Several FDA-approved medications available to treat EGFR-positive and ALK-positive lung adenocarcinoma, including:

- EFGR inhibitors - Tarceva (erlotinib), Gilotrif (afatinib), Iressa (gefitinib), Tagrisso (osimertinib), and Portrazza (necitumumab)
- ALK inhibitors - Xalkori (crizotinib), Zykadia (ceritinib), and Alecensa (alectinib)
- ROS1 inhibitor - Xalkori (crizotinib)

Application of VEGF inhibitor: VEGF (Vascular endothelial growth factor) can activate angiogenesis through different signaling pathways. VEGF inhibitors are drugs that block the ability of tumors to form new blood vessels, and hence, grow and spread. Some currently available medications include bevacizumab, ramucirumab, axitinib, endostar and anlotinib.

KPS score: A performance measure for rating the ability of a person to perform usual activities, evaluating a patient's progress after a therapeutic procedure, and determining a patient's suitability for therapy. It is used most commonly in the

prognosis of cancer therapy, usually after chemotherapy and customarily administered before and after therapy. It was named for Dr. David A. Karnofsky, an American specialist in cancer chemotherapy. Patients with more than 80 scores had better postoperative status and longer survival time. And patients with more than 70 scores can suffer from chemoradiotherapy.

Smoking: Smoker refers to continuous or cumulative smoking > 1 cigarette/day over a lifetime of more than 6 months. (1997, WHO)

Hypertension: Hypertension is defined as a repeatedly elevated blood pressure exceeding 140 over 90 mmHg.

Diabetes: Diabetes is a group of metabolic diseases characterized by hyperglycemia. And fasting blood glucose more than 7.0 mmol/l or blood glucose more than 11.1 mmol/l within 2hoursafter meal can be diagnosed diabetes.

Hyperlipemia: Hyperlipemia means the presence of excess fat or lipids in the blood. And total cholesterol ≥ 6.2 mmol/L, low density lipoprotein cholesterin ≥ 4.1 mmol/L, triglyceride ≥ 2.3 mmol/L, high density lipoprotein cholesterin < 1.0 mmol/L can be diagnosed hyperlipemia.

Heart failure: The information was recorded through history taking and verified after hospitalization. Heart failure means inability of the heart to keep up with the demands on it and, specifically, failure of the heart to pump blood with normal efficiency. Heart failure may be due to failure of the right or left or both ventricles. The signs and symptoms depend upon which side of the heart is failing. They can include shortness of breath (dyspnea), asthma due to the heart (cardiac asthma), pooling of blood (stasis) in the general body (systemic) circulation or in the liver's (portal) circulation, swelling (edema), blueness or duskiness (cyanosis), and enlargement (hypertrophy) of the heart.

ACS: Acute coronary syndrome is a term for a group of conditions that suddenly stop or severely reduce blood from flowing to the heart. When blood cannot flow to the heart, the heart muscle can become damaged. Heart attack and unstable angina are both acute coronary syndromes (ACS).

Withdraw treatment: Reasons for patients withdrew from treatment were listed below: 1) High cost of the treatment: In China, the cost of hormone therapy can be up to 3000 RMB a month if without health insurance. 2) Poor patient compliance: Some patients discontinue treatment because they do not comply with the treatment plan prescribed by their doctor.

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Part II. Report of sample size assessment

PASS 15.0.5

Tests for Two Survival Curves Using Cox's Proportional Hazards Model

Numeric Results with Ha: HR ≠ 1

Power	Total Sample Size N	Control Sample Size N1	Trtmnt Sample Size N2	Prop'n Control N1/N P1	Hazard Ratio h2/h1 HR	Control Prob Event Pev1	Trtmnt Prob Event Pev2	Control Events E1	Trtmnt Events E2	Alpha	Beta
0.9005	103	51	52	0.500	0.500	0.750	0.950	38.3	49.4	0.050	0.100

References

Chow, S.C., Shao, J., Wang, H. 2008. Sample Size Calculations in Clinical Research, 2nd Edition. Chapman & Hall/CRC.

Schoenfeld, David A. 1983. 'Sample Size Formula for the Proportional-Hazards Regression Model', Biometrics, Volume 39, Pages 499-503.

Report Definitions

Power is the probability of rejecting a false null hypothesis. Power should be close to one.
N is the total sample size.
N1 and N2 are the sample sizes of the control and treatment groups.
P1 is the proportion of the total sample that is in the control group, group 1.
HR is the hazard ratio: h2/h1.
Pev1 and Pev2 are the probabilities of an event in the control and the treatment groups.
E1 and E2 are the number of events required in the control and the treatment groups.
Alpha is the probability of a type one error: rejecting a true null hypothesis.
Beta is the probability of a type two error: failing to reject a false null hypothesis.

Summary Statements

A two-sided test of whether the hazard ratio is one with an overall sample size of 103 subjects (of which 51 are in the control group and 52 are in the treatment group) achieves 90% power at a 0.050 significance level when the hazard ratio is actually 0.500. The number of events required to achieve this power is 87.7. It is anticipated that proportions of subjects having the event during the study is 0.750 for the control group and 0.950 for the treatment group. These results assume that the hazard ratio is constant throughout the study and that Cox proportional hazards regression is used to analyze the data.

Procedure Input Settings

Autosaved Template File

\\Mac\Home\Documents\PASS 15\Procedure Templates\Autosave\Tests for Two Survival Curves Using Cox's

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Proportional Hazards Model - Autosaved 2020_1_24-9_57_39.t92

Design Tab

Solve For:	Sample Size
Alternative Hypothesis:	Ha: HR ≠ 1
Power:	0.90
Alpha:	0.05
Group Allocation:	Equal (N1 = N2)
Pev1 (Probability of a Control Event):	0.750
Pev2 (Probability of a Treatment Event):	0.950
HR (Actual Hazard Ratio = h2/h1):	0.5

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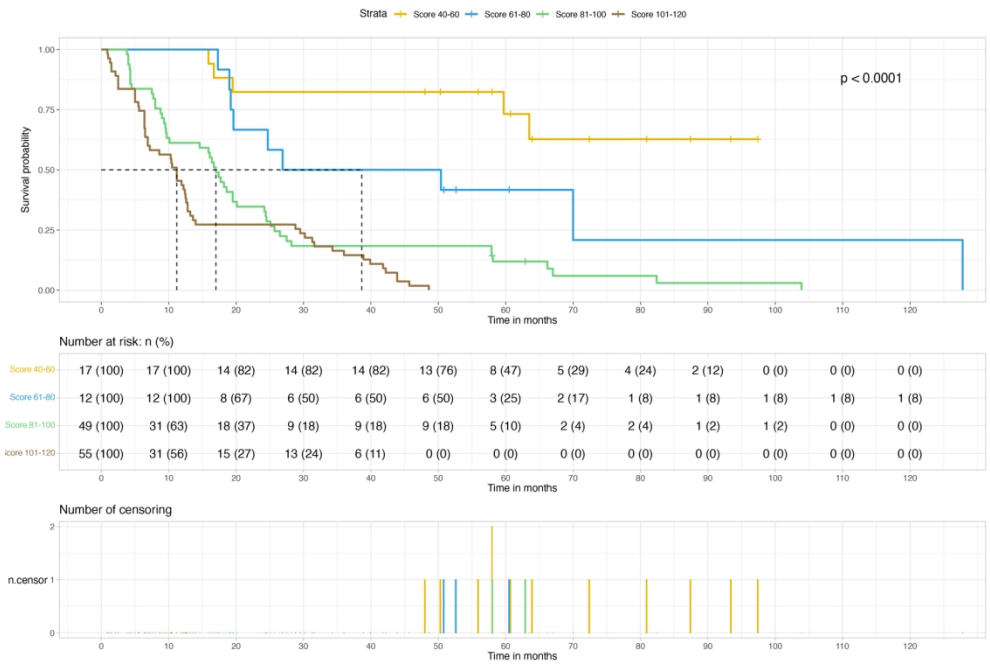
Figure S1a



160x109mm (300 x 300 DPI)

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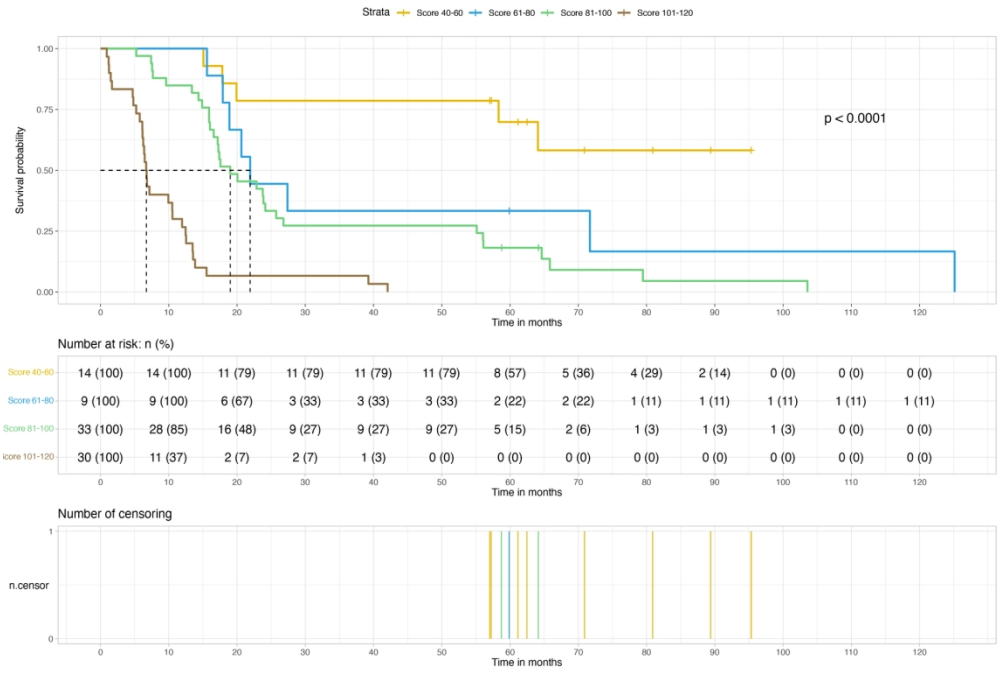
Figure S1b



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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			**5**
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*6*
Bias	9	Describe any efforts to address potential sources of bias	7
Study size	10	Explain how the study size was arrived at	7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7
		(*b*) Describe any methods used to examine subgroups and interactions	7
		(*c*) Explain how missing data were addressed	7
		(*d*) If applicable, explain how loss to follow-up was addressed	7
		(*e*) Describe any sensitivity analyses	7
Results			

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8
		(b) Give reasons for non-participation at each stage	8
		(c) Consider use of a flow diagram	8
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9
		(b) Indicate number of participants with missing data for each variable of interest	9
		(c) Summarise follow-up time (eg, average and total amount)	9
Outcome data	15*	Report numbers of outcome events or summary measures over time	10
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10
		(*b*) Report category boundaries when continuous variables were categorized	11
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	11
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	12
Discussion			
Key results	18	Summarise key results with reference to study objectives	13-14
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15
Generalisability	21	Discuss the generalisability (external validity) of the study results	15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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BMJ Open

Prognostic value of MiR-219-5p in relation to cancer-related mortality in patients with small cell lung cancer: a retrospective observational cohort study in China

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Primary Subject Heading:	Oncology
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Keywords:	Risk management < HEALTH SERVICES ADMINISTRATION & MANAGEMENT, ONCOLOGY, Gene therapy < ONCOLOGY, Molecular aspects < ONCOLOGY, Oncogenes < ONCOLOGY

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Prognostic value of MiR-219-5p in relation to cancer-related mortality in patients with small cell lung cancer: a retrospective observational cohort study in China

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Running title: MiR-219-5p decrease the risk of SCLC patients

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ABSTRACT

Objectives Small cell lung cancer (SCLC) is a lethal human malignancy, and previous studies support the contribution of microRNA (miRNA) to cancer progression. The prognostic value of miR-219-5p in SCLC patients remains unclear. This study evaluated the role of MiR-219-5p for SCLC and created a prediction model for them.

Design Retrospective observational cohort study.

Setting and participants The program has yielded a database of all patients with SCLC in 2 defined geographical regions of China. We did a real-world study, including data from 133 patients with SCLC between Mar 1, 2010, and June 1, 2015 in the Suzhou Xiangcheng People's Hospital. External data from 86 SCLC patients from Sichuan Cancer Hospital and the First affiliated hospital of Soochow University was conducted.

Primary and secondary outcome measures MiR-219-5p was recorded during the admission. Cox proportional hazard model was applied for survival analyses and for analyzing risk factors for cancer-related mortality and to create a nomogram for prediction. The accuracy of the model was evaluated by C-index and calibration curve.

Results In our data, the mortality in the group with a high miR-219-5p level (≥ 1.50) (n=67) was 74.6%, while the mortality in the low group (n=66) was 100.0%. Based on univariate analysis, we put factors (P <0.05) into a multivariate regression model, patients with high miR-219-5p level (HR=0.39, 95%CI=[0.26-0.59], P <0.001), immunotherapy (HR=0.44, 95%CI=[0.23-0.84], P <0.001), PNI score >47.9 (HR=0.45, 95%CI=[0.24-0.83], P =0.01) remained statistically factors for better overall survival (OS). Nomogram revealed good accuracy in estimating the risk, with a bootstrap-corrected C index of 0.691. External validation displayed an AUC of 0.749 (0.709-0.788).

Conclusions MiR-219-5p was associated with a reduced risk of cancer-related mortality. Nomogram demonstrated good accuracy in estimating the risk of overall mortality. Prospective validation of the prognostic nomogram is needed in the future.

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Keywords: small cell lung cancer, miR-219-5p, overall survival, nomogram, prediction model

Strengths and limitations of this study

- ► The study established the first risk nomogram for predicting the 3-year and 5-year specific mortality probability for SCLC
- ► Based on the retrospective sample, the nomogram can improve the ability of clinicians in predicting survival probabilities in individual patients.
- ► The model is not comprehensive enough because the epidemiology and end results database does not include all prognostic factors for SCLC.
- ► The available data on treatment status are not detailed enough to distinguish the impact of various treatment plans.
- ► The model needs to be prospectively studied to confirm its reliability

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INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide, with millions of new cases diagnosed each year[1]. Small cell lung cancer (SCLC) is a kind of neuroendocrine malignant tumor with poor prognosis, accounting for about 15% of lung cancer patients[2]. SCLC is generally divided into limited disease (LD-SCLC) and extensive disease (ED-SCLC). Platinum combined with etoposide is the first-line therapeutic strategy of SCLC, and most patients are easy to receive initial chemotherapy[3]. However, the 5-year survival rates of LD-SCLC and ED-SCLC are only 15% and 3%, respectively[4]. Therefore, improvement in early diagnosis and prognostic prediction of SCLC is vital.

MicroRNAs (miRNAs) are endogenous non-coding RNAs (~ 22 nt), which regulate mRNA activity by hybridization with 3 '- untranslated region (UTR) of specific genes[5]. Many studies have shown that miRNAs could participate in a variety of cell biological processes, including cell growth, differentiation and apoptosis[6, 7]. In addition, researches have demonstrated that miRNAs are frequently dysregulated in cancers[8, 9], and some miRNAs can serve as diagnostic and prognostic biomarkers for caners[10]. Recently, several miRNAs have been proved to participant in the occurrence and development of SCLC, but few of them are likely to be a biomarker or therapeutic target for SCLC.

Recently, miR-219-5p has been found to be abnormally expressed and play a significant role in different cancers. Ma et al. found that the expression of miR-219-5p was significantly decreased in esophageal squamous cell carcinoma (ESCC) tissues compared with normal tissues[11]. A study of Gong et al. revealed a tumor suppressive role for miR-219-5p by targeting glypican-3 in hepatocellular carcinoma (HCC)[12]. On the contrary, Yang et al. indicated that miR-219-5p could promote cell growth and metastasis of HCC and serve as a prognostic marker for HCC patients[13]. A research investigated by Wei et al. suggested that miR-219-5p could inhibit proliferation, migration and invasion of epithelial ovarian cancer through downregulation of the Wnt signaling pathway, and it could serve as a diagnostic

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biomarker and therapeutic target for epithelial ovarian cancer[14]. However, the biological functions of miR-219-5p and its potential prognostic role for biomarker in SCLC are still unknown.

In this study, we aimed to examine the variation in the expression levels of miR-219-5p in patients with SCLC and explored the potential prognostic role of miR-219-5p for SCLC. We also displayed a nomogram that could provide individualized, evidence-based, highly accurate risk estimates. Nomograms were easy to performed and could facilitate management-related decision making.

METHODS

Study Design and Patient Characteristics

We did a real-world study, including data obtained from 133 patients with SCLC between Mar 2010 and June 2015, in the Suzhou Xiangcheng People's Hospital. Those participants who lacked information on complement components data, withdrew from treatment or lacked follow-up information were excluded. Clinical information of patients, including gender, age, BMI, neutrophils count, lymphocytes count, serum CEA level, CRP level, albumin level, hemoglobin level, stage of SCLC, platelet count, PNI score, KPS score, NLR, pathologic type, immunotherapy, therapy of radiation, application of platinum, application of VEGF inhibitor, target therapy, application of TKI, smoking, ACS, diabetes, heart failure and hyperlipemia, were recorded. Diagnosis of SCLC was confirmed by histopathological examination. The median length of follow-up was 23.6 months. Median was used as the cut-off value. The definition and details of all the variables above were provided in Supplemental Materials Part I. Data from 86 patients with NSCLC at Sichuan Cancer Hospital and

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the First affiliated hospital of Soochow University were applied for external validation. Inform, and consent was obtained from all patients or their immediate family members. All protocols were in line with the guidelines with the ethic committee of Suzhou Xiangcheng People's Hospital, Sichuan Cancer Hospital, the First affiliated hospital of Soochow University and following the Declaration of Helsinki.

Assays for Detection of MiR-219-5p Levels

The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was conducted for the detection of miR-219-5p expression levels.

Total RNA from tissues was isolated and extracted using miRcute Extraction and Separation of miRNAs kit (Tiangen Biotech Co., Ltd., Beijing, China), and then reversely transcribed into cDNA by PrimeScript™ Ⅱ 1st strand cDNA synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co., Ltd.) was used for qRT-PCR. The thermocycling conditions were as follows: one cycle at 95˚C for 3 min (initial denaturation), 40 cycles at 95˚C for 15 s and 60˚C for 30 s. U6 small nuclear RNA (U6) served as the respective internal control. The relative expression of miR-219-5p was quantified by the $2^{-\Delta\Delta Ct}$ methods, and normalized to the U6. The following primers were used: miR-219-5p forward, 5'-ACACTCCAGCTGGGTGATTGTCCAAACGCAAT-3' and reverse, 5'-CTCAACTGGTGTCGTGGA-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The experiments were repeated at least 3 times.

Statistical Analysis

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Sample size assessment was performed using NCSS-PASS software version 11.0 (https://www.ncss.com/software/pass/). Power was set as 0.99, and alpha was 0.5. The mortalities of both miR-219-5p high-level group and miR-219-5p low-level group in our previous data (2008-2009) (0.750 and 0.950) were entered into the PASS. The Actual Hazard Ratio was set as 0.50. Then the sample size was calculated using PASS, and the minimum sample size was 103 (control = 51, experiment = 43). Our sample size was 133 (66 and 67 for each group), which was suitable. The report of sample size assessment was displayed in Supplemental Material Part II. The missing data (<5.0%) were estimated by random forest algorithm using the mice package in RStudio (R version 3.6.1). Categorical variates were presented as percentages and compared via the κ^2 test. Continuous variates with skewed and normal distributions were presented as median with interquartile ranges and mean ± standard deviation. The Mann-Whitney U test and the unpaired t-test were applied for comparison between Groups. Cumulative mortality was showed by the Kaplan-Meier curve and analyzed by the log-rank test. Univariate and multivariate survival analyses for OS were conducted using the Cox regression model. The forest plots were used to visualize the significance of covariates to the prognosis. The restricted cubic spline analyses were performed with Harrell's Regression Modelling Strategies (rms) package.

We screened multifactor analysis for statistically significant indicators for inclusion in the prediction model. To build the nomogram, find the position of each variable on the corresponding axis, draw a line to the points axis for the number of points, add the points from all the variables, and draw a line from the total points axis

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to determine the OS probabilities at the lower line of the nomogram. The contribution of each covariate was quantified and visualized in a prognostic nomogram with internal validation via 1000-times bootstrapping. The consistency of the resulting model was assessed by the calibration assay. Decision curve analyses were performed to evaluate net clinical benefits of the model compared with conventional prognostic scores. The scatter plots were applied for visualization of the consistency of each model. A 1000-time bootstrapping was employed as indicated. The association between miR-219-5p class and survival endpoints was evaluated by Kaplan-Meier curves and log-rank test. Statistical analysis was performed using the RStudio (R version 3.6.1) with the following packages: 'ggplot2', 'rms', 'PredictABLE', 'risk regression', and 'survminer'.

Patient and public involvement

This study was conducted without patient involvement. Patients were not invited to comment on the study design and were not consulted to develop patient-relevant outcomes or interpret the results. Moreover, patients were not allowed to contribute to the writing or editing of this document for readability or accuracy.

RESULTS

Baseline Characteristics

A total of 133 SCLC patients who were diagnosed between Mar 2010 and June 2015 were included in this study. A flow chart of the screening process was shown in figure 1. The median age of these patients was 64 years old (58-70), and it contained 106 (80.0%) males. Median serum CEA and CRP level was 3.43 ng/ml and 7.83 μmol/L, respectively. For the stage of SCLC, 51 (38.0%) patients were diagnosed with limited disease, 82 (61.0%) patients were extensive disease. 25 (19.0%) patients accepted the immunotherapy therapy. 54 (41.0%) patients got the therapy of radiation. In addition,

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platinum was applied for 131 (98.0%) patients, and TKI was used for 15 (12.0%) patients. KPS score of these patients was examined, and the results revealed that 107 (81.0%) patients got 80 or higher points. The distribution of basic diseases was also assessed in our data. Diabetes was found in 17 (13.0%) patients, while hyperlipemia was 13 (10.0%). Cardiovascular diseases such as heart failure and ACS were found in 3 (2.0%) and 4 (3.0%) patients, respectively. 13 (10.0%) patients suffered from hypertension. In addition, 82 (62.0%) patients had a habit of smoking among all the patients. The baseline characteristics of these patients were listed in table 1.

Among all the 133 patients, the overall mortality was 87.2%. The mortality in high miR-219-5p level group (n=67) was 74.6%, , while the mortality in the low group (n=66) was 100.0%. Moreover, in the high miR-219-5p level group, patients with extensive disease were 35 (52.0%), while the low group was 47 (71.0%) (table 1).

Table 1. Study Participant Characteristics at Enrollment

Variation	Total (n=133)	Cohort, median (IQR)		
		mir-219-5p<1.50, (n=66)	mir-219-5p≥1.50, (n=67)	p.value
Age, (year)	64(58,70)	65(59,70)	63(56.5,68)	0.276
BMI, (kg/m2)	23.12±3.09	22.99±3.22	23.26±2.98	0.619
Serum CEA level, (ng/ml)	3.43(1.96,9.26)	3.34(1.92,10.05)	3.43(1.99,8.11)	0.87
Serum CRP level, (μmol/L)	7.83(1.68,12.78)	10.68(2.78,13.15)	5.64(1.2,12.16)	0.107
Albumin level, (g/L)	39.46±5.2	38.86±4.68	40.05±5.65	0.188
Neutrophils count, (10^9/L)	4.55(3.59,5.88)	4.54(3.77,5.75)	4.55(3.48,5.92)	0.975
Lymphocytes count, (10^9/L)	1.63(1.31,1.95)	1.5(1.21,1.78)	1.73(1.38,2.08)	0.031*
Hemoglobin level, (g/L)	133(125,145)	134(124,145)	132(125,143.5)	0.564
Platelet count, (10^9/L)	233(184,288)	244.5(180,293.75)	226(184.5,273.5)	0.306
PNI score	47.9(43.95,51.85)	45.98(42.51,50.38)	48.7(44.92,54)	0.026*
NLR	2.66(1.99,4.19)	2.75(2.11,4.57)	2.59(1.92,3.7)	0.232
Gender, (n%)				0.211
Female	27(20)	10(15)	17(25)	
Male	106(80)	56(85)	50(75)	
Metastasis, n(%)				0.299
No	45(34)	19(29)	26(39)	
Yes	88(66)	47(71)	41(61)	
Stage of SCLC				0.038*

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Limited disease	51(38)	19(29)	32(48)	
Extensive disease	82(62)	47(71)	35(52)	
Immunotherapy, (n%)				0.197
No	108(81)	57(86)	51(76)	
Yes	25(19)	9(14)	16(24)	
Therapy of radiation, (n%)				0.417
No	79(59)	42(64)	37(55)	
Yes	54(41)	24(36)	30(45)	
Application of platinum, (n%)				0.244
No	2(2)	2(3)	0(0)	
Yes	131(98)	64(97)	67(100)	
Chemotherapy				0.45
AP	28(21)	12(18)	16(24)	
DP	15(11)	6(9)	9(13)	
EP	71(53)	35(53)	36(54)	
GP	3(2)	2(3)	1(1)	
Others	16(12)	11(17)	5(7)	
Target therapy, (n%)				0.627
No	116(87)	59(89)	57(85)	
Yes	17(13)	7(11)	10(15)	
Application of TKI, (n%)				0.449
No	118(89)	60(91)	58(87)	
TKI I	9(7)	4(6)	5(7)	
TKI II	1(1)	1(2)	0(0)	
TKI III	5(4)	1(2)	4(6)	
Application of VEGF inhibitor, n(%)				0.645
No	114(86)	58(88)	56(84)	
Yes	19(14)	8(12)	11(16)	
KPS score, n(%)				0.678
40	2(2)	0(0)	2(3)	
50	5(4)	3(5)	2(3)	
60	7(5)	3(5)	4(6)	
70	12(9)	8(12)	4(6)	
80	29(22)	14(21)	15(22)	
90	56(42)	29(44)	27(40)	
100	22(17)	9(14)	13(19)	
Smoking, n(%)				0.255
No	51(38)	29(44)	22(33)	
Yes	82(62)	37(56)	45(67)	
Hypertension, n(%)				1
No	80(60)	40(61)	40(60)	
Yes	53(40)	26(39)	27(40)	
Diabetes, n(%)				1
No	116(87)	58(88)	58(87)	

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Yes	17(13)	8(12)	9(13)	
Hyperlipemia, n(%)				0.579
No	120(90)	61(92)	59(88)	
Yes	13(10)	5(8)	8(12)	
Heart failure, n(%)				1
No	130(98)	65(98)	65(97)	
Yes	3(2)	1(2)	2(3)	
ACS, n(%)				0.619
No	129(97)	65(98)	64(96)	
Yes	4(3)	1(2)	3(4)	

Abbreviation: IQR, interquartile range; CRP, C-reactive protein; PNI, neutrophil lymphocyte ratio; NLR, neutrophil lymphocyte ratio; SCLC, small-cell lung cancer; TKI, Tyrosine Kinase Inhibitor; VEGF, vascular endothelial growth factor; KPS, Karnofsky Performance Status; ACS, acute coronary syndrome. ***p<0.001, **p<0.01, *p<0.05.

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MiR-219-5p Expression Level, and Clinical Risk Factors Predict the Development of SCLC Patients

According to the univariate analysis, high miR-219-5p levels (≥ 1.50) was a strong protective predictor of cancer-related mortality (HR 0.36, 95% CI 0.25-0.53, *P* <0.001) (table 2). Kaplan Meier curve showed that patients in the high miR-219-5p group had a decreased cumulative rate of death than those in the low miR-219-5p group (log-rank *P* <0.001) (figure 2A). Meanwhile, patients who accepted immunotherapy also showed a low morality compared to those patients without accepting immunotherapy in the survival curve (HR 0.28, 95% CI 0.15-0.52, *P* <0.001) (figure 2B).

In addition, gender, age, serum CRP level, albumin level, lymphocytes count, PNI score, immunotherapy, heart failure, and KPS score were also correlated with overall mortality (table 2). When adjusted by age and gender, patients in the miR-219-5p high-level group also displayed a low cumulative rate death compared to those in the low-level group.

Table 2. Cox Regression Analysis of Hazard Ratio on SCLC patients

Variation	Non-adjustment		Model 1	
	Hazard Ratio (95% CI)	p.value	Hazard Ratio (95% CI)	p.value
Gender, male vs. female	1.66 [1.02, 2.69]	0.041*	-	-

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Age (year), ≥60 vs. < 60	1.52 [1.03, 2.26]	0.036*	-	-
BMI, ≥23.12 kg/m2 vs. < 22.86kg/m2	0.99 [0.69, 1.43]	0.97	0.95 [0.66, 1.38]	0.806
Serum CEA level, >3.43 ng/ml vs. ≤3.43 ng/ml	1.01 [0.70, 1.46]	0.954	1.00 [0.69, 1.45]	0.999
Serum CRP level, >7.83 μmol/L vs. ≤7.83 μmol/L	1.91 [1.31, 2.78]	0.001**	1.92 [1.32, 2.79]	0.001**
Albumin level, >39.46 g/L vs. ≤39.46 g/L	1.66 [1.15, 2.40]	0.007**	1.60 [1.10, 2.31]	0.013*
Neutrophils count, >4.55x10^9/L vs. ≤ 4.55x10^9/L	1.18 [0.82, 1.71]	0.367	1.15 [0.79, 1.66]	0.464
Lymphocytes count, >1.63x10^9/L vs. ≤ 1.63x10^9/L	0.59 [0.40, 0.85]	0.005**	0.61 [0.42, 0.89]	0.01*
Hemoglobin level, >133 g/L vs. ≤ 133 g/L	0.80 [0.56, 1.16]	0.244	0.72 [0.49, 1.05]	0.089
Platelet count, >233x10^9/L vs. ≤ 233x10^9/L	1.23 [0.85, 1.78]	0.275	1.23 [0.85, 1.78]	0.275
PNI score, >47.9 vs. ≤47.9	0.54 [0.37, 0.78]	0.001**	0.53 [0.37, 0.77]	0.001**
NLR, >2.66 vs. ≤2.66	1.18 [0.82, 1.71]	0.367	1.20 [0.83, 1.74]	0.341
Metastasis, Yes vs. No	1.05 [0.70, 1.57]	0.81	1.10 [0.73, 1.64]	0.654
Stage of NSCLC, IV, III vs. II and I	0.94 [0.50, 1.75]	0.838	1.05 [0.56, 1.98]	0.868
Stage of SCLC, Yes vs. No	1.33 [0.90, 1.96]	0.154	1.43 [0.97, 2.12]	0.074
Immunotherapy, Yes vs. No	0.28 [0.15, 0.52]	<0.001***	0.30 [0.16, 0.55]	<0.001***
Therapy of radiation, Yes vs. No	0.88 [0.60, 1.27]	0.488	0.79 [0.54, 1.16]	0.235
Application of platinum, Yes vs. No	0.61 [0.15, 2.47]	0.483	0.66 [0.16, 2.70]	0.562
Target therapy, Yes vs. No	0.75 [0.42, 1.33]	0.323	0.81 [0.45, 1.45]	0.481
Application of TKI, Yes vs. No	0.77 [0.41, 1.44]	0.415	0.82 [0.44, 1.53]	0.534
Application of VEGF inhibitor, Yes vs. No	0.83 [0.47, 1.45]	0.518	0.90 [0.51, 1.59]	0.723
Chemotherapy, AP vs. others	0.61 [0.37, 1.00]	0.052	0.66 [0.40, 1.09]	0.104
Smoking, Yes vs. No	1.23 [0.84, 1.79]	0.292	0.90 [0.56, 1.43]	0.645
Hypertension, Yes vs. No	1.13 [0.78, 1.64]	0.531	1.10 [0.75, 1.60]	0.622
Diabetes, Yes vs. No	0.90 [0.51, 1.61]	0.726	0.97 [0.54, 1.75]	0.927
Hyperlipemia, Yes vs. No	0.79 [0.42, 1.48]	0.461	0.77 [0.40, 1.46]	0.421
Heart failure, Yes vs. No	5.61 [1.71, 18.42]	0.004**	6.43 [1.94, 21.26]	0.002**
ACS, Yes vs. No	0.74 [0.23, 2.35]	0.612	0.69 [0.21, 2.21]	0.53
KPS score, >80 vs. ≤ 80	0.52 [0.35, 0.75]	0.001**	0.51 [0.35, 0.74]	<0.001***
miR-219-5p ≥ 1.50 vs. < 1.50	0.36 [0.25, 0.53]	<0.001***	0.37 [0.25, 0.55]	<0.001***

Abbreviation: HR, hazard risk; BMI, Body Mass Index; CRP, C-reactive protein; PNI, neutrophil lymphocyte ratio; NLR, neutrophil lymphocyte ratio; SCLC, small-cell lung cancer; TKI, Tyrosine Kinase Inhibitor; VEGF, vascular endothelial growth factor; KPS, Karnofsky Performance Status; ACS, acute coronary syndrome. ***p<0.001, **p<0.01, *p<0.05.

Model 1: Adjusted by age and gender

Independent Prognostic Factors for OS of Patients With SCLC

After multivariate adjustment, the miR-219-5p high level (HR 0.39, 95% CI 0.26-0.59, $P < 0.001$) was also associated with a low increase in the risk of death (figure 3). Meanwhile, gender, PNI score, immunotherapy and heart failure were also the independent risk factors for OS.

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Development and Validation of an OS-predicting Nomogram

The independently related risk factors derived from the multivariate analysis were used to create an OS estimation nomogram (figure 4). The prognostic model was internally validated according to the bootstrap validation method. With an unadjusted C index of 0.691 and a bootstrap-corrected C index of 0.691, the nomogram displayed excellent accuracy in estimating the risk of OS. In the validation cohort, the nomogram showed a C index of 0.691 for the estimation of OS. A suitable calibration curve for risk estimation was also displayed (R^2=0.455, LR chi2=80.55) (figure 4B). We collected 86 NSCLC patients from Sichuan Cancer Hospital in the external validation step. The ROC curve showed an AUC of 0.783 (0.743-0.822) for predicting 5-year overall survival, compared with an AUC of 0.749 (0.709-0.788) for the external validation data (figure 5). We calculated the total score using Nomogram for patients in the training and validation sets, respectively, and divided them into four groups according to 40-60,61-80,81-100,101-120, and performed Kaplan-Meier analysis and plotted survival curves, which were found to have good separation and were statistically significant (Figure S1a, S1b).

Discussion

In this study, we detected the expression of miR-219-5p in a large cohort of SCLC patients at a single institution, between Mar 2010 and June 2015. The results suggested that reduced expression of miR-219-5p was significantly correlated with unfavorable clinical features. Moreover, patients in high miR-219-5p expression group displayed better OS compared with those in low miR-219-5p expression group. The multivariate analysis demonstrated miR-219-5p an independent prognostic factor for OS. In addition, to propose, and retrospectively verify in an independent cohort of patients, these independent risk factors were applied to establish a nomogram for OS estimation. The nomogram revealed good accuracy in estimating the risk of OS.

Carcinogenesis involves multiple biological processes which are related to many key genes[15, 16]. The characteristics of cancer occurrence represent properties that a

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cell acquire a certain ability to become and maintain itself as a cancer cell[17]. The key genes guide the cellular signaling pathways related to occurrence and progression of cancers[18, 19]. Using miRNA expression to predict the clinical diagnosis and prognosis of cancer has more advantages than mRNAs, as miRNAs are proved to be the vital posttranscriptional regulators of gene expression[20, 21]. In comparison with mRNAs, these vital gene regulators are highly conserved among species[22].

It has been reported that miRNAs were related to the initiation and progression of various cancers, and many miRNAs have been identified as a promising biomarker for prognostic prediction of cancer[10, 23]. Recently, some miRNAs have been proved to be a novel prognostic biomarker for SCLC[24, 25]. A study of Yu et al. indicated that miR-92a-2 was significantly higher in SCLC patients group compared to healthy control, and detection of miR-92a-2 levels could be a potential biomarker for patients with SCLC[26]. As a promising biomarker, miR-219-5p has been identified as a prognostic factor for different cancers. Long et al. found that miR-219-5p expression level was distinctly decreased in melanoma tissues and cell lines, and the modulation of miR-219-5p expression could be a prognostic biomarker and treatment strategy in melanoma[27]. A study from Huang et al. suggested a role of miR-219-5p for prognostic prediction and therapeutic strategy in colorectal cancer[28]. However, there is no studies exploring the role of miR-219-5p for biomarker in patients with SCLC. To the best of our knowledge, this study was the first attempt ever made to comprehensively evaluate the role for prognostic prediction based on miR-219-5p expression in patients with SCLC. In the current study, we initially examined the expression levels of miR-219-5p in SCLC patients. We, for the first time, demonstrated a correlation of the altered miR-219-5p expression with

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available clinical parameters. We found that miR-219-5p was significantly associated with lymphocytes count, PNI score and stage of SCLC. The univariate analysis indicated that increased miR-219-5p expression was a protective predictor for mortality. Kaplan-Meier curve displayed that patients with elevated miR-219-5p expression levels or accepted immunotherapy had low cumulative incidence of death compared to those with reduced miR-219-5p expression or unaccepted immunotherapy, respectively. In addition, gender, age, serum CRP level, albumin level, lymphocytes count, PNI score, immunotherapy, heart failure, KPS score and miR-219-5p level were associated with overall mortality. The multivariate analysis showed that miR-219-5p, gender, PNI score, immunotherapy and heart failure could predict OS as the independent risk factors.

Nomograms are applied for visualization of statistical models, graphical evaluation of variable significance and examination of predicted values[29, 30]. They have been widely performed to predict cancer risks and therapeutic outcomes[31, 32]. Most recently, several studies have successfully established a prognostic nomogram that combined a miRNA with clinical-related variables for OS estimation in different cancers[33-35]. Although nomogram is becoming increasingly popular, no studies have built prognostic models using combination of miR-219-5p and clinical risk factors in SCLC patients. In this study, based on the combination of miR-219-5p and independent clinicopathological variables, we created a nomogram model that could provide an individual prognostic prediction for OS estimation in SCLC patients. The results indicated excellent accuracy in estimating the risk of OS. There was a suitable calibration curve for risk estimation, indicating a well-performed nomogram, and good agreements between observation and prediction. To further verify the accuracy and efficiency of the model, an external date containing 86 patients from Sichuan Cancer Hospital was conducted. The results indicated that the prognositc model could

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accurately predict the prognosis of SCLC patients. Hence, this was the first prognostic nomogram for patients with SCLC that considered clinical parameters in addition to miR-219-5p. This nomogram could provide comprehensive information for patients, as well as a better guidance for clinical therapy. Based on the model, the potential high-risk patients with low survival rate could be more accurately selected for a specific therapeutic strategy.

CONCLUSIONS

In conclusion, we found that the miR-219-5p expression levels were significantly correlated with clinical parameters of SCLC patients. Furthermore, miR-219-5p was proved to be an independent factor for prognostic prediction in patients with SCLC. Moreover, nomogram based on multivariate analysis showed excellent accuracy in estimating the risk of OS. However, the prospective validation of the prognostic nomogram is needed in the future.

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Contributors ZHX and XMW designed the study. ZJC, XHZ, JGZ and MQX collected and analysed the data. ZJC, JGZ and XHZ drafted the initial manuscript. ZHX and XMW reviewed and edited the article. All authors read and approved the final manuscript.

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Competing interests The authors declare that they have no competing interests.

Patient consent for publication Not required.

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Ethics approval

The study was approved by ethics committee of Suzhou Xiangcheng People's Hospital. The reference number was 20140193. All procedures performed in the present study were in accordance with the principles outlined in the 1964 Helsinki Declaration and its later amendments.

Provenance and peer review Not commissioned; externally peer reviewed.

Data Availability Statement The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Figure Legends

Figure 1 A flow chart of the screening process.

Figure 2 Overall survival (OS) of SCLC patients in different levels of miR-219-5p and different treatments. (A) OS of SCLC patients with high or low level of miR-219-5p. (B) OS of SCLC patients with different treatments (immunotherapy vs non-immunotherapy).

Figure 3 Multivariate cox regression analysis of 5-year overall survival on data in the

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SCLC patients.

Figure 4 Nomogram for overall survival (OS) risk estimation of SCLC patients and its predictive performance. (A) Nomogram to estimate the OS risk of SCLC patients in different variations. (B) Validity of the predictive performance of the nomogram in estimating the OS risk of SCLC patients.

Figure 5 External validation of the prognostic model.

Table 1 Study participant characteristics at enrollment.

Table 2 Univariate cox regression analysis of overall survival on SCLC patients.

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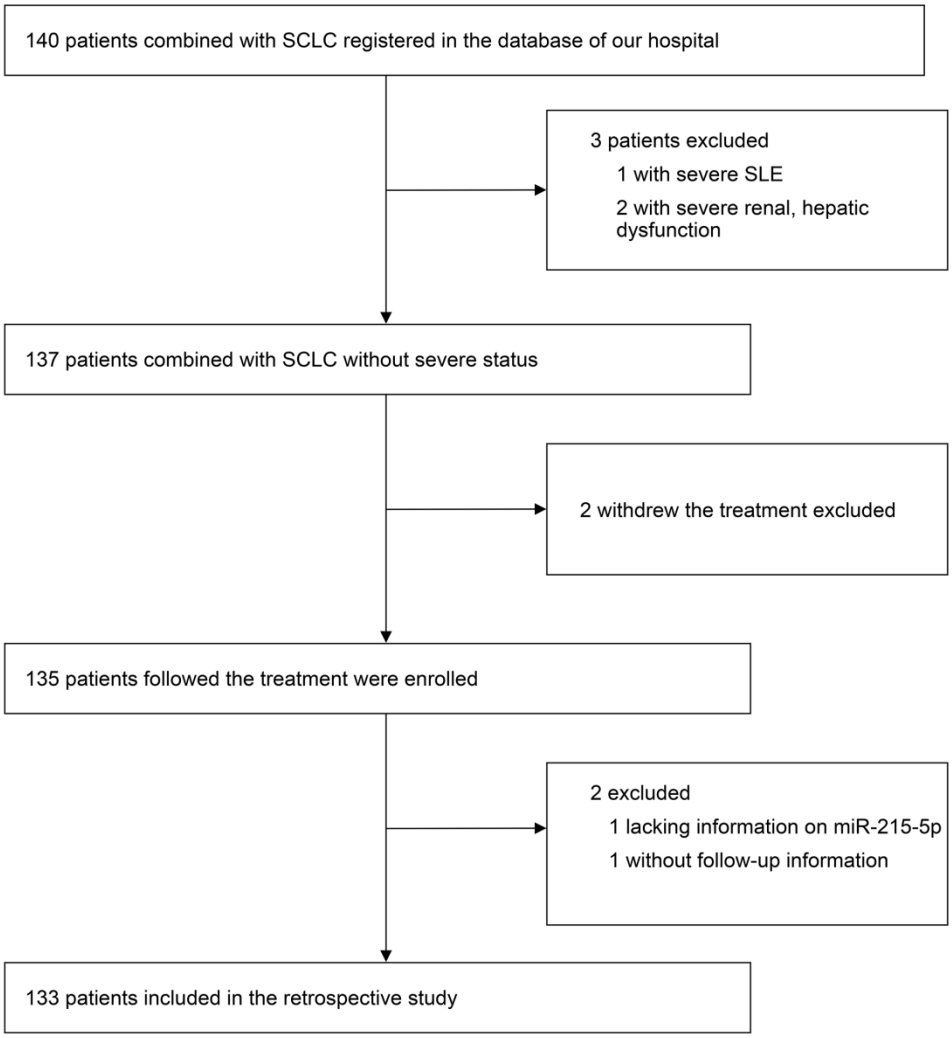

Figure 1 A flow chart of the screening process.

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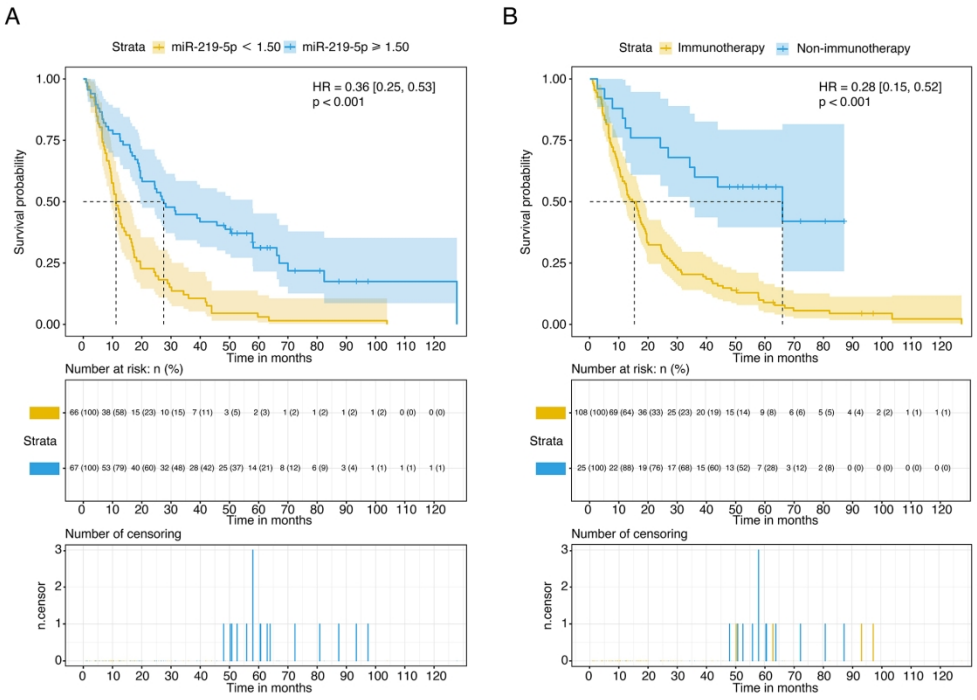

Figure 2 Overall survival (OS) of SCLC patients in different levels of miR-219-5p and different treatments. (A) OS of SCLC patients with high or low level of miR-219-5p. (B) OS of SCLC patients with different treatments (immunotherapy vs non-immunotherapy).

180x126mm (300 x 300 DPI)

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Figure 3

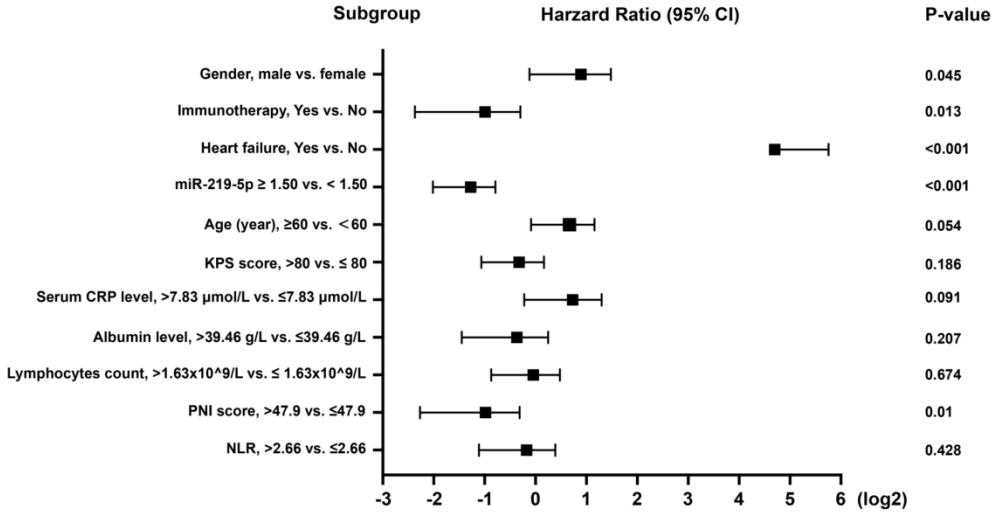

Figure 3 Multivariate cox regression analysis of 5-year overall survival on data in the SCLC patients.

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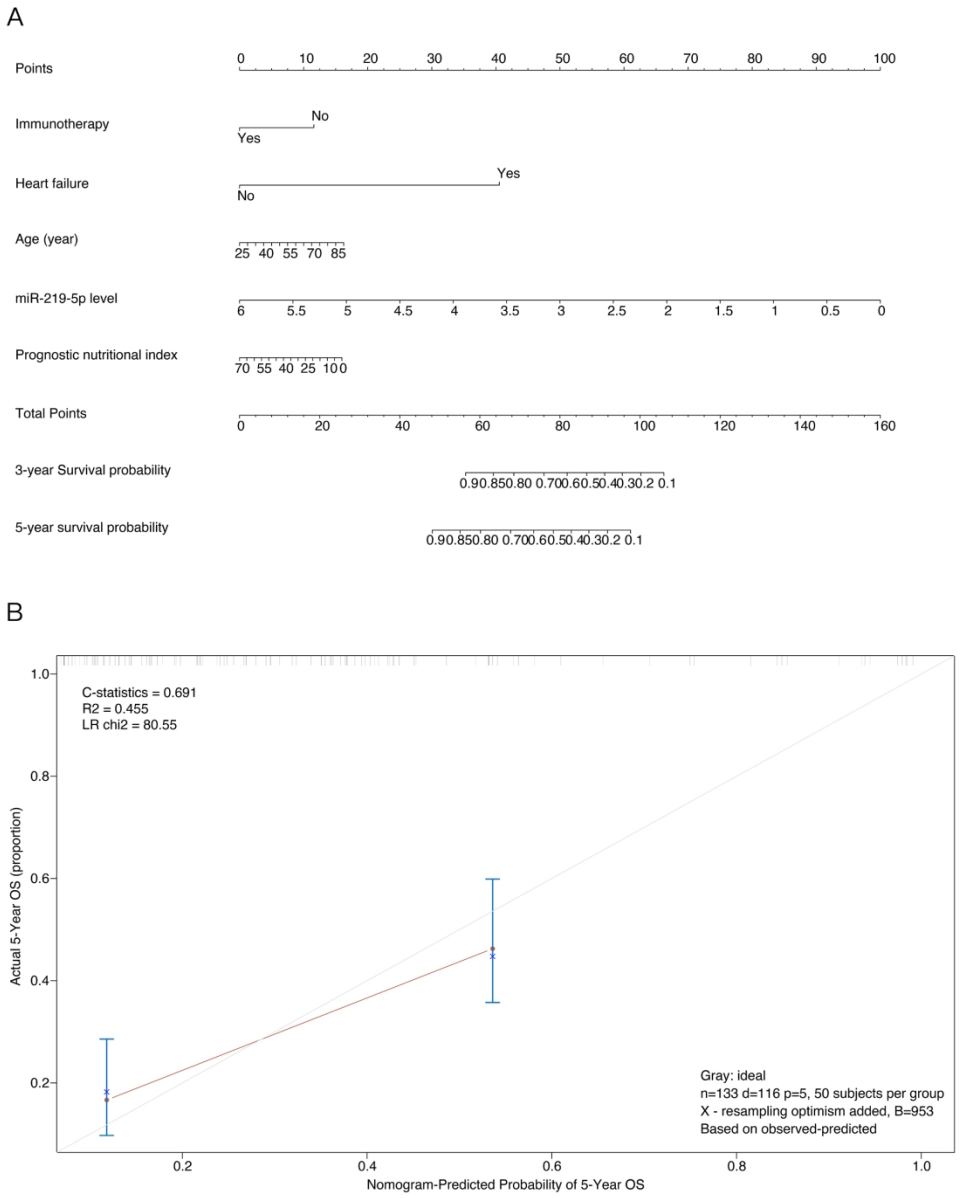

Figure 4 Nomogram for overall survival (OS) risk estimation of SCLC patients and its predictive performance. (A) Nomogram to estimate the OS risk of SCLC patients in different variations. To build the nomogram, find the position of each variable on the corresponding axis, draw a line to the points axis for the number of points, add the points from all of the variables, and draw a line from the total points axis to determine the OS probabilities at the lower line of the nomogram. (B) Validity of the predictive performance of the nomogram in estimating the OS risk of SCLC patients.

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Figure 5

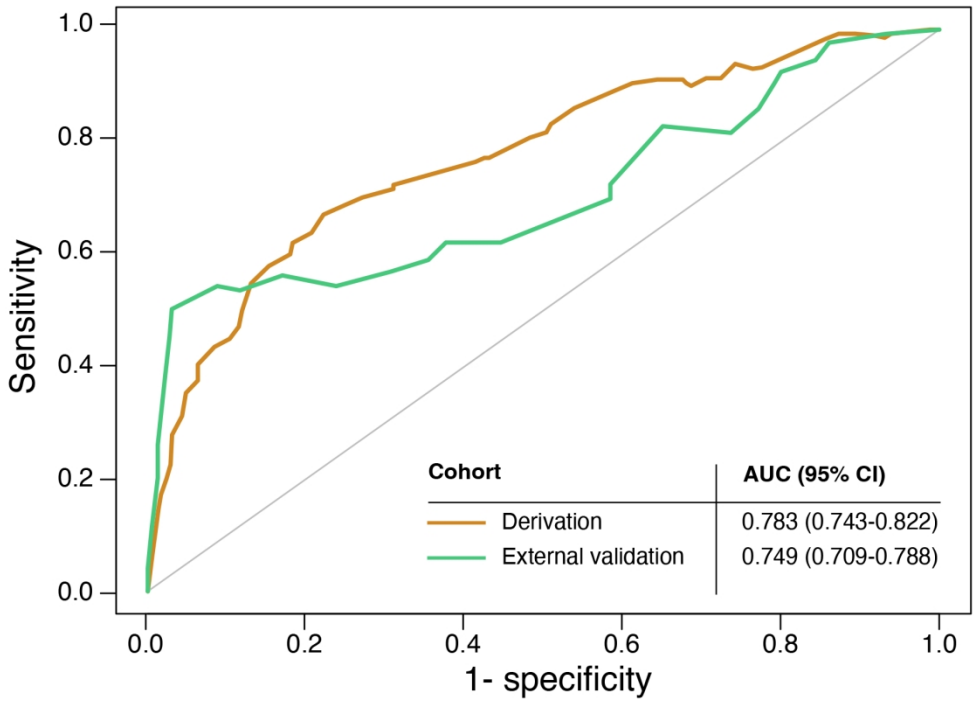

Figure 5 External validation of the prognostic model.

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Supplement Materials

Part I. Details of variants

Patients' age: The age of patients was based on the time of diagnosis of NSCLC.

Body mass index: The equation of body mass index (BMI) is calculated as follow: BMI (kg/m^2) = weight (kg) / height $(m)^2$. The data of BMI was recorded before clinical treatment.

Serum CEA level: The serum level of carcinoembryonic antigen (CEA) is one of tumor marks， the higher serum *CEA* level than that of healthy individuals led to its clinical application as a diagnostic biomarker for cancer.

Serum CRP level: C-reactive protein (CRP) is detected by olorimetric determination through fasting blood collection and CRP is one of the inflamation markers.

Albumin level: Serum albumin is detected by colorimetric determination through fasting blood collection. And serum albumin is one of the indicators of nutritional status.

Neutrophils count, lymphocytes count:, hemoglobin level, platelet count: All these indexes were extracted from blood routine examination to evaluate preoperative status of patients.

PNI score: Prognostic nutritional index (PNI) is an index to evaluate the nutritional status of surgical patients, to predict the risk of surgery and to help make prognostic judgment. The equation is listed as below: PNI = serum albumin (g/L) + 5 * lymphocytes ($*10^9/L$).

NLR: Neutrophil to Lymphocyte Ratio (NLR) is an index of inflammation markers to predict the prognosis of various cancer, including lung cancer.

Pathologic type: Non-small cell lung cancer can be classified as adenocarcinoma, squamous cell carcinoma, large cell lung cancer, or others, depending on surgical or tumor biopsy specimens

Stage of NSCLC: Non-small cell lung cancer was staged according to the seventh edition of international union against cancer (UICC) TNM was published in 2009

Surgery: For early and locally advanced NSCLC, which is generally acceptable, there is a history of surgical resection.

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Therapy of radiation: Radiation therapy is a medical procedure that uses the delivery of high-energy radiation to kill cancer cells and shrink tumors, including external beam radiation therapy, internal radiation therapy and stereotactic body radiotherapy (SBRT). It aims to cure small tumors, treat lung cancer both locally and nearby lymph nodes, treat metastases, relieve symptoms, and also at prevention.

Application of platinum: Platinum-based chemotherapy may be used as a first or second line therapy for advanced lung cancer, after surgery (adjuvant chemotherapy), or before surgery to reduce the size of a tumor (neoadjuvant therapy). The most commonly used platinum drugs are cisplatin, carboplatin and nedaplatin. Treatment of non-small cell lung cancer begins with either cisplatin or carboplatin combined with another medication, such as docetaxel, pemetrexed, paclitaxel, gemcitabine and gemcitabine.

Target therapy: Targeted therapies for lung cancer are drugs that are tailored to attack certain mutations of cancer cells. These mutations lead to the production of abnormal proteins which guide the growth and development of a cancer cell. Common driver mutations include EGFR mutations, ALK rearrangements, ROS1 rearrangements, MET amplifications, KRAS mutations, HER2 mutations, BRAF mutations and so on.

Application of TKI: Tyrosine kinase inhibitors (TKI) act as competitive inhibitors of ATP binding to tyrosine kinases, or as tyrosine analogs that block tyrosine kinase activity and inhibit cell proliferation. Epithelial growth factor receptor (EGFR) is a protein that is present on the surface of both normal cells and lung cancer cells. Mutations in EGFR can occur at different locations on exon 18 to 21. ALK-positive lung cancer refers to people who have lung cancer that tests positive for the EML4-ALK fusion mutation. Several FDA-approved medications available to treat EGFR-positive and ALK-positive lung adenocarcinoma, including:

- EFGR inhibitors - Tarceva (erlotinib), Gilotrif (afatinib), Iressa (gefitinib), Tagrisso (osimertinib), and Portrazza (necitumumab)
- ALK inhibitors - Xalkori (crizotinib), Zykadia (ceritinib), and Alecensa (alectinib)
- ROS1 inhibitor - Xalkori (crizotinib)

Application of VEGF inhibitor: VEGF (Vascular endothelial growth factor) can activate angiogenesis through different signaling pathways. VEGF inhibitors are drugs that block the ability of tumors to form new blood vessels, and hence, grow and spread. Some currently available medications include bevacizumab, ramucirumab, axitinib, endostar and anlotinib.

KPS score: A performance measure for rating the ability of a person to perform usual activities, evaluating a patient's progress after a therapeutic procedure, and determining a patient's suitability for therapy. It is used most commonly in the

prognosis of cancer therapy, usually after chemotherapy and customarily administered before and after therapy. It was named for Dr. David A. Karnofsky, an American specialist in cancer chemotherapy. Patients with more than 80 scores had better postoperative status and longer survival time. And patients with more than 70 scores can suffer from chemoradiotherapy.

Smoking: Smoker refers to continuous or cumulative smoking > 1 cigarette/day over a lifetime of more than 6 months. (1997, WHO)

Hypertension: Hypertension is defined as a repeatedly elevated blood pressure exceeding 140 over 90 mmHg.

Diabetes: Diabetes is a group of metabolic diseases characterized by hyperglycemia. And fasting blood glucose more than 7.0 mmol/l or blood glucose more than 11.1 mmol/l within 2hoursafter meal can be diagnosed diabetes.

Hyperlipemia: Hyperlipemia means the presence of excess fat or lipids in the blood. And total cholesterol ≥ 6.2 mmol/L, low density lipoprotein cholesterin ≥ 4.1 mmol/L, triglyceride ≥ 2.3 mmol/L, high density lipoprotein cholesterin < 1.0 mmol/L can be diagnosed hyperlipemia.

Heart failure: The information was recorded through history taking and verified after hospitalization. Heart failure means inability of the heart to keep up with the demands on it and, specifically, failure of the heart to pump blood with normal efficiency. Heart failure may be due to failure of the right or left or both ventricles. The signs and symptoms depend upon which side of the heart is failing. They can include shortness of breath (dyspnea), asthma due to the heart (cardiac asthma), pooling of blood (stasis) in the general body (systemic) circulation or in the liver's (portal) circulation, swelling (edema), blueness or duskiness (cyanosis), and enlargement (hypertrophy) of the heart.

ACS: Acute coronary syndrome is a term for a group of conditions that suddenly stop or severely reduce blood from flowing to the heart. When blood cannot flow to the heart, the heart muscle can become damaged. Heart attack and unstable angina are both acute coronary syndromes (ACS).

Withdraw treatment: Reasons for patients withdrew from treatment were listed below: 1) High cost of the treatment: In China, the cost of hormone therapy can be up to 3000 RMB a month if without health insurance. 2) Poor patient compliance: Some patients discontinue treatment because they do not comply with the treatment plan prescribed by their doctor.

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Part II. Report of sample size assessment

PASS 15.0.5

Tests for Two Survival Curves Using Cox's Proportional Hazards Model

Numeric Results with Ha: HR ≠ 1

Power	Total Sample Size N	Control Sample Size N1	Trtmnt Sample Size N2	Prop'n Control N1/N P1	Hazard Ratio h2/h1 HR	Control Prob Event Pev1	Trtmnt Prob Event Pev2	Control Events E1	Trtmnt Events E2	Alpha	Beta
0.9005	103	51	52	0.500	0.500	0.750	0.950	38.3	49.4	0.050	0.100

References

Chow, S.C., Shao, J., Wang, H. 2008. Sample Size Calculations in Clinical Research, 2nd Edition. Chapman & Hall/CRC.

Schoenfeld, David A. 1983. 'Sample Size Formula for the Proportional-Hazards Regression Model', Biometrics, Volume 39, Pages 499-503.

Report Definitions

Power is the probability of rejecting a false null hypothesis. Power should be close to one.
N is the total sample size.
N1 and N2 are the sample sizes of the control and treatment groups.
P1 is the proportion of the total sample that is in the control group, group 1.
HR is the hazard ratio: h2/h1.
Pev1 and Pev2 are the probabilities of an event in the control and the treatment groups.
E1 and E2 are the number of events required in the control and the treatment groups.
Alpha is the probability of a type one error: rejecting a true null hypothesis.
Beta is the probability of a type two error: failing to reject a false null hypothesis.

Summary Statements

A two-sided test of whether the hazard ratio is one with an overall sample size of 103 subjects (of which 51 are in the control group and 52 are in the treatment group) achieves 90% power at a 0.050 significance level when the hazard ratio is actually 0.500. The number of events required to achieve this power is 87.7. It is anticipated that proportions of subjects having the event during the study is 0.750 for the control group and 0.950 for the treatment group. These results assume that the hazard ratio is constant throughout the study and that Cox proportional hazards regression is used to analyze the data.

Procedure Input Settings

Autosaved Template File

\\Mac\Home\Documents\PASS 15\Procedure Templates\Autosave\Tests for Two Survival Curves Using Cox's

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Proportional Hazards Model - Autosaved 2020_1_24-9_57_39.t92

Design Tab

Solve For:	Sample Size
Alternative Hypothesis:	Ha: HR ≠ 1
Power:	0.90
Alpha:	0.05
Group Allocation:	Equal (N1 = N2)
Pev1 (Probability of a Control Event):	0.750
Pev2 (Probability of a Treatment Event):	0.950
HR (Actual Hazard Ratio = h2/h1):	0.5

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Figure S1a

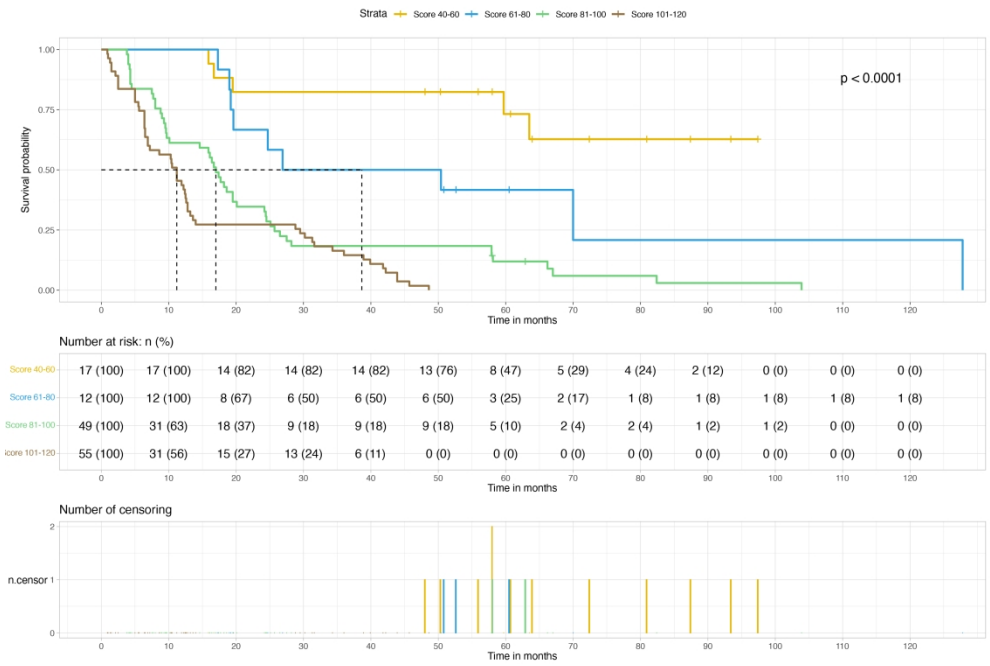

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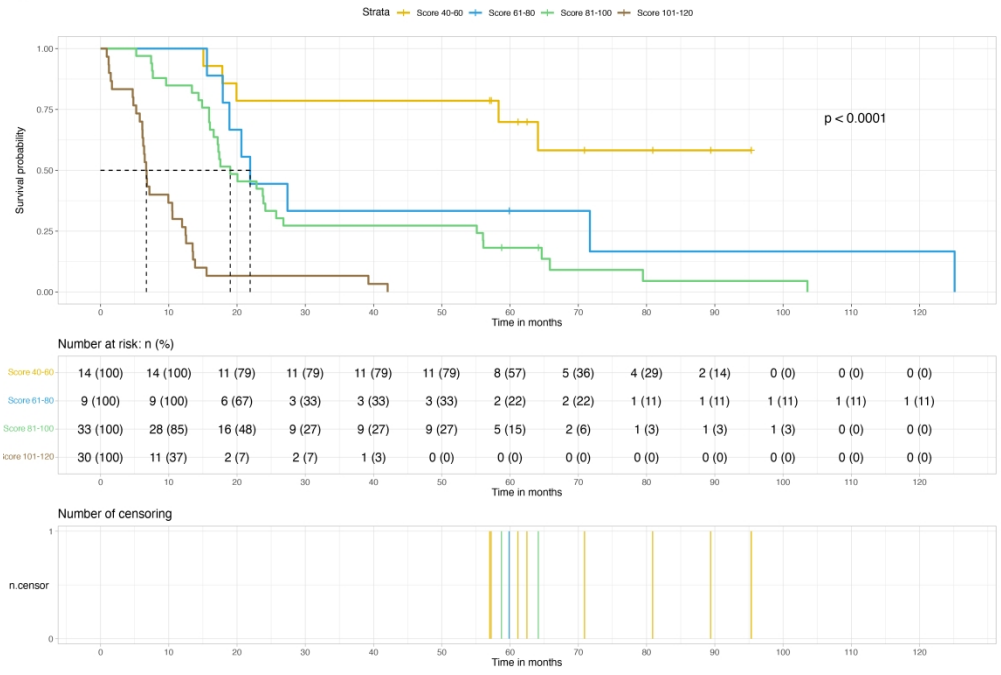
Figure S1b



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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			**5**
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*6*
Bias	9	Describe any efforts to address potential sources of bias	7
Study size	10	Explain how the study size was arrived at	7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7
		(*b*) Describe any methods used to examine subgroups and interactions	7
		(*c*) Explain how missing data were addressed	7
		(*d*) If applicable, explain how loss to follow-up was addressed	7
		(*e*) Describe any sensitivity analyses	7
Results			

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8
		(b) Give reasons for non-participation at each stage	8
		(c) Consider use of a flow diagram	8
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9
		(b) Indicate number of participants with missing data for each variable of interest	9
		(c) Summarise follow-up time (eg, average and total amount)	9
Outcome data	15*	Report numbers of outcome events or summary measures over time	10
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10
		(*b*) Report category boundaries when continuous variables were categorized	11
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	11
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	12
Discussion			
Key results	18	Summarise key results with reference to study objectives	13-14
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15
Generalisability	21	Discuss the generalisability (external validity) of the study results	15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Prognostic value of MiR-219-5p in relation to mortality in patients with small cell lung cancer: a retrospective, observational cohort study in China

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Primary Subject Heading:	Oncology
Secondary Subject Heading:	Surgery
Keywords:	Risk management < HEALTH SERVICES ADMINISTRATION & MANAGEMENT, ONCOLOGY, Gene therapy < ONCOLOGY, Molecular aspects < ONCOLOGY, Oncogenes < ONCOLOGY

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Prognostic value of MiR-219-5p in relation to mortality in patients with small cell lung cancer: a retrospective, observational cohort study in China

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ABSTRACT

Objectives Small cell lung cancer (SCLC) is a lethal human malignancy, and previous studies support the contribution of microRNA (miRNA) to cancer progression. The prognostic value of miR-219-5p in SCLC patients remains unclear. This study aimed to evaluate the predictive value of miR-219-5p with respect to mortality in patients with SCLC and to incorporate miR-219-5p level into a prediction model and nomogram for mortality.

Design Retrospective observational cohort study.

Setting and participants Our main cohort included data from 133 patients with SCLC between Mar 1, 2010, and June 1, 2015 from the Suzhou Xiangcheng People's Hospital. Data from 86 patients with NSCLC at Sichuan Cancer Hospital and the First Affiliated Hospital of Soochow University were used for external validation.

Outcome measures Tissue samples were taken during admission and stored and miR-219-5p levels were measured at a later date. A Cox proportional hazard model was used for survival analyses and for analyzing risk factors to create a nomogram for mortality prediction. The accuracy of the model was evaluated by C-index and calibration curve.

Results Mortality in patients with a high miR-219-5p level (≥ 1.50) (n=67) was 74.6%, while mortality in the low-level group (n=66) was 100.0%. Based on univariate analysis, we included significant factors (P <0.05) in a multivariate regression model: patients with high miR-219-5p level (HR 0.39, 95%CI 0.26-0.59, P <0.001), immunotherapy (HR 0.44, 95%CI 0.23-0.84, P <0.001), PNI score >47.9 (HR=0.45, 95%CI 0.24-0.83, P =0.01) remained statistically significant factors for improved overall survival (OS). The nomogram had good accuracy in estimating the risk, with a bootstrap-corrected C index of 0.691. External validation indicated an AUC of 0.749 (0.709-0.788).

Conclusions MiR-219-5p level was associated with a reduced risk of mortality in patients with SCLC. A nomogram incorporating MiR-219-5p level and clinical factors demonstrated good accuracy in estimating the risk of overall mortality. Prospective validation of the prognostic nomogram is needed.

Keywords: small cell lung cancer, miR-219-5p, overall survival, nomogram,

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prediction model

Strengths and limitations of this study

► The study utilizes databases of all patients with small cell lung cancer (SCLC) in two defined geographical regions of China.

► The study included the creation of a nomogram for predicting survival probabilities in individual patients.

► However, the model is not comprehensive since the database does not include all prognostic factors for SCLC.

► Additionally, the available data on treatment status are not adequately detailed to distinguish the impact of various treatment plans.

► The model needs to be prospectively assessed to determine its reliability.

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INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide, with millions of new cases diagnosed each year[1]. Small cell lung cancer (SCLC) is a kind of neuroendocrine malignant tumor with poor prognosis, accounting for about 15% of lung cancer patients[2]. SCLC is generally divided into limited disease (LD-SCLC) and extensive disease (ED-SCLC). Platinum combined with etoposide is the first-line therapeutic strategy of SCLC, and most patients are easy to receive initial chemotherapy[3]. However, the 5-year survival rates of LD-SCLC and ED-SCLC are only 15% and 3%, respectively[4]. Therefore, improvement in early diagnosis and prognostic prediction of SCLC is vital.

MicroRNAs (miRNAs) are endogenous non-coding RNAs (~ 22 nt), which regulate mRNA activity by hybridization with 3 '- untranslated region (UTR) of specific genes[5]. Many studies have shown that miRNAs could participate in a variety of cell biological processes, including cell growth, differentiation and apoptosis[6, 7]. In addition, researches have demonstrated that miRNAs are frequently dysregulated in cancers[8, 9], and some miRNAs can serve as diagnostic and prognostic biomarkers for caners[10]. Recently, several miRNAs have been proved to participant in the occurrence and development of SCLC, but few of them are likely to be a biomarker or therapeutic target for SCLC.

Recently, miR-219-5p has been found to be abnormally expressed and play a significant role in different cancers. Ma et al. found that the expression of miR-219-5p was significantly decreased in esophageal squamous cell carcinoma (ESCC) tissues compared with normal tissues[11]. A study of Gong et al. revealed a tumor suppressive role for miR-219-5p by targeting glypican-3 in hepatocellular carcinoma (HCC)[12]. On the contrary, Yang et al. indicated that miR-219-5p could promote cell growth and metastasis of HCC and serve as a prognostic marker for HCC patients[13]. A research investigated by Wei et al. suggested that miR-219-5p could inhibit proliferation, migration and invasion of epithelial ovarian cancer through downregulation of the Wnt signaling pathway, and it could serve as a diagnostic biomarker and therapeutic target

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for epithelial ovarian cancer[14]. However, the biological functions of miR-219-5p and its potential prognostic role for biomarker in SCLC are still unknown.

In this study, we aimed to examine the variation in the expression levels of miR-219-5p in patients with SCLC, to evaluate the predictive value of miR-219-5p with respect to mortality in patients with SCLC, and to incorporate miR-219-5p level into a prediction model and nomogram for mortality.

METHODS

Study design and patients

The study utilizes databases of all patients with small cell lung cancer (SCLC) in two defined geographical regions of China. Our main cohort included data obtained from 133 patients with SCLC between Mar 2010 and June 2015, in the Suzhou Xiangcheng People's Hospital. Tissue samples were taken during admission and stored and the miR-219-5p levels were measured at a later date. Those participants who lacked information on complement components data, withdrew from treatment or lacked follow-up information were excluded. Clinical information of patients, including gender, age, BMI, neutrophils count, lymphocytes count, serum CEA level, CRP level, albumin level, hemoglobin level, stage of SCLC, platelet count, PNI score, KPS score, NLR, pathologic type, immunotherapy, therapy of radiation, application of platinum, application of VEGF inhibitor, target therapy, application of TKI, smoking, ACS, diabetes, heart failure and hyperlipemia, were recorded. Diagnosis of SCLC was confirmed by histopathological examination. The median length of follow-up was 23.6 months. Median was used as the cut-off value. The definition and details of all the variables above were provided in Supplemental Materials Part I. Data from 86 patients

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with NSCLC at Sichuan Cancer Hospital and the First Affiliated Hospital of Soochow University were used for external validation. For both the main cohort and the validation cohort, informed consent was obtained from all patients or their immediate family members for the collection and storage of samples and their use for future scientific research. All procedures were in line with the guidelines of the ethics committee of Suzhou Xiangcheng People's Hospital, Sichuan Cancer Hospital, and the First Affiliated Hospital of Soochow University and the study was performed in accordance with the Declaration of Helsinki.

Assays for detection of MiR-219-5p levels

The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was conducted for the detection of miR-219-5p expression levels.

Total RNA from tissues was isolated and extracted using miRcute Extraction and Separation of miRNAs kit (Tiangen Biotech Co., Ltd., Beijing, China), and then reversely transcribed into cDNA by PrimeScript™ Ⅱ 1st strand cDNA synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co., Ltd.) was used for qRT-PCR. The thermocycling conditions were as follows: one cycle at 95˚C for 3 min (initial denaturation), 40 cycles at 95˚C for 15 s and 60˚C for 30 s. U6 small nuclear RNA (U6) served as the respective internal control. The relative expression of miR-219-5p was quantified by the $2^{-\Delta\Delta Ct}$ methods, and normalized to the U6. The following primers were used: miR-219-5p forward, 5'-ACACTCCAGCTGGGTGATTGTCCAAACGCAAT-3' and reverse, 5'-CTCAACTGGTGTCGTGGA-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The experiments were repeated at least 3

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times.

Statistical analysis

Sample size assessment was performed using NCSS-PASS software version 11.0 (https://www.ncss.com/software/pass/). Power was set as 0.99, and alpha was 0.5. The mortality in both the miR-219-5p high-level group and miR-219-5p low-level group in our previous data (2008-2009) (0.750 and 0.950) were entered into the PASS. The Actual Hazard Ratio was set as 0.50. Then the sample size was calculated using PASS, and the minimum sample size was 103 (control = 51, experiment = 43). Our sample size was 133 (66 and 67 for each group), which was suitable. The report of sample size assessment was displayed in Supplemental Material Part II. The missing data (<5.0%) were estimated by random forest algorithm using the mice package in RStudio (R version 3.6.1). Categorical variates were presented as percentages and compared via the κ^2 test. Continuous variates with skewed and normal distributions were presented as median with interquartile ranges and mean ± standard deviation. The Mann-Whitney U test and the unpaired t-test were applied for comparison between Groups. Cumulative mortality was showed by the Kaplan-Meier curve and analyzed by the log-rank test. Univariate and multivariate survival analyses for OS were conducted using the Cox regression model. The forest plots were used to visualize the significance of covariates to the prognosis. The restricted cubic spline analyses were performed with Harrell's Regression Modelling Strategies (rms) package.

We screened multifactor analysis for statistically significant indicators for inclusion in the prediction model. To build the nomogram, find the position of each

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variable on the corresponding axis, draw a line to the points axis for the number of points, add the points from all the variables, and draw a line from the total points axis to determine the OS probabilities at the lower line of the nomogram. The contribution of each covariate was quantified and visualized in a prognostic nomogram with internal validation via 1000-times bootstrapping. The consistency of the resulting model was assessed by the calibration assay. Decision curve analyses were performed to evaluate net clinical benefits of the model compared with conventional prognostic scores. The scatter plots were applied for visualization of the consistency of each model. A 1000-time bootstrapping was employed as indicated. The association between miR-219-5p class and survival endpoints was evaluated by Kaplan-Meier curves and log-rank test. Statistical analysis was performed using the RStudio (R version 3.6.1) with the following packages: 'ggplot2', 'rms', 'PredictABLE', 'risk regression', and 'survminer'.

Patient and public involvement

None.

RESULTS

Baseline characteristics

A total of 133 SCLC patients who were diagnosed between Mar 2010 and June 2015 were included in the main cohort. A flowchart of the screening process was shown in figure 1. The median age of these patients was 64 years old (58-70), and it contained 106 (80.0%) males. Median serum CEA and CRP level was 3.43 ng/ml and 7.83 μmol/L, respectively. For the stage of SCLC, 51 (38.0%) patients were diagnosed with limited disease, 82 (61.0%) patients were extensive disease. 25 (19.0%) patients accepted the immunotherapy therapy. 54 (41.0%) patients got the therapy of radiation. In addition,

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platinum was applied for 131 (98.0%) patients, and TKI was used for 15 (12.0%) patients. KPS score of these patients was examined, and the results revealed that 107 (81.0%) patients got 80 or higher points. The distribution of basic diseases was also assessed in our data. Diabetes was found in 17 (13.0%) patients, while hyperlipemia was 13 (10.0%). Cardiovascular diseases such as heart failure and ACS were found in 3 (2.0%) and 4 (3.0%) patients, respectively. 13 (10.0%) patients suffered from hypertension. In addition, 82 (62.0%) patients had a habit of smoking among all the patients. The baseline characteristics of these patients were listed in table 1.

Among all the 133 patients, overall mortality was 87.2%. The mortality in high miR-219-5p level group (n=67) was 74.6%, while the mortality in the low-level group (n=66) was 100.0%. Moreover, in the high miR-219-5p level group, patients with extensive disease were 35 (52.0%), while the low group was 47 (71.0%) (table 1).

Table 1. Study participant characteristics at enrollment

Variation	Total (n=133)	Cohort, median (IQR)		
		mir-219-5p<1.50, (n=66)	mir-219-5p≥1.50, (n=67)	p.value
Age, (year)	64(58,70)	65(59,70)	63(56.5,68)	0.276
BMI, (kg/m2)	23.12±3.09	22.99±3.22	23.26±2.98	0.619
Serum CEA level, (ng/ml)	3.43(1.96,9.26)	3.34(1.92,10.05)	3.43(1.99,8.11)	0.87
Serum CRP level, (μmol/L)	7.83(1.68,12.78)	10.68(2.78,13.15)	5.64(1.2,12.16)	0.107
Albumin level, (g/L)	39.46±5.2	38.86±4.68	40.05±5.65	0.188
Neutrophils count, (10^9/L)	4.55(3.59,5.88)	4.54(3.77,5.75)	4.55(3.48,5.92)	0.975
Lymphocytes count, (10^9/L)	1.63(1.31,1.95)	1.5(1.21,1.78)	1.73(1.38,2.08)	0.031*
Hemoglobin level, (g/L)	133(125,145)	134(124,145)	132(125,143.5)	0.564
Platelet count, (10^9/L)	233(184,288)	244.5(180,293.75)	226(184.5,273.5)	0.306
PNI score	47.9(43.95,51.85)	45.98(42.51,50.38)	48.7(44.92,54)	0.026*
NLR	2.66(1.99,4.19)	2.75(2.11,4.57)	2.59(1.92,3.7)	0.232
Gender, (n%)				0.211
Female	27(20)	10(15)	17(25)	
Male	106(80)	56(85)	50(75)	
Metastasis, n(%)				0.299
No	45(34)	19(29)	26(39)	
Yes	88(66)	47(71)	41(61)	
Stage of SCLC				0.038*
Limited disease	51(38)	19(29)	32(48)	

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Extensive disease	82(62)	47(71)	35(52)	
Immunotherapy, (n%)				0.197
No	108(81)	57(86)	51(76)	
Yes	25(19)	9(14)	16(24)	
Therapy of radiation, (n%)				0.417
No	79(59)	42(64)	37(55)	
Yes	54(41)	24(36)	30(45)	
Application of platinum, (n%)				0.244
No	2(2)	2(3)	0(0)	
Yes	131(98)	64(97)	67(100)	
Chemotherapy				0.45
AP	28(21)	12(18)	16(24)	
DP	15(11)	6(9)	9(13)	
EP	71(53)	35(53)	36(54)	
GP	3(2)	2(3)	1(1)	
Others	16(12)	11(17)	5(7)	
Target therapy, (n%)				0.627
No	116(87)	59(89)	57(85)	
Yes	17(13)	7(11)	10(15)	
Application of TKI, (n%)				0.449
No	118(89)	60(91)	58(87)	
TKI I	9(7)	4(6)	5(7)	
TKI II	1(1)	1(2)	0(0)	
TKI III	5(4)	1(2)	4(6)	
Application of VEGF inhibitor, n(%)				0.645
No	114(86)	58(88)	56(84)	
Yes	19(14)	8(12)	11(16)	
KPS score, n(%)				0.678
40	2(2)	0(0)	2(3)	
50	5(4)	3(5)	2(3)	
60	7(5)	3(5)	4(6)	
70	12(9)	8(12)	4(6)	
80	29(22)	14(21)	15(22)	
90	56(42)	29(44)	27(40)	
100	22(17)	9(14)	13(19)	
Smoking, n(%)				0.255
No	51(38)	29(44)	22(33)	
Yes	82(62)	37(56)	45(67)	
Hypertension, n(%)				1
No	80(60)	40(61)	40(60)	
Yes	53(40)	26(39)	27(40)	
Diabetes, n(%)				1
No	116(87)	58(88)	58(87)	
Yes	17(13)	8(12)	9(13)	

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Hyperlipemia, n(%)				0.579
No	120(90)	61(92)	59(88)	
Yes	13(10)	5(8)	8(12)	
Heart failure, n(%)				1
No	130(98)	65(98)	65(97)	
Yes	3(2)	1(2)	2(3)	
ACS, n(%)				0.619
No	129(97)	65(98)	64(96)	
Yes	4(3)	1(2)	3(4)	

Abbreviation: IQR, interquartile range; CRP, C-reactive protein; PNI, neutrophil lymphocyte ratio; NLR, neutrophil lymphocyte ratio; SCLC, small cell lung cancer; TKI, Tyrosine Kinase Inhibitor; VEGF, vascular endothelial growth factor; KPS, Karnofsky Performance Status; ACS, acute coronary syndrome. ***p<0.001, **p<0.01, *p<0.05.

MiR-219-5p expression level and clinical risk factors

According to the univariate analysis, high miR-219-5p levels (≥ 1.50) was a strong protective predictor of mortality (HR 0.36, 95% CI 0.25-0.53, *P* <0.001) (table 2). Kaplan Meier curve showed that patients in the high miR-219-5p group had a decreased cumulative rate of death than those in the low miR-219-5p group (log-rank *P* <0.001) (figure 2A). Meanwhile, patients who accepted immunotherapy also showed a low morality compared to those patients without accepting immunotherapy in the survival curve (HR 0.28, 95% CI 0.15-0.52, *P* <0.001) (figure 2B).

In addition, gender, age, serum CRP level, albumin level, lymphocytes count, PNI score, immunotherapy, heart failure, and KPS score were also correlated with overall mortality (table 2). When adjusted by age and gender, patients in the miR-219-5p high-level group also displayed a low cumulative rate death compared to those in the low-level group.

Table 2. Results of univariate Cox regression analysis for overall mortality

Variation	Non-adjustment		Model 1	
	Hazard Ratio (95% CI)	p.value	Hazard Ratio (95% CI)	p.value
Gender, male vs. female	1.66 [1.02, 2.69]	0.041*	-	-
Age (year), ≥60 vs. < 60	1.52 [1.03, 2.26]	0.036*	-	-
BMI, ≥23.12 kg/m2 vs. < 22.86kg/m2	0.99 [0.69, 1.43]	0.97	0.95 [0.66, 1.38]	0.806
Serum CEA level, >3.43 ng/ml vs. ≤3.43 ng/ml	1.01 [0.70, 1.46]	0.954	1.00 [0.69, 1.45]	0.999
Serum CRP level, >7.83 μmol/L vs. ≤7.83 μmol/L	1.91 [1.31, 2.78]	0.001**	1.92 [1.32, 2.79]	0.001**
Albumin level, >39.46 g/L vs. ≤39.46 g/L	1.66 [1.15, 2.40]	0.007**	1.60 [1.10, 2.31]	0.013*

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Neutrophils count, >4.55x10^9/L vs. ≤ 4.55x10^9/L	1.18 [0.82, 1.71]	0.367	1.15 [0.79, 1.66]	0.464
Lymphocytes count, >1.63x10^9/L vs. ≤ 1.63x10^9/L	0.59 [0.40, 0.85]	0.005**	0.61 [0.42, 0.89]	0.01*
Hemoglobin level, >133 g/L vs. ≤ 133 g/L	0.80 [0.56, 1.16]	0.244	0.72 [0.49, 1.05]	0.089
Platelet count, >233x10^9/L vs. ≤ 233x10^9/L	1.23 [0.85, 1.78]	0.275	1.23 [0.85, 1.78]	0.275
PNI score, >47.9 vs. ≤47.9	0.54 [0.37, 0.78]	0.001**	0.53 [0.37, 0.77]	0.001**
NLR, >2.66 vs. ≤2.66	1.18 [0.82, 1.71]	0.367	1.20 [0.83, 1.74]	0.341
Metastasis, Yes vs. No	1.05 [0.70, 1.57]	0.81	1.10 [0.73, 1.64]	0.654
Stage of NSCLC, IV, III vs. II and I	0.94 [0.50, 1.75]	0.838	1.05 [0.56, 1.98]	0.868
Stage of SCLC, Yes vs. No	1.33 [0.90, 1.96]	0.154	1.43 [0.97, 2.12]	0.074
Immunotherapy, Yes vs. No	0.28 [0.15, 0.52]	<0.001***	0.30 [0.16, 0.55]	<0.001***
Therapy of radiation, Yes vs. No	0.88 [0.60, 1.27]	0.488	0.79 [0.54, 1.16]	0.235
Application of platinum, Yes vs. No	0.61 [0.15, 2.47]	0.483	0.66 [0.16, 2.70]	0.562
Target therapy, Yes vs. No	0.75 [0.42, 1.33]	0.323	0.81 [0.45, 1.45]	0.481
Application of TKI, Yes vs. No	0.77 [0.41, 1.44]	0.415	0.82 [0.44, 1.53]	0.534
Application of VEGF inhibitor, Yes vs. No	0.83 [0.47, 1.45]	0.518	0.90 [0.51, 1.59]	0.723
Chemotherapy, AP vs. others	0.61 [0.37, 1.00]	0.052	0.66 [0.40, 1.09]	0.104
Smoking, Yes vs. No	1.23 [0.84, 1.79]	0.292	0.90 [0.56, 1.43]	0.645
Hypertension, Yes vs. No	1.13 [0.78, 1.64]	0.531	1.10 [0.75, 1.60]	0.622
Diabetes, Yes vs. No	0.90 [0.51, 1.61]	0.726	0.97 [0.54, 1.75]	0.927
Hyperlipemia, Yes vs. No	0.79 [0.42, 1.48]	0.461	0.77 [0.40, 1.46]	0.421
Heart failure, Yes vs. No	5.61 [1.71, 18.42]	0.004**	6.43 [1.94, 21.26]	0.002**
ACS, Yes vs. No	0.74 [0.23, 2.35]	0.612	0.69 [0.21, 2.21]	0.53
KPS score, >80 vs. ≤ 80	0.52 [0.35, 0.75]	0.001**	0.51 [0.35, 0.74]	<0.001***
miR-219-5p ≥ 1.50 vs. < 1.50	0.36 [0.25, 0.53]	<0.001***	0.37 [0.25, 0.55]	<0.001***

Abbreviation: HR, hazard risk; BMI, Body Mass Index; CRP, C-reactive protein; PNI, neutrophil lymphocyte ratio; NLR, neutrophil lymphocyte ratio; SCLC, small cell lung cancer; TKI, Tyrosine Kinase Inhibitor; VEGF, vascular endothelial growth factor; KPS, Karnofsky Performance Status; ACS, acute coronary syndrome. ***p<0.001, **p<0.01, *p<0.05.

Model 1: Adjusted by age and gender

Independent prognostic factors for OS

After multivariate adjustment, the miR-219-5p high level (HR 0.39, 95% CI 0.26-0.59, $P < 0.001$) was also associated with a low increase in the risk of death (figure 3). Meanwhile, gender, PNI score, immunotherapy and heart failure were also the independent risk factors for OS.

Development and validation of an OS-prediction nomogram

The independently related risk factors derived from the multivariate analysis were used to create an OS estimation nomogram (figure 4A). The prognostic model was internally

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validated according to the bootstrap validation method. With an unadjusted C index of 0.691 and a bootstrap-corrected C index of 0.691, the nomogram displayed excellent accuracy in estimating the risk of OS. In the validation cohort, the nomogram showed a C index of 0.691 for the estimation of OS. A suitable calibration curve for risk estimation was also displayed (R^2=0.455, LR chi2=80.55) (figure 4B). We collected 86 NSCLC patients from Sichuan Cancer Hospital in the external validation step. The ROC curve showed an AUC of 0.783 (0.743-0.822) for predicting 5-year overall survival, compared with an AUC of 0.749 (0.709-0.788) for the external validation data (figure 5). We calculated the total score using Nomogram for patients in the training and validation sets, respectively, and divided them into four groups according to 40-60,61-80,81-100,101-120, and performed Kaplan-Meier analysis and plotted survival curves, which were found to have good separation and were statistically significant (Supplementary Figures S1a, S1b).

Discussion

In this study, we detected the expression of miR-219-5p in a large cohort of SCLC patients at a single institution, between Mar 2010 and June 2015. The results suggested that reduced expression of miR-219-5p was significantly correlated with unfavorable clinical features. Moreover, patients in high miR-219-5p expression group displayed better OS compared with those in low miR-219-5p expression group. The multivariate analysis demonstrated miR-219-5p an independent prognostic factor for OS. In addition, to propose, and retrospectively verify in an independent cohort of patients, these independent risk factors were applied to establish a nomogram for OS estimation. The nomogram revealed good accuracy in estimating the risk of OS.

Carcinogenesis involves multiple biological processes which are related to many key genes[15, 16]. The characteristics of cancer occurrence represent properties that a cell acquire a certain ability to become and maintain itself as a cancer cell[17]. The key genes guide the cellular signaling pathways related to occurrence and progression of cancers[18, 19]. Using miRNA expression to predict the clinical diagnosis and

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prognosis of cancer has more advantages than mRNAs, as miRNAs are proved to be the vital posttranscriptional regulators of gene expression[20, 21]. In comparison with mRNAs, these vital gene regulators are highly conserved among species[22].

It has been reported that miRNAs were related to the initiation and progression of various cancers, and many miRNAs have been identified as a promising biomarker for prognostic prediction of cancer[10, 23]. Recently, some miRNAs have been proved to be a novel prognostic biomarker for SCLC[24, 25]. A study of Yu et al. indicated that miR-92a-2 was significantly higher in SCLC patients group compared to healthy control, and detection of miR-92a-2 levels could be a potential biomarker for patients with SCLC[26]. As a promising biomarker, miR-219-5p has been identified as a prognostic factor for different cancers. Long et al. found that miR-219-5p expression level was distinctly decreased in melanoma tissues and cell lines, and the modulation of miR-219-5p expression could be a prognostic biomarker and treatment strategy in melanoma[27]. A study from Huang et al. suggested a role of miR-219-5p for prognostic prediction and therapeutic strategy in colorectal cancer[28]. However, there is no studies exploring the role of miR-219-5p for biomarker in patients with SCLC. To the best of our knowledge, this study was the first attempt ever made to comprehensively evaluate the role for prognostic prediction based on miR-219-5p expression in patients with SCLC. In the current study, we initially examined the expression levels of miR-219-5p in SCLC patients. We, for the first time, demonstrated a correlation of the altered miR-219-5p expression with available clinical parameters. We found that miR-219-5p was significantly associated with lymphocytes count, PNI score and stage of SCLC. The univariate analysis indicated that increased miR-219-5p

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expression was a protective predictor for mortality. Kaplan-Meier curve displayed that patients with elevated miR-219-5p expression levels or accepted immunotherapy had low cumulative incidence of death compared to those with reduced miR-219-5p expression or unaccepted immunotherapy, respectively. In addition, gender, age, serum CRP level, albumin level, lymphocytes count, PNI score, immunotherapy, heart failure, KPS score and miR-219-5p level were associated with overall mortality. The multivariate analysis showed that miR-219-5p, gender, PNI score, immunotherapy and heart failure could predict OS as the independent risk factors.

Nomograms are applied for visualization of statistical models, graphical evaluation of variable significance and examination of predicted values[29, 30]. They have been widely performed to predict cancer risks and therapeutic outcomes[31, 32]. Most recently, several studies have successfully established a prognostic nomogram that combined a miRNA with clinical-related variables for OS estimation in different cancers[33-35]. Although nomogram is becoming increasingly popular, no studies have built prognostic models using combination of miR-219-5p and clinical risk factors in SCLC patients. In this study, based on the combination of miR-219-5p and independent clinicopathological variables, we created a nomogram model that could provide an individual prognostic prediction for OS estimation in SCLC patients. The results indicated excellent accuracy in estimating the risk of OS. There was a suitable calibration curve for risk estimation, indicating a well-performed nomogram, and good agreements between observation and prediction. To further verify the accuracy and efficiency of the model, an external date containing 86 patients from Sichuan Cancer Hospital was conducted. The results indicated that the prognostic model could accurately predict the prognosis of SCLC patients. Hence, this was the first prognostic nomogram for patients with SCLC that considered clinical parameters in addition to miR-219-5p. This nomogram could provide comprehensive information for patients, as well as a better guidance for clinical therapy. Based on the model, the potential high-

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risk patients with low survival rate could be more accurately selected for a specific therapeutic strategy.

Strengths and limitations

We screened valid variables by Cox regression to construct a survival prediction model for SCLC and collected data for external validation in a logical manner. However, there are some limitations in this article. Firstly, experimental research explaining the biological processes of miR-219-5p is needed. Thus, the molecular mechanism of miR-219-5p should be investigated in further research. Secondly, the prognostic nomogram needs to be further assessed in a prospective and large-scale multicenter study before it can be applied to clinical practice. Finally, our data lacked some of the risk factors associated with SCLC for inclusion, such as the determination of some of the high-risk genes and the patient's previous chemotherapy and specific targeted therapies, which will require further analysis in our future studies.

CONCLUSIONS

In conclusion, we found that the miR-219-5p expression levels were significantly correlated with clinical parameters of SCLC patients. Furthermore, miR-219-5p was proved to be an independent factor for prognostic prediction in patients with SCLC. Moreover, a nomogram based on multivariate analysis and including miR-219-5p expression levels showed excellent accuracy in estimating the risk of OS. However, the prospective validation of the prognostic nomogram is needed in the future.

** ** **

Contributors

ZHX and XMW designed the study. ZJC, XHZ, JGZ and MQX collected and analysed the data. ZJC, JGZ and XHZ drafted the initial manuscript. ZHX and XMW reviewed and edited the article. All authors read and approved the final manuscript.

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None.

Competing interests

The authors declare that they have no competing interests.

Patient consent for publication

Not applicable.

Ethics approval

The study was approved by ethics committee of Suzhou Xiangcheng People's Hospital (reference number 20140193). All procedures performed in the present study were in accordance with the principles outlined in the 1964 Helsinki Declaration and its later amendments.

Provenance and peer review

Not commissioned; externally peer reviewed.

Data availability statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Figure titles

Figure 1. Study screening flowchart

Figure 2. Overall survival (OS) of SCLC patients with different levels of miR-219-5p and different treatments

(A) OS of SCLC patients with high or low level of miR-219-5p. (B) OS of SCLC patients with different treatments (immunotherapy vs non-immunotherapy).

Figure 3. Multivariate Cox regression analysis of 5-year overall survival

Figure 4. Nomogram for overall survival (OS) risk estimation and its predictive performance

(A) Nomogram to estimate the OS risk of SCLC patients. (B) Validity of the predictive performance of the nomogram in estimating the OS risk.

Figure 5. External validation of the prognostic model

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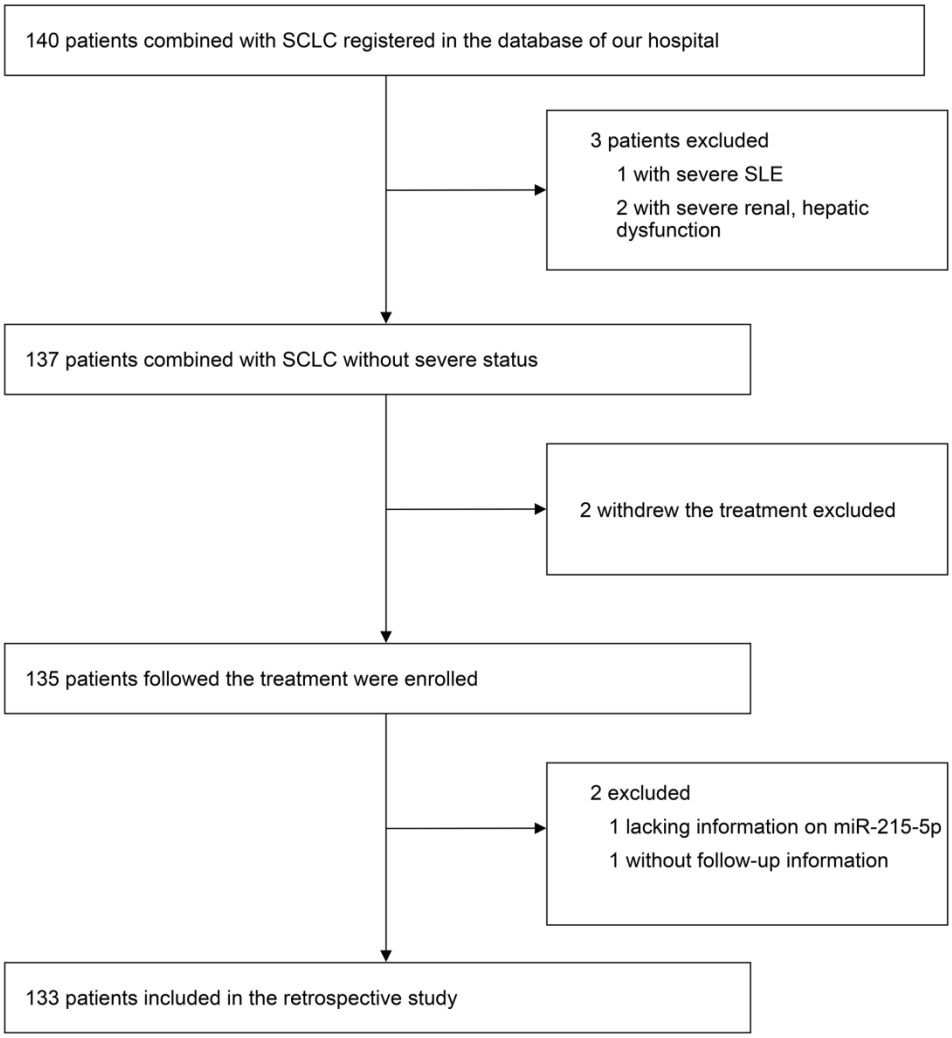

Figure 1 A flow chart of the screening process.

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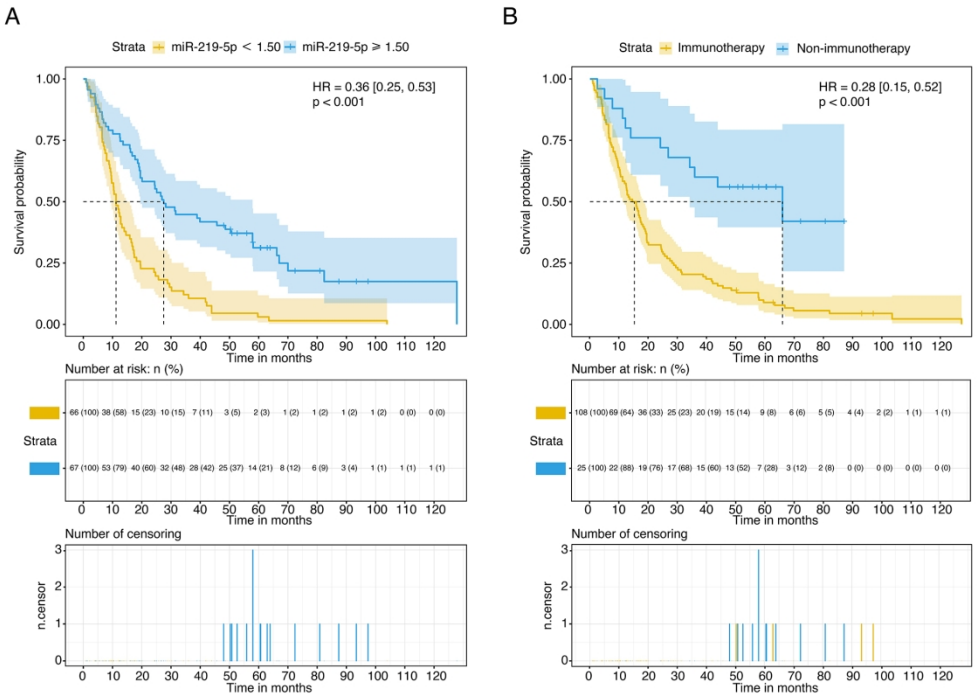

Figure 2 Overall survival (OS) of SCLC patients in different levels of miR-219-5p and different treatments. (A) OS of SCLC patients with high or low level of miR-219-5p. (B) OS of SCLC patients with different treatments (immunotherapy vs non-immunotherapy).

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Figure 3

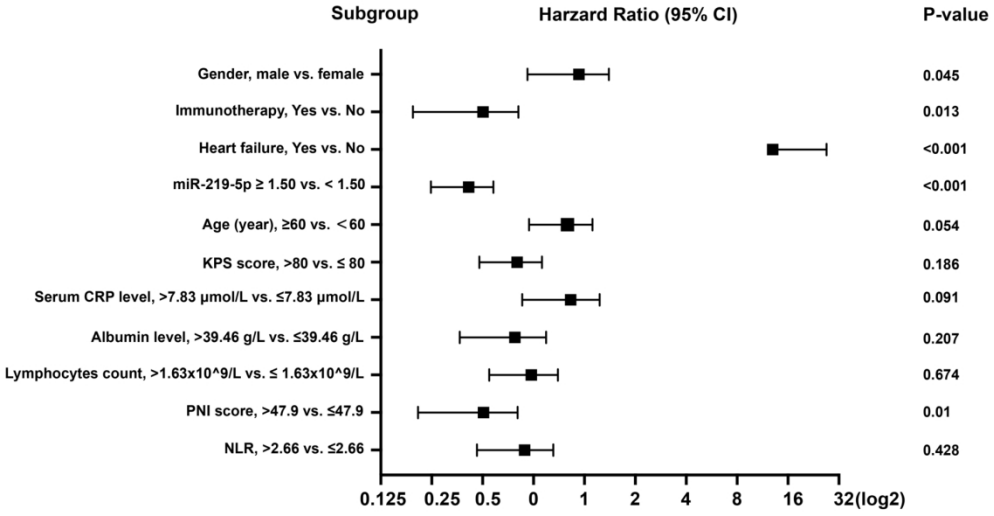

Figure 3 Multivariate cox regression analysis of 5-year overall survival on data in the SCLC patients.

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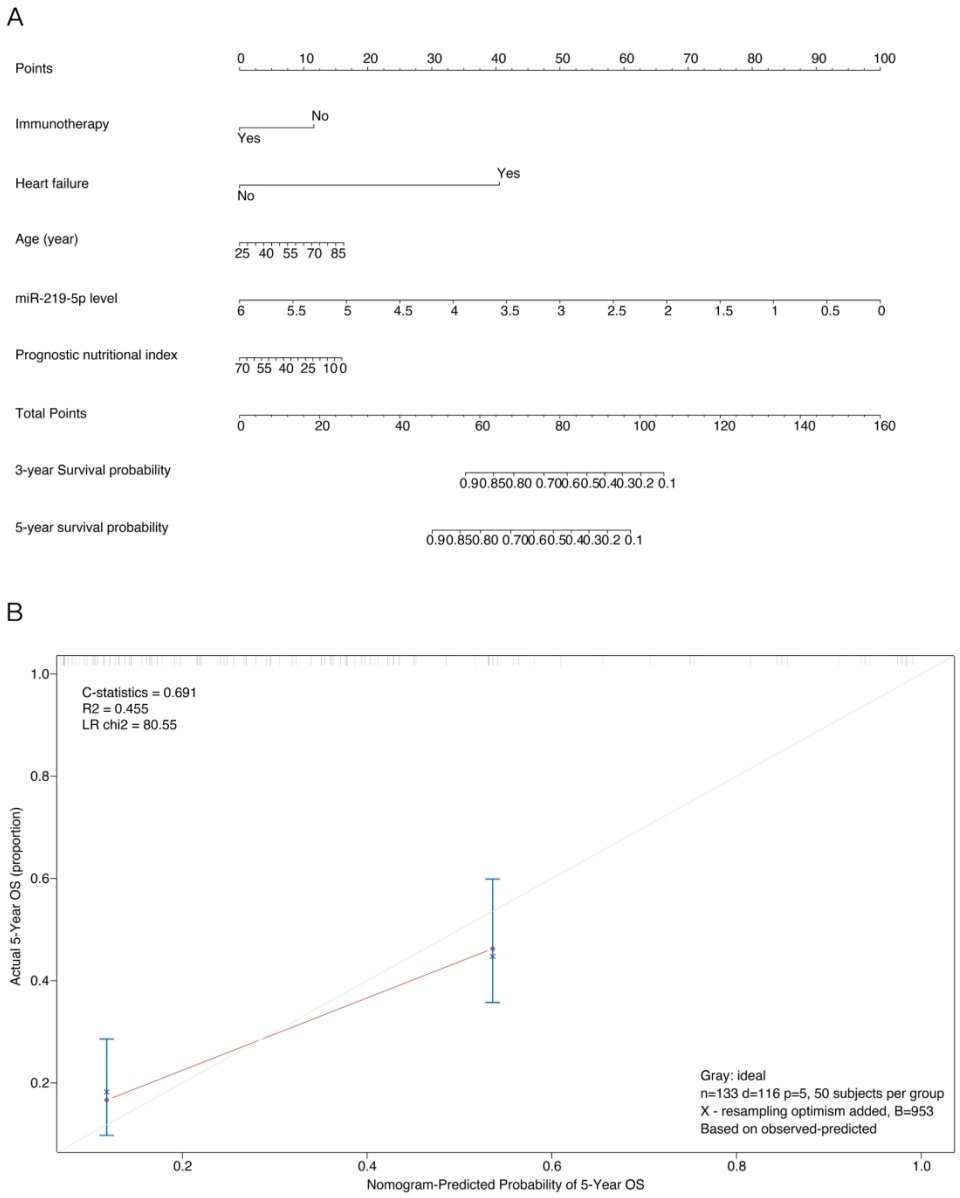

Figure 4 Nomogram for overall survival (OS) risk estimation of SCLC patients and its predictive performance. (A) Nomogram to estimate the OS risk of SCLC patients in different variations. To build the nomogram, find the position of each variable on the corresponding axis, draw a line to the points axis for the number of points, add the points from all of the variables, and draw a line from the total points axis to determine the OS probabilities at the lower line of the nomogram. (B) Validity of the predictive performance of the nomogram in estimating the OS risk of SCLC patients.

180x216mm (300 x 300 DPI)

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Figure 5

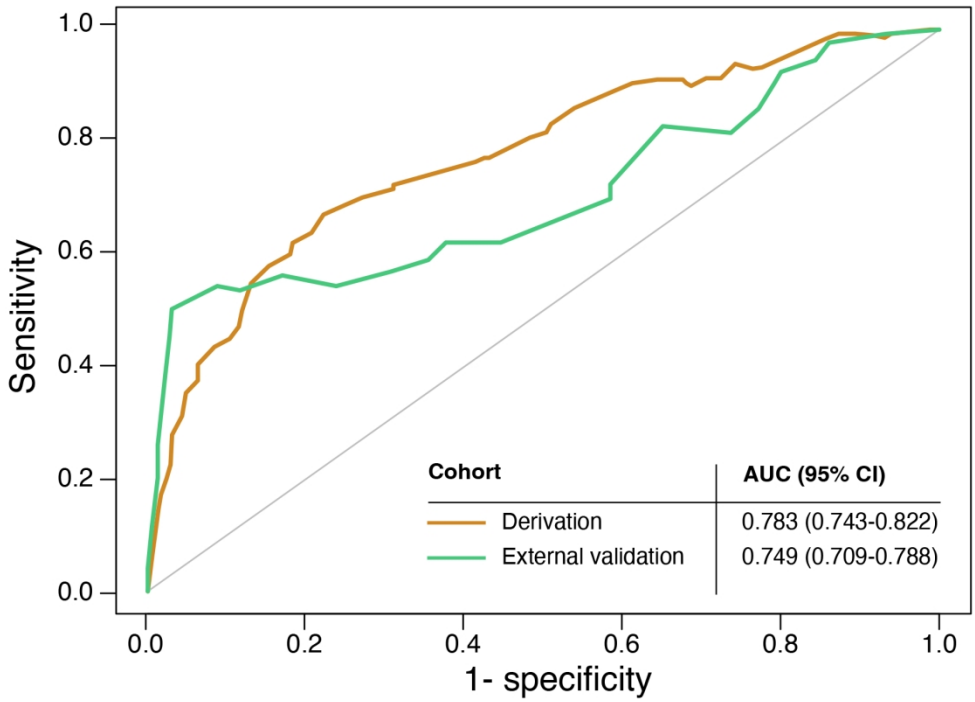

Figure 5 External validation of the prognostic model.

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Supplement Materials

Part I. Details of variants

Patients' age: The age of patients was based on the time of diagnosis of NSCLC.

Body mass index: The equation of body mass index (BMI) is calculated as follow: BMI (kg/m^2) = weight (kg) / height $(m)^2$. The data of BMI was recorded before clinical treatment.

Serum CEA level: The serum level of carcinoembryonic antigen (CEA) is one of tumor marks， the higher serum *CEA* level than that of healthy individuals led to its clinical application as a diagnostic biomarker for cancer.

Serum CRP level: C-reactive protein (CRP) is detected by olorimetric determination through fasting blood collection and CRP is one of the inflamation markers.

Albumin level: Serum albumin is detected by colorimetric determination through fasting blood collection. And serum albumin is one of the indicators of nutritional status.

Neutrophils count, lymphocytes count:, hemoglobin level, platelet count: All these indexes were extracted from blood routine examination to evaluate preoperative status of patients.

PNI score: Prognostic nutritional index (PNI) is an index to evaluate the nutritional status of surgical patients, to predict the risk of surgery and to help make prognostic judgment. The equation is listed as below: PNI = serum albumin (g/L) + 5 * lymphocytes ($*10^9/L$).

NLR: Neutrophil to Lymphocyte Ratio (NLR) is an index of inflammation markers to predict the prognosis of various cancer, including lung cancer.

Pathologic type: Non-small cell lung cancer can be classified as adenocarcinoma, squamous cell carcinoma, large cell lung cancer, or others, depending on surgical or tumor biopsy specimens

Stage of NSCLC: Non-small cell lung cancer was staged according to the seventh edition of international union against cancer (UICC) TNM was published in 2009

Surgery: For early and locally advanced NSCLC, which is generally acceptable, there is a history of surgical resection.

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Therapy of radiation: Radiation therapy is a medical procedure that uses the delivery of high-energy radiation to kill cancer cells and shrink tumors, including external beam radiation therapy, internal radiation therapy and stereotactic body radiotherapy (SBRT). It aims to cure small tumors, treat lung cancer both locally and nearby lymph nodes, treat metastases, relieve symptoms, and also at prevention.

Application of platinum: Platinum-based chemotherapy may be used as a first or second line therapy for advanced lung cancer, after surgery (adjuvant chemotherapy), or before surgery to reduce the size of a tumor (neoadjuvant therapy). The most commonly used platinum drugs are cisplatin, carboplatin and nedaplatin. Treatment of non-small cell lung cancer begins with either cisplatin or carboplatin combined with another medication, such as docetaxel, pemetrexed, paclitaxel, gemcitabine and gemcitabine.

Target therapy: Targeted therapies for lung cancer are drugs that are tailored to attack certain mutations of cancer cells. These mutations lead to the production of abnormal proteins which guide the growth and development of a cancer cell. Common driver mutations include EGFR mutations, ALK rearrangements, ROS1 rearrangements, MET amplifications, KRAS mutations, HER2 mutations, BRAF mutations and so on.

Application of TKI: Tyrosine kinase inhibitors (TKI) act as competitive inhibitors of ATP binding to tyrosine kinases, or as tyrosine analogs that block tyrosine kinase activity and inhibit cell proliferation. Epithelial growth factor receptor (EGFR) is a protein that is present on the surface of both normal cells and lung cancer cells. Mutations in EGFR can occur at different locations on exon 18 to 21. ALK-positive lung cancer refers to people who have lung cancer that tests positive for the EML4-ALK fusion mutation. Several FDA-approved medications available to treat EGFR-positive and ALK-positive lung adenocarcinoma, including:

- EFGR inhibitors - Tarceva (erlotinib), Gilotrif (afatinib), Iressa (gefitinib), Tagrisso (osimertinib), and Portrazza (necitumumab)
- ALK inhibitors - Xalkori (crizotinib), Zykadia (ceritinib), and Alecensa (alectinib)
- ROS1 inhibitor - Xalkori (crizotinib)

Application of VEGF inhibitor: VEGF (Vascular endothelial growth factor) can activate angiogenesis through different signaling pathways. VEGF inhibitors are drugs that block the ability of tumors to form new blood vessels, and hence, grow and spread. Some currently available medications include bevacizumab, ramucirumab, axitinib, endostar and anlotinib.

KPS score: A performance measure for rating the ability of a person to perform usual activities, evaluating a patient's progress after a therapeutic procedure, and determining a patient's suitability for therapy. It is used most commonly in the

prognosis of cancer therapy, usually after chemotherapy and customarily administered before and after therapy. It was named for Dr. David A. Karnofsky, an American specialist in cancer chemotherapy. Patients with more than 80 scores had better postoperative status and longer survival time. And patients with more than 70 scores can suffer from chemoradiotherapy.

Smoking: Smoker refers to continuous or cumulative smoking > 1 cigarette/day over a lifetime of more than 6 months. (1997, WHO)

Hypertension: Hypertension is defined as a repeatedly elevated blood pressure exceeding 140 over 90 mmHg.

Diabetes: Diabetes is a group of metabolic diseases characterized by hyperglycemia. And fasting blood glucose more than 7.0 mmol/l or blood glucose more than 11.1 mmol/l within 2hoursafter meal can be diagnosed diabetes.

Hyperlipemia: Hyperlipemia means the presence of excess fat or lipids in the blood. And total cholesterol ≥ 6.2 mmol/L, low density lipoprotein cholesterin ≥ 4.1 mmol/L, triglyceride ≥ 2.3 mmol/L, high density lipoprotein cholesterin < 1.0 mmol/L can be diagnosed hyperlipemia.

Heart failure: The information was recorded through history taking and verified after hospitalization. Heart failure means inability of the heart to keep up with the demands on it and, specifically, failure of the heart to pump blood with normal efficiency. Heart failure may be due to failure of the right or left or both ventricles. The signs and symptoms depend upon which side of the heart is failing. They can include shortness of breath (dyspnea), asthma due to the heart (cardiac asthma), pooling of blood (stasis) in the general body (systemic) circulation or in the liver's (portal) circulation, swelling (edema), blueness or duskiness (cyanosis), and enlargement (hypertrophy) of the heart.

ACS: Acute coronary syndrome is a term for a group of conditions that suddenly stop or severely reduce blood from flowing to the heart. When blood cannot flow to the heart, the heart muscle can become damaged. Heart attack and unstable angina are both acute coronary syndromes (ACS).

Withdraw treatment: Reasons for patients withdrew from treatment were listed below: 1) High cost of the treatment: In China, the cost of hormone therapy can be up to 3000 RMB a month if without health insurance. 2) Poor patient compliance: Some patients discontinue treatment because they do not comply with the treatment plan prescribed by their doctor.

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Part II. Report of sample size assessment

PASS 15.0.5

Tests for Two Survival Curves Using Cox's Proportional Hazards Model

Numeric Results with Ha: HR ≠ 1

Power	Total Sample Size N	Control Sample Size N1	Trtmnt Sample Size N2	Prop'n Control N1/N P1	Hazard Ratio h2/h1 HR	Control Prob Event Pev1	Trtmnt Prob Event Pev2	Control Events E1	Trtmnt Events E2	Alpha	Beta
0.9005	103	51	52	0.500	0.500	0.750	0.950	38.3	49.4	0.050	0.100

References

Chow, S.C., Shao, J., Wang, H. 2008. Sample Size Calculations in Clinical Research, 2nd Edition. Chapman & Hall/CRC.

Schoenfeld, David A. 1983. 'Sample Size Formula for the Proportional-Hazards Regression Model', Biometrics, Volume 39, Pages 499-503.

Report Definitions

Power is the probability of rejecting a false null hypothesis. Power should be close to one.
N is the total sample size.
N1 and N2 are the sample sizes of the control and treatment groups.
P1 is the proportion of the total sample that is in the control group, group 1.
HR is the hazard ratio: h2/h1.
Pev1 and Pev2 are the probabilities of an event in the control and the treatment groups.
E1 and E2 are the number of events required in the control and the treatment groups.
Alpha is the probability of a type one error: rejecting a true null hypothesis.
Beta is the probability of a type two error: failing to reject a false null hypothesis.

Summary Statements

A two-sided test of whether the hazard ratio is one with an overall sample size of 103 subjects (of which 51 are in the control group and 52 are in the treatment group) achieves 90% power at a 0.050 significance level when the hazard ratio is actually 0.500. The number of events required to achieve this power is 87.7. It is anticipated that proportions of subjects having the event during the study is 0.750 for the control group and 0.950 for the treatment group. These results assume that the hazard ratio is constant throughout the study and that Cox proportional hazards regression is used to analyze the data.

Procedure Input Settings

Autosaved Template File

\\Mac\Home\Documents\PASS 15\Procedure Templates\Autosave\Tests for Two Survival Curves Using Cox's

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Proportional Hazards Model - Autosaved 2020_1_24-9_57_39.t92

Design Tab

Solve For:	Sample Size
Alternative Hypothesis:	Ha: HR ≠ 1
Power:	0.90
Alpha:	0.05
Group Allocation:	Equal (N1 = N2)
Pev1 (Probability of a Control Event):	0.750
Pev2 (Probability of a Treatment Event):	0.950
HR (Actual Hazard Ratio = h2/h1):	0.5

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Figure S1a

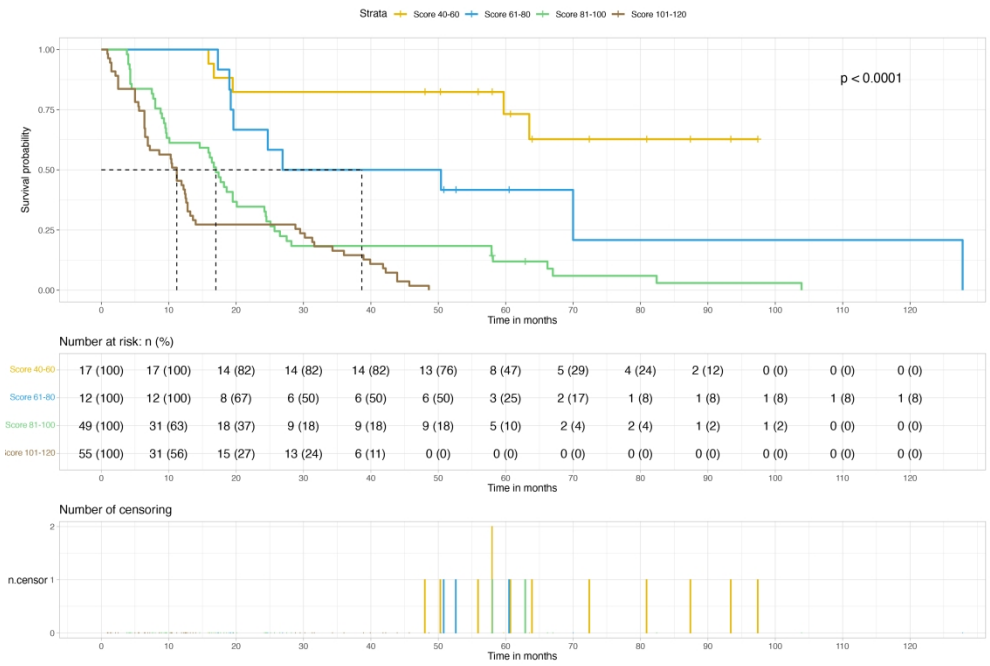

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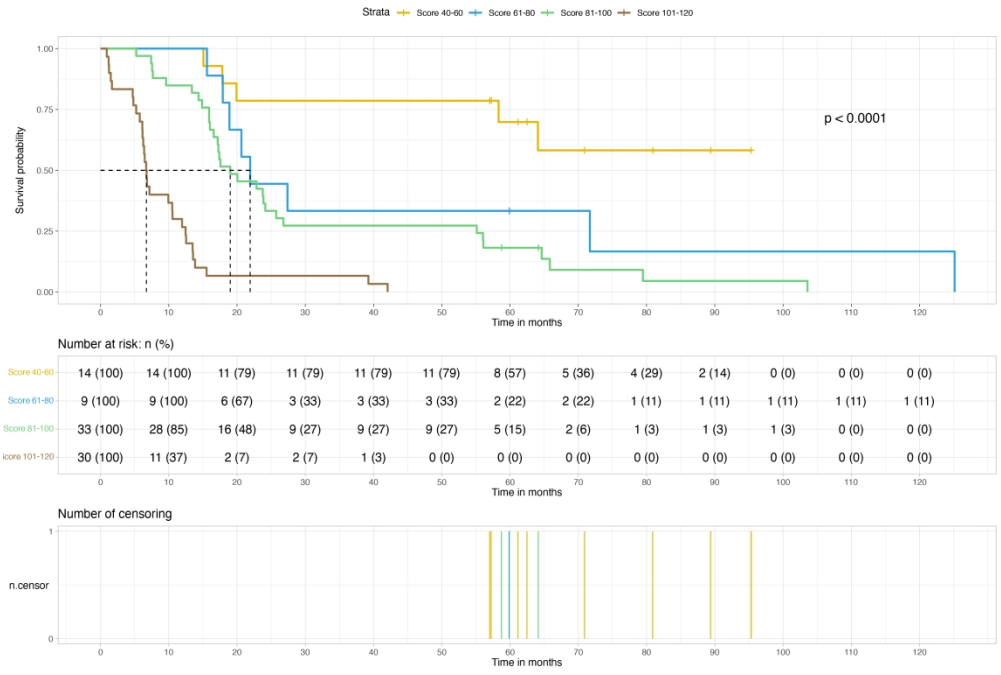
Figure S1b



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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			**5**
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*6*
Bias	9	Describe any efforts to address potential sources of bias	7
Study size	10	Explain how the study size was arrived at	7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7
		(*b*) Describe any methods used to examine subgroups and interactions	7
		(*c*) Explain how missing data were addressed	7
		(*d*) If applicable, explain how loss to follow-up was addressed	7
		(*e*) Describe any sensitivity analyses	7
Results			

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8
		(b) Give reasons for non-participation at each stage	8
		(c) Consider use of a flow diagram	8
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9
		(b) Indicate number of participants with missing data for each variable of interest	9
		(c) Summarise follow-up time (eg, average and total amount)	9
Outcome data	15*	Report numbers of outcome events or summary measures over time	10
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10
		(*b*) Report category boundaries when continuous variables were categorized	11
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	11
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	12
Discussion			
Key results	18	Summarise key results with reference to study objectives	13-14
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15
Generalisability	21	Discuss the generalisability (external validity) of the study results	15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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