Original Paper

Clinical Significance of miR-210 and its Prospective Signaling Pathways in Non-Small Cell Lung Cancer: Evidence from Gene Expression Omnibus and the Cancer Genome Atlas Data Mining with 2763 Samples and Validation via Real-Time Quantitative PCR

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Key Words

Nsclc • MiR-210 • RT-qPCR • Diagnosis • Prognosis • Bioinformatics

Abstract

Background/Aims: Since the function of microRNA (miR)-210 in non-small cell lung cancer (NSCLC) remains unclear, we aimed to explore the clinical significance of miR-210 in NSCLC. **Methods:** NSCLC-related data from 1673 samples on Gene Expression Omnibus and 1090 samples on The Cancer Genome Atlas were obtained and analyzed. The expression level of miR-210 was validated via real-time quantitative PCR analysis with 125 paired clinical samples. A meta-analysis was performed to generate a comprehensive understanding of miR-210 expression and its clinical significance in NSCLC. In addition, bioinformatics analysis was also conducted to reveal the potential underlying mechanism of miR-210 action in NSCLC. **Results:** miR-210 expression was consistently elevated in NSCLC solid tissue samples. However, its expression was controversial in easily obtained body fluids (i.e., blood, plasma, and serum). Moreover, an overall pooled meta-analysis implied a comparatively higher level of miR-210 expression in NSCLC cancerous tissue than in normal control tissue ($P < 0.001$). In addition, a meta-analysis of outcome revealed a significant diagnostic capacity of miR-210 in NSCLC by detecting its expression in serum and sputum (area under the summary receiver operating

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characteristic curve 0.82 and 0.81, respectively). miR-210 overexpression was associated with poor progression-free survival (PFS) in NSCLC and was negatively related to overall survival and disease-free survival. Bioinformatic gene enrichment and annotation analyses showed that the target genes of miR-210 were greatly enriched in cell adhesion and plasma membrane, and three pathways were considered to be the main functional circuits of miR-210: renin secretion, the cGMP-PKG signaling pathway, and cell adhesion molecules. **Conclusion:** In NSCLC, miR-210 expression was elevated and overexpression indicated poor PFS. Expression level of miR-210 in serum and sputum showed significant diagnostic value for NSCLC.

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Introduction

Lung cancer, comprising the main two types of non-small cell lung cancer (NSCLC) and small cell lung cancer, is the primary cause of malignant tumor-related mortality worldwide, the incidence of which is continuing to increase. The three main classifications of NSCLC—squamous cell carcinoma, adenocarcinoma, and large cell carcinoma—account for 80%–85% of all cases of lung cancer [1]. In 2017, there were 222, 500 and 155, 870 estimated new lung cancer cases and deaths, respectively, and the overall 5-year survival rate was 18% [2]. The low survival rate is mainly due to diagnosis at a late stage, with the 5-year survival rate being only 4% in such patients [2]. Hence, the early diagnosis and treatment of lung cancer is imperative and also challenging. Despite all of the advances of recent years, there is still a large number of patients with lung cancer who are diagnosed at an advanced stage. In addition, the molecular biological processes by which lung tumors develop have yet to be determined. Thus, there is an urgent need to clarify the possible mechanisms underlying the development of NSCLC, discover novel biomarkers for early screening, and develop therapeutic targets.

MicroRNAs (miRNAs) are endogenous small-noncoding RNAs of 20–25 nucleotides that can inhibit gene expression at either the mRNA or protein level [3, 4]. miRNAs can promote or inhibit cell proliferation, differentiation, apoptosis, and necrosis by either inhibiting mRNA transcription or stalling translation [5–7]. Aberrant miRNA expression is associated with the occurrence of cancer in humans, such as colorectal cancer [8] and NSCLC [9]. Recently, miRNA (miR)-210, which is located on chromosome 11p15.5, was found to have multiple roles in various biological processes, such as angiogenesis and regulation of the immune system, by modulating cell proliferation, differentiation, degradation, and necrosis [10], and miR-210 was involved in the myocardial energy metabolism regulating the energy shift in cardiomyocytes during oxidative stress [11]. Deregulation of miR-210 may aberrantly activate oncogenes, deactivate tumor suppressor genes, inhibit DNA repair, and promote angiogenesis, ultimately accelerating the process of carcinogenesis, especially in a hypoxic environment [12]. miR-210 is aberrantly expressed in several cancers, such as malignant melanoma [13], pancreatic tumors [14], mammary cancer [15], and kidney carcinomas [16]. Several studies have reported the up-regulation of miR-210 expression using multiple microarrays [17–19]. In addition, a meta-analysis revealed the potential diagnostic capacity of miR-210 in NSCLC patients by detecting its expression level in blood and sputum [20].

In several previous studies, miR-210 expression was explored by analyzing fold changes in its levels, along with many other miRNAs, with the use of multiple microarrays. Because the specific expression level and clinical significance of miR-210 in NSCLC has not been fully elucidated, we decided to perform a comprehensive meta-analysis to reveal the specific expression level and clinical significance of miR-210 in NSCLC. In this study, we explored the expression level of miR-210 in NSCLC by mining data from 1673 samples on Gene Expression Omnibus (GEO) and 1090 samples on The Cancer Genome Atlas (TCGA), and then validated these findings via real-time quantitative PCR (RT-qPCR) analysis conducted on 125 paired clinical samples. To fully understand appreciate the clinical role of miR-210 in NSCLC, we also performed a meta-analysis by pooling the findings of previous studies: eligible GEO and TCGA datasets, RT-qPCR results, and qualified references. We then evaluated the diagnostic

and prognostic value of miR-210 in NSCLC, and performed bioinformatics analysis to illustrate the function of miR-210 in NSCLC by predicting its potential target genes and functional pathways.

Materials and Methods

Screening strategy for microarrays in the GEO datasets and references

The microarray profiles and datasets of NSCLC were acquired from GEO (<http://www.ncbi.nlm.nih.gov/geo/>). Public references in PubMed, Wiley Online Library, Web of Science, EMBASE, Chinese CNKI, Wan Fang, and Chong Qing VIP were screened for eligible studies that provided the expression level and prognostic value of miR-210 in NSCLC patients. The following search strategy was used: (miR-210 OR miR210 OR microRNA-210 OR microRNA210 OR miRNA-210 OR miRNA210) AND (lung OR pulmonary OR bronchi OR respiratory OR alveoli) AND (cancer OR carcinoma OR tumor OR neoplas* OR malignan* OR adenocarcinoma OR squamous).

Inclusion criteria and data extraction

The inclusion criteria were: (1) research participants with a diagnosis of NSCLC; (2) case control studies or studies including both a cancer group and control group (normal healthy participants or adjacent noncancerous tissue) with each containing at least 2 samples; (3) specific expression data of miR-210 in the case and control groups given as mean and standard deviation (SD); and (4) human research participants.

The required data were extracted independently from all eligible datasets and studies by two experienced investigators. Disagreements were settled by discussion with a third arbitral researcher. Detailed information was extracted from each eligible study: citation (ref.), unique PMID, year, country, region, sample type, data source, platform, miR-210 expression values, and sample size. For the selection of microarray expression data, the average value of the probes was regarded as the expression value of miR-210 when more than 1 probe was used for detection.

Data obtained from TCGA and preprocessing

Level 3 expression data for miR-210 together with all corresponding accessible clinical information were retrieved for 999 NSCLCs and 91 normal control patients from TCGA. The corresponding paired tumor samples of 91 adjacent noncancerous tissue samples were selected from 999 NSCLC samples (45 lung squamous cell carcinoma samples and 46 lung adenocarcinoma samples) for paired-design analysis that would give a more precise outcome.

Clinical NSCLC tissue samples and RT-qPCR analysis

To validate the results of the online GEO and TCGA microarray databases, a total of 125 cancerous and corresponding adjacent normal lung tissues were collected from the Department of Pathology, First Affiliated Hospital of the Guangxi Medical University (Nanning, Guangxi, China) with the permission of the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University.

Total RNA was extracted using a TRIzol reagent RNeasy FFPE Kit (QIAGEN, KJ Venlo, Netherlands). The OD₂₆₀/OD₂₈₀ ratio was obtained to measure the concentration and purity of extracted total RNA. RT-qPCR was conducted using a TaqMan MicroRNA Reverse Transcription Kit (Cat. No. 4366596; Tideradar Beijing Technology Co., Ltd., Beijing, China). Specific primers for miR-210 and the combination of miR-191 and miR-103 (internal control) were designed using Prime 3.0 and were provided by Invitrogen (Carlsbad, CA). A LightCycler 480 (Roche, Shanghai, China) was applied to analyze the PCR reactions. PCR efficiency values (E) for each gene were calculated using the formula $E = 10(-1 / \text{slope})$. The expression level of miR-210 was calculated using the 2-ΔCq method.

Target gene prediction and bioinformatics analysis

For further analysis, differentially expressed genes (DE-genes) were determined from GEO microarrays (GSE33247 and GSE43622). The expression variation of specific genes was considered statistically significant when the absolute value of Log2 fold change was greater than 1.0 and the false discovery rate of the P value was less than 0.05. Since the analysis of eligible literature and microarray datasets revealed

a trend for miR-210 to be overexpressed in NSCLC, miR-210 target genes were likely to be among the DE-genes with a comparatively lower expression level. Thus, down-regulated DE-genes in GSE33247 and GSE43622 were selected for further analysis.

Online prediction software (miRWalk, RNAhybrid, MicroT4, PITA, miRDB, miRanda, mirbridge, miRMAP, miRMap, Pictar2, RNA22, and TargetScan) and validated software (TarBase, miRTarBase, and miRecords) in miRWalk2.0 (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>) were respectively used to acquire predicted and validated target genes of miR-210. Then, we removed the repetitions and selected the genes that appeared in more than two of the above online prediction tools to overlap with the selected down-regulated DE-genes. The overlapping genes, which were regarded as potential targets of miR-210, were aggregated and annotated through biological process, cellular component, and molecular function in gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Both GO and KEGG analysis in The Database for Annotation, Visualization, and Integrated Discovery 6.7 (DAVID 6.7; <https://david.ncifcrf.gov/>) were used. After that, we employed Microsoft Excel 2016 to draw pie charts of the most significant 10 GO terms and KEGG pathways. Cytoscape was used to visualize the functional annotation and signaling pathways. Finally, the hub genes were input to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; <http://www.string-db.org/>) for the construction of the protein-protein interaction relationship network. We also analyzed the expression levels of the final potential target genes in GEPIA (<http://gepia.cancer-pku.cn/>), and the protein expression levels of 6 genes were assessed using the Human Protein Atlas (HPA; <https://www.proteinatlas.org/>). Corresponding scatter point plots of the target genes were constructed. Disease ontology analysis was conducted by using the ClusterProfiler package of the R language. Q and P values less than 0.05 were considered to indicate statistical significance. Moreover, further alterations in the levels of genes by miR-210 across NSCLC samples were examined using cBioPortal (<http://cbioportal.org>).

Statistical analysis

Means and SDs were estimated using SPSS 22.0. An independent-sample t-test was applied for random design studies, while a paired-sample t-test was used for the paired-design studies. Kaplan–Meier curve analysis was conducted for survival analysis, and multivariate Cox regression analysis was performed to evaluate the connection between the miR-210 expression state and prognostic outcome of NSCLC patients.

Overall standard mean deviation (SMD) was used as an overall effect size to assess the expression level of miR-210 in NSCLC. Q and I^2 values were estimated to evaluate inter-study heterogeneity. Significant heterogeneity was defined when $P < 0.1$ or $I^2 > 50\%$ [21, 22]. Consequently, a random effects model was selected; otherwise, a fixed effects model was applied [23, 24]. In addition, if significant heterogeneity was observed, subgroup analysis was performed to explore the possible sources of heterogeneity. To evaluate the stability of the overall estimates, sensitivity analysis was conducted by omitting one study at a time [25]. Furthermore, the existence of publication bias was estimated by generating a funnel plot and Egger's and Begg's test [26].

A bivariate model was used to combine the summary diagnostic values of the sensitivity and specificity of miR-210 expression for NSCLC [26], and a SROC curve was also constructed. The Midas package [27] was installed and applied in STATA 14.0 for analysis [28]. In addition, hazard ratios (HRs) along with 95% confidence intervals (CIs) and other information from the published references were retrieved directly or extracted from the given Kaplan–Meier curve using Engauge 4.1. Then, the combined HR was generated in STATA 14.0 to evaluate the prognostic value of miR-210 in NSCLC.

All analyses were conducted by using SPSS 22.0 and STATA 14.0.

Results

Acquisition of all eligible data

The raw expression data for miR-210 were acquired from GEO and TCGA datasets and we also searched for relevant published research papers. In addition, RT-qPCR was performed on 125 paired cancer and adjacent noncancerous tissue samples collected from NSCLC patients to validate the results obtained from the GEO and TCGA datasets. The expression level of miR-210 in NSCLC was estimated by SMD, which was obtained by merging all of

Fig. 1. Summary of study structure and flow chart of GEO datasets and reference screening.

the accessible data derived from the above sources. To evaluate the capability of miR-210 for the diagnosis and prognosis of NSCLC, a receiver operating characteristic (ROC) curve and prognostic forest plots were constructed, respectively. The details of the dataset and reference screening processes and the overall design of the subsequent analyses are shown in Fig. 1.

In total, 28 eligible records and references were included in the analysis: GEO datasets ($n = 24$), TCGA ($n = 1$), in-house RT-qPCR ($n = 1$), and qualified references ($n = 2$ [29, 30]). Their characteristics are summarized in Table 1. Overall, the 28 enrolled studies included 2551 NSCLC samples and 970 control samples with sample type being mainly dissected solid tissue (17 tissue, 5 blood, 3 plasma, and 3 serum) and the enrolled subjects were mainly from "Euramerica" (9 USA, 4 Germany, 1 Finland, 1 Netherlands, 1 Norway, and 1 Switzerland) and Asia (6 China and 4 Japan). In addition, 125 paired clinical cases of cancerous and para-cancerous NSCLC tissues were collected from the First Affiliated Hospital of Guangxi Medical University with the approval of the hospital ethics committee and consent of the patients.

Data processing

Since raw expression data was acquired from GEO and TCGA databases, means and SDs were calculated to estimate the expression state of miR-210 in NSCLC by using SPSS 22.0. An independent-sample *t* test was applied for random design studies, while a paired-samples *t* test was used for the paired design studies. The results for these analyses are shown in Table 1 and intuitive scatter point plots are shown in Figs. 2-4.

As shown in Fig. 2, solid NSCLC tissues exhibited a consistently higher expression level of miR-210 than normal tissue in most GEO datasets. The analysis of 91 TCGA paired NSCLC samples and our own RT-qPCR results of 125 clinical paired NSCLC tissue samples validated this finding (Fig. 3). For the TCGA paired samples, the mean $\pm$ SD of tumor and adjacent normal tissues were 11.69 ± 1.84 versus 7.42 ± 1.48 , respectively ($P < 0.001$), while RT-qPCR analysis of 125 clinical paired tissues gave values of 6.24 ± 3.50 versus 3.51 ± 2.76 , respectively ($P < 0.001$). These findings indicate that miR-210 expression was considerably higher in NSCLC tissue than in adjacent normal tissue.

The expression of miR-210 in easily obtained body fluids (i.e., blood, plasma, and serum) is shown in Fig. 4, while its expression in tissue is shown in Fig. 2. The expression of miR-210

Table 1. Details of the datasets used in the present meta-analysis of NSCLC: eligible GEO datasets, results of RT-qPCR and TCGA analyses, and qualified references. P < 0.05 was considered as significant. N0, N1: number of enrolled samples in the normal control group and experimental group, respectively. Ref NO.: The sequential reference numbers of citations. Citations of microarray datasets are displayed in the end of References list, and there are no citations available for the following 4 datasets: GSE19945 [101], GSE74190 [108], GSE77380 [109], GSE93300 [119], thus website address was affiliated

Study ID	First author	Year	Ref NO.	Country	Nation	Sample type	Control N0.	Experiment N1.	T value	P
GSE14936	Seike M	2009	[98]	USA	Euramerican	tissue	26	26	-2.445	0.018
GSE15008	Tan X	2010	[99]	China	Asian	tissue	112	112	-11.960	0.000
GSE16025	Raponi M	2009	[100]	USA	Euramerican	tissue	10	61	-6.518	0.000
GSE19945	Ohba T	2015	[101]	Japan	Asian	tissue	8	20	-6.024	0.000
GSE25508	Nymark P	2011	[102]	Finland	Euramerican	tissue	26	26	-6.468	0.000
GSE47525	van Jaarsveld MT	2013	[103]	Netherlands	Euramerican	tissue	14	18	-1.809	0.080
GSE48414	Bjaanaes MM	2014	[104]	Norway	Euramerican	tissue	20	154	-12.519	0.000
GSE51853	Arima C	2014	[105]	Japan	Asian	tissue	5	124	-4.750	0.000
GSE63805	Robles AI	2015	[106]	USA	Euramerican	tissue	30	30	-4.325	0.000
GSE72526	Gasparini P	2015	[107]	Switzerland	Euramerican	tissue	18	52	-2.785	0.008
GSE74190	Jin Y	2015	[108]	China	Asian	tissue	38	38	-8.090	0.000
GSE77380	Yoshimoto T	2016	[109]	Japan	Asian	tissue	12	12	-3.423	0.003
GSE2564	Lu J	2005	[110]	USA	Euramerican	tissue	4	14	2.078	0.054
GSE29248	Ma L	2012	[111]	China	Asian	tissue	6	6	-1.700	0.151
GSE56036	Fujita Y	2017	[112]	Japan	Asian	tissue	29	29	-9.600	0.000
TCGA	NR	NR	NR	NR	NR	tissue	91	999	-28.679	0.000
TCGA	NR	NR	NR	NR	NR	tissue	91	91	-24.171	0.000
PCR	NR	NR	NR	China	Asian	tissue	125	125	-6.55	0.000
GSE17681	Keller A	2009	[113]	Germany	Euramerican	blood	10	17	-0.985	0.335
GSE27486	Patnaik SK	2011	[114]	USA	Euramerican	blood	23	22	-0.684	0.001
GSE31568	Keller A	2011	[115]	Germany	Euramerican	blood	70	32	-1.620	0.109
GSE40738	Patnaik SK	2012	[116]	USA	Euramerican	blood	56	81	0.113	0.181
GSE61741	Keller A	2014	[117]	Germany	Euramerican	blood	94	72	-1.210	0.228
GSE68951	Leidinger P	2015	[118]	Germany	Euramerican	plasma	12	26	1.774	0.085
GSE93300	Liu X	2017	[119]	USA	Euramerican	plasma	4	9	0.945	0.365
PMID:27499953	Wang X	2016	[29]	China	Asian	plasma	59	59	-3.669	0.001
GSE16512	Lodes MJ	2009	[120]	USA	Euramerican	serum	14	3	-2.690	0.017
GSE46729	Godfrey A	2014	[121]	USA	Euramerican	serum	24	24	-2.039	0.048
PMID:24065453	Li ZH	2014	[30]	China	Asian	serum	30	60	NR	<0.005

in blood, plasma, and serum of NSCLC patients varied, with 1 dataset exhibiting significantly low miR-210 expression in blood, 2 exhibiting significantly low miR-210 expression in blood, and the remaining 6 exhibiting no conspicuous difference. It is unfortunate that TCGA microarray analysis was conducted using only solid tissue from NSCLC patients because the corresponding expression data of miR-210 in blood and blood-component samples are lacking. Further validation via RT-qPCR was not performed due to the lack of easily obtained samples from clinical patients. Therefore, further study is needed to draw a conclusion on the expression level of miR-210 in easily obtained body fluids.

Clinical significance of miR-210 in NSCLC

Because the expression of miR-210 was varied in easily obtained body fluids and tissues in the GEO datasets, a meta-analysis was used to assess all of the data from the GEO datasets, the results from TCGA, RT-qPCR, and eligible publications to give an overall conclusion. The results are given in Table 2 and shown visually in Fig. 5A-5D. A forest plot merging all of the qualified data indicated that the expression level of miR-210 in NSCLC samples was

comparatively higher than in normal control samples (overall pooled SMD = 0.963, 95% CI: 0.536-1.389, $P < 0.001$) and significant heterogeneity was detected ($I^2 = 95\%$). Therefore, a random model was applied, and exploring the source of the high heterogeneity became an imperative process for the present meta-analysis.

To investigate the potential source of heterogeneity, subgroup analysis was performed. As shown in Fig. 5B and 5C, we first conducted subgroup analysis stratified according to sample type. There was a dramatic drop of heterogeneity in the easily obtained body fluid groups—blood ($I^2 = 74.00\%$), plasma ($I^2 = 81.80\%$), and serum ($I^2 = 60.10\%$)—and a small drop in the tissue-only group ($I^2 = 93.90\%$). A significant difference was seen in the serum-only subgroup (SMD = 1.091, 95% CI: 0.447-1.735, $P = 0.001$) and tissue-only subgroup (SMD = 1.425, 95% CI: 0.915-1.9350, $P < 0.001$), but not in the blood-only or plasma-only meta-analysis ($P > 0.05$). The results of nation-based subgroup analysis are shown in Fig. 5D. Both the Euramerica-only group (SMD = 0.470, 95% CI: 0.080-0.860, $P = 0.018$) and Asian-only group (SMD = 1.522, 95% CI: 1.33-1.911, $P < 0.001$) showed significant outcomes, and there was a clear drop in heterogeneity in each group ($I^2 = 86.90\%$ and 81.80% , respectively). These findings indicate that differences caused by sample type and nation might, in part, be the sources of heterogeneity. Unfortunately, each platform used in the dataset was different; thus, sub-group analysis according to different detection approaches was not possible.

In the subsequent meta-analysis investigating miR-210 expression in NSCLC patients with different clinical pathologies (Table 2), miR-210 expression was comparatively higher in ever-smoking NSCLC cases than in non-smoking cases, while no significant differences were seen for the other clinical parameters (age, sex, lymph node metastasis, tumor, mode, metastasis stage, and tumor size; $P > 0.05$).

Sensitivity analysis indicated valid inter-study stability (Fig. 6A). A funnel plot (Fig. 6B) and Egger's and Begg's test (Table 2) indicated no significant publication bias ($P > 0.05$).

Fig. 2. Scatter point plots exhibiting miR-210 expression in tissue.

Fig. 3. TCGA and RT-qPCR analyses of paired samples.

Diagnostic role of miR-210 in NSCLC

A diagnosis meta-analysis was performed to assess the diagnostic value of miR-210 to distinguish NSCLC patients from normal controls. A total of 40 records were included and 4741 patients were involved in this meta-analysis: 13 published articles [20, 29-40], 24 raw data GEO datasets and TCGA data, and our own RT-qPCR results for clinical patients. Among these records, details were provided on

Fig. 4. Scatter point plots exhibiting miR-210 expression in blood, plasma, and serum.

Fig. 5. A: NSCLC vs. normal, random-effects model. B: Subgroup analysis layered by sample type, blood and tissue, random-effects model. C: Subgroup analysis layered by sample type, plasma and serum, random-effects model. D: Subgroup analysis, subgrouped into the Euramerican and Asian groups, random-effects model.

Table 2. Expression level of miR-210 and clinical assessment: forest plots and publication bias analysis. Random: random-effects model; fixed: fixed-effects model; SMD: standard mean deviation; 95% CI: 95% confidence interval; LL: lower limit; UL: upper limit; NSCLC: non-small cell lung cancer; LNM: lymph node metastasis; TNM: tumor, node, metastasis; Het X²: X² of heterogeneity Q test; P_{het}: P-value of heterogeneity; Egger's P_{bias}, Begg's P_{bias}: P value of Egger's test and Begg's test, respectively

Clinical parameters	Study	Samples	Analysis	Overall estimate				Heterogeneity			Metabias	
	number	number	model	SMD	95%CI(LL-UL)	Z	P	Het X ²	I ² (%)	P _{het}	Egger P _{bias}	Begg P _{bias}
normal/NSCLC (overall)	28	3221	Random	0.963	(0.536-1.389)	4.42	0.000	535.95	95.00%	0.000	0.361	0.707
normal/NSCLC(blood only)	5	477	Random	-0.038	(-0.431-0.355)	0.19	0.849	15.37	74.00%	0.004	0.725	0.806
normal/NSCLC(serum only)	3	141	Random	1.091	(0.447-1.735)	3.32	0.001	5.01	60.10%	0.082	0.866	1.000
normal/NSCLC (plasma only)	3	183	Random	-0.047	(-0.991-0.897)	1.69	0.100	10.97	81.80%	0.004	0.312	1.000
normal/NSCLC (tissue only)	17	2420	Random	1.425	(0.915-1.935)	5.48	0.000	261.68	93.90%	0.000	0.298	0.592
normal/NSCLC (Asian only)	10	1009	Random	1.522	(1.133-1.911)	7.67	0.000	49.40	81.80%	0.000	0.098	0.474
normal/NSCLC (Euramerican only)	17	1122	Random	0.470	(0.080-0.860)	2.36	0.018	122.60	86.90%	0.000	0.548	0.733
age <60 vs. ≥60	9	1374	Random	0.050	(-0.213-0.314)	0.37	0.709	19.68	59.30%	0.012	0.251	0.426
Female vs. Male	11	1457	Random	0.166	(-0.061-0.393)	1.43	0.152	21.24	52.90%	0.020	0.473	0.755
Non-LNM vs. LNM	3	1122	Random	0.101	(-0.337-0.540)	0.45	0.651	11.35	82.40%	0.003	0.877	1.000
Smoking	4	1033	fixed	0.274	(0.136-0.413)	3.88	0.000	1.97	0.00%	0.579	0.464	0.734
TNM stage I vs. II	3	840	fixed	0.049	(-0.095-0.193)	0.67	0.502	3.62	44.80%	0.163	0.486	1.000
TNM stage I/II vs. III/IV	4	1160	Random	0.190	(-0.143-0.523)	1.12	0.246	7.68	60.90%	0.053	0.272	0.734
Tumor size <3cm vs. ≥3cm	3	225	fixed	0.299	(0.018-0.579)	2.09	0.037	0.60	0.00%	0.742	0.608	1.000

miR-210 expression for solid tissue (n = 17), blood (n = 5), plasma (n = 6), serum (n = 6), and sputum (n = 6), as shown in Table 3.

An ROC curve of every dataset was generated and the 4-fold table data were also estimated. The ROC curves of datasets with easily obtained sample types (Fig. 7) show varied diagnostic value. Overall, the ROC curve of datasets with dissected solid tissue showed relatively high diagnostic value (Fig. 8). The ROC curve of 125 clinical paired NSCLC tissues and 91 TCGA paired NSCLC samples confirmed the positive results of the GEO datasets with dissected solid tissue (Fig. 9). Because there was some controversy in the diagnostic value of miR-210, SROC plots containing all of the data from the GEO datasets and the results of TCGA and RT-qPCR analyses, as well as eligible publications, were constructed to draw a credible conclusion.

The overall combined area under the curve (AUC) was 0.83 (95% CI: 0.79-0.86), and sensitivity and specificity were 0.72 and 0.80, respectively (Table 4 and Fig. 10). To consider the potential application of miR-210 for clinical diagnosis, we excluded records providing the expression level of miR-210 in solid tissue and merged the data that were obtained from the easily obtained body fluids. The combined AUC of the tissue-excluded data was 0.77 (95% CI: 0.73-0.80), with sensitivity and specificity of 0.65 and 0.76, respectively. However, significant heterogeneity was observed in the overall estimation for only the easily obtained body fluid estimation (tissue-excluded estimation) with large Q and I² values. Thus, subgroup analysis was performed to investigate the potential sources of this heterogeneity.

Fig. 6. A: Funnel plot. B: Visualized graph of influence analysis.

Subgroup analysis according to the sample type of the records revealed overall AUCs for blood, plasma, serum (Fig. 11A) and sputum (Fig. 11B) were 0.61, 0.73, 0.82, and 0.81, respectively. As shown in Table 4, the heterogeneity in the plasma and sputum groups declined dramatically with the I^2 value being reduced to 0.00%, while that in the blood and serum groups showed only a smaller drop with an I^2 value of 98%. Thus, the expression of miR-210 in sputum was relatively consistent ($I^2 = 0.00\%$) and had high diagnostic capacity (0.81, 95% CI: 0.78-0.84).

Fig. 7. ROC curves exhibiting the diagnostic value of miR-210 in blood, plasma, serum, and sputum.

Prognostic significance of miR-210 in NSCLC

Forest plots were generated to evaluate the prognostic role of miR-210 in NSCLC. There were a total of 2 GEO datasets and 4 public studies [35, 41-43], as well as our own RT-qPCR results with an overall number of 396 patients included in this meta-analysis (Table 5). A fixed model was selected for estimation as no significantly high heterogeneity was observed. All of the effects for overall survival (OS), disease-free survival (DFS), and progression-free survival (PFS) showed significant differences in survival outcome with HRs of 0.602 (OS, 95% CI: 0.410-0.884), 0.360 (DFS, 95% CI: 0.194-0.667), and 2.020 (PFS, 95% CI: 1.366-2.988; Table

6 and Fig. 12A-12C). This indicates that miR-210 overexpression was associated with poor PFS in NSCLC, while it was negatively related to OS and DFS. Moreover, no instability was seen in the constructed influence plot (Fig. 13A), and no publication bias was detected in a funnel plot (Fig. 13B) or Begg's and Egger's test.

Bioinformatics analysis of miR-210 in NSCLC

After integrating the outcomes of the 12 online prediction software tools, there were 17,010 potential target genes. We selected 8645 genes that were identified in more than two prediction tools to overlap with the DE-genes (Fig. 14). Eventually, a total of 709 genes were considered as candidate target genes of miR-210. Bioinformatics analysis was then employed for these genes using DAVID 6.7.

Ten GO terms were

Fig. 8. A and B: ROC curves exhibiting the diagnostic value of miR-210 in tissue.

Fig. 9. ROC curves of TCGA and RT-qPCR paired-design studies.

Table 3. ROC 4-fold table for all available data. N0, N1: number of enrolled samples in the normal control group and experimental group, respectively; AUC: area under the receiver operating characteristic curve; TP: true positive value; FN: false negative value; FP: false positive value; TN: true negative value; NR: information not reported. Ref NO.: The sequential reference numbers of citations. Citations of microarray datasets are displayed in the end of References list, and there are no citations available for the following 4 datasets: GSE19945 [101], GSE74190 [108], GSE77380 [109], GSE93300 [119], thus website address was affiliated. Study1 (Xia et al, [34]) and Study2 (Zhou et al, [35]) are the codes for two Chinese articles; the corresponding articles are displayed at the end of References section

ID	Author	Year	Ref NO.	Country	Nation	Sample type	N0	N1	AUCs	Thresholds	Sensitivity	Specificity	TP	FN	FP	TN
GSE72526	Gasparini P	2015	[107]	Switzerland	Euramerican	tissue	18	52	0.698	4.500	0.596	0.788	31	21	4	14
GSE2564	Lu J	2005	[110]	USA	Euramerican	tissue	4	14	0.214	7.700	0.286	0.500	4	10	2	2
GSE77380	Yoshimoto T	2016	[109]	Japan	Asian	tissue	12	12	0.826	5.936	0.750	0.917	9	3	1	11
GSE47525	van Jaarsveld MT	2013	[103]	Netherlands	Euramerican	tissue	14	18	0.594	5.469	0.333	1.000	6	12	0	14
GSE19945	Ohba T	2015	[101]	Japan	Asian	tissue	8	20	0.981	2.872	0.950	1.000	19	1	0	8
GSE74190	Jin Y	2015	[108]	China	Asian	tissue	38	38	0.892	4.807	0.868	0.026	33	5	37	1
GSE48414	Bjaanaes MM	2014	[104]	Norway	Euramerican	tissue	20	154	0.918	-0.820	0.799	1.000	123	31	0	20
GSE16025	Raponi M	2009	[100]	USA	Euramerican	tissue	10	61	0.974	6.590	0.885	1.000	54	7	0	10
GSE51853	Arima C	2014	[105]	Japan	Asian	tissue	5	124	0.958	-0.004	0.935	1.000	116	8	0	5
GSE63805	Robles AI	2015	[106]	USA	Euramerican	tissue	30	30	0.792	4.526	0.733	0.833	22	8	5	25
GSE15008	Tan X	2010	[99]	China	Asian	tissue	112	112	0.865	12.423	0.821	0.839	92	20	18	94
GSE14936	Seike M	2009	[98]	USA	Euramerican	tissue	26	26	0.686	9.504	0.538	0.846	14	12	4	22
GSE29248	Ma L	2012	[111]	China	Asian	tissue	6	6	0.750	0.578	1.000	0.500	6	0	3	3
GSE56036	Fujita Y	2017	[112]	Japan	Asian	tissue	29	29	0.966	7.233	0.862	0.966	25	4	1	28
GSE25508	Nymark P	2011	[102]	Finland	Euramerican	tissue	26	26	0.891	7.670	0.808	0.885	21	5	3	23
TCGA	NR	NR	NR	NR	NR	tissue	91	999	0.978	9.938	0.919	0.956	918	81	4	87
PCR	NR	NR	NR	China	Asian	tissue	125	125	0.762	2.500	0.920	0.528	115	10	59	66
GSE61741	Keller A	2014	[117]	Germany	Euramerican	blood	94	72	0.548	9.988	0.361	0.755	26	46	23	71
GSE40738	Patnaik SK	2012	[116]	USA	Euramerican	blood	56	81	0.430	2.639	0.148	0.911	12	69	5	51
GSE31568	Keller A	2011	[115]	Germany	Euramerican	blood	70	32	0.572	10.130	0.375	0.839	12	20	11	59
GSE27486	Patnaik SK	2011	[114]	USA	Euramerican	blood	23	22	0.253	-0.215	0.955	0.043	21	1	22	1
GSE17681	Keller A	2009	[113]	Germany	Euramerican	blood	10	17	0.647	8.610	0.529	0.800	9	8	2	8
PMID:24053570	Anjuman N	2013	[31]	USA	Euramerican	plasma	42	39	0.767	NR	0.692	0.762	27	12	10	32
PMID:21116241	Shen J	2011	[32]	USA	Euramerican	plasma	29	58	0.746	NR	0.741	0.690	43	15	9	20
PMID:21864403	Shen J	2011	[33]	USA	Euramerican	plasma	33	32	0.691	2.001	0.563	0.727	18	14	9	24
PMID:27499953	Wang X	2016	[29]	China	Asian	plasma	59	59	0.751	3.341	0.746	0.746	44	15	15	44
GSE68951	Leidinger P	2015	[118]	Germany	Euramerican	plasma	12	26	0.340	3.496	0.346	0.677	9	17	4	8
GSE93300	Liu X	2017	[119]	USA	Euramerican	plasma	4	9	0.333	-7.113	0.889	0.250	8	1	3	1
Study1	Xia XB	2017	[34]	China	Asian	serum	46	58	0.824	NR	0.759	0.804	44	14	9	37
PMID:24065453	Li ZH	2014	[30]	China	Asian	serum	30	60	0.775	1.307	0.787	0.740	47	13	8	22
Study2	Zhou JL	2018	[35]	China	Asian	serum	40	48	0.845	NR	0.741	0.782	36	12	9	31
PMID:27093275	Zhu WY	2016	[20]	China	Asian	serum	104	112	0.616	0.107	0.339	1.000	38	74	0	104
GSE46729	Godfrey A	2014	[121]	USA	Euramerican	serum	24	24	0.651	3.785	0.833	0.417	20	4	14	10
GSE16512	Lodes MJ	2009	[120]	USA	Euramerican	serum	14	3	0.905	1.110	1.000	0.786	3	0	3	11
PMID:24305007	Shen J	2014	[36]	USA	Euramerican	sputum	68	66	0.780	NR	0.658	0.738	43	23	18	50
PMID:24305007	Shen J	2014	[36]	USA	Euramerican	sputum	73	64	0.790	NR	0.626	0.718	40	24	21	52
PMID:20526284	Xing L	2010	[37]	USA	Euramerican	sputum	48	48	0.748	1.640	0.583	0.792	28	20	10	38
PMID:25593345	Xing L	2015	[38]	USA	Euramerican	sputum	62	60	0.853	NR	0.753	0.859	45	15	9	53
PMID:24281335	Li N	2014	[39]	USA	Euramerican	sputum	40	35	0.730	NR	0.571	0.900	20	15	4	36
PMID:27777637	Su Y	2016	[40]	China	Asian	sputum	171	144	0.840	NR	0.766	0.812	110	34	32	139

identified in which the target genes were greatly enriched in biological process, cellular component, and molecular function (Fig. 15A-15C). Cell adhesion was the most significant GO term of biological process, while plasma membrane, integral component of plasma membrane, and proteinaceous extracellular matrix were largely enriched in cellular component. As for molecular function, Ras guanyl-nucleotide exchange factor activity and actin binding were the two most relevant GO terms. We also identified 10 significant pathways in KEGG (Fig. 15D), among which renin secretion, the cGMP-PKG signaling pathway, and cell adhesion molecules showed extraordinary significance. Additionally, we used Cytoscape to visualize the functional annotation and signaling pathways (Fig. 16A-16C). The protein-protein interaction network, which was constructed using STRING, contained 80 hub genes (Fig. 17) and suggested that 6 target genes might be key

Fig. 10. Overall SROC graph evaluating the capacity of miR-210 to distinguish NSCLC patients from normal controls.

Fig. 11. SROC graph evaluating the capacity of miR-210 to distinguish NSCLC patients from normal controls. A: Serum-only samples. B: Sputum-only samples.

Table 4. ROC analysis of all available data. AUC: area under receiver operating characteristic curve; 95% CI: 95% confidence interval; LL: lower limit; UL: upper limit. Q: heterogeneity Q test; P_{het} : P value of heterogeneity

Sample type	Study	Enrolled	Overall estimate				Heterogeneity			Threshold
	number	number	AUC	95%CI(LL-UL)	sensitivity	specificity	Q	I ² (%)	P _{het}	effect
Overall	40	4741	0.83	(0.79 - 0.86)	0.72	0.80	354.195	99.00%	0.000	8.00%
Tissue	17	2420	0.90	(0.87 - 0.92)	0.81	0.89	116.931	98.00%	0.000	0.00%
Tissue-excluded	23	2321	0.77	(0.73 - 0.80)	0.65	0.76	190.259	99.00%	0.000	60.00%
Blood	5	477	0.61	(0.56 - 0.65)	0.50	0.67	97.754	98.00%	0.000	100.00%
Plasma	6	402	0.73	(0.69 - 0.76)	0.66	0.72	3.163	37.00%	0.103	100.00%
Serum	6	563	0.82	(0.79 - 0.85)	0.72	0.84	92.465	98.00%	0.000	100.00%
Sputum	6	879	0.81	(0.78 - 0.84)	0.68	0.79	0.173	0.00%	0.459	100.00%

Table 5. Available studies with miR-210-related survival analysis. OS: overall survival; DFS: disease-free survival; PFS: progression-free survival; HR: hazard ratio; LL: lower limit of the 95% confidence interval; UL: upper limit of the 95% confidence interval; LUSC: lung squamous cell carcinoma; NSCLC: non-small cell lung cancer; ADC: adenocarcinoma; LUAD: lung adenocarcinoma. Ref NO.: The sequential reference numbers of citations. Citations of microarray datasets are displayed in the end of References list, and there are no citations available for the following 4 datasets: GSE19945 [101], GSE74190 [108], GSE77380 [109], GSE93300 [119], thus website address was affiliated. Study2 (Zhou et al., [35]) and Study3 (Lu et al., [42]) are the codes for two Chinese articles; the corresponding articles are displayed at the end of References section

Data source	Author	Year	Ref NO.	Country	Nation	Cancer type	Total enroll	Sample type	Analysis method	Event type	HR	LL	UL
GSE16025	Raponi M	2009	[100]	USA	Euramerica	LUSC	61	tissue	Multivariate analysis	OS	0.938	0.473	1.861
GSE63805	Robles AI	2015	[106]	USA	Euramerica	NSCLC	30	tissue	Multivariate analysis	OS	0.367	0.127	1.063
PMID:25733977	Osugi J	2015	[41]	Japan	Asian	ADC	62	tissue	Multivariate analysis	OS	0.36	0.13	1.03
Study3	Lu S	2015	[42]	China	Asian	ADC	62	tissue	Multivariate analysis	OS	0.36	0.13	1.03
PCR	NR	NR	NR	China	Asian	NSCLC	57	tissue	Multivariate analysis	OS	0.765	0.371	1.577
PMID:25733977	Osugi J	2015	[41]	Japan	Asian	ADC	62	tissue	Multivariate analysis	DFS	0.36	0.15	0.85
Study3	Lu S	2015	[42]	China	Asian	ADC	62	tissue	Multivariate analysis	DFS	0.36	0.15	0.87
PMID:26687391	Shi SB	2015	[43]	China	Asian	LUAD	76	blood	Univariate analysis	PFS	2.06	1.19	3.57
Study2	Zhou JL	2017	[35]	China	Asian	NSCLC	48	serum	Multivariate analysis	PFS	1.98	0.99	3.02

target genes of miR-210: interleukin 6 (IL-6), C-X-C motif chemokine ligand 12 (CXCL12), G protein subunit γ 11 (GNG11), adenylate cyclase 9 (ADCY9), adrenoceptor β 2 (ADRB2), and cholinergic receptor muscarinic 2 (CHRM2).

Disease ontology analysis was conducted, and the top 10 items of the miR-210-related disease spectrum are shown in Fig. 18. In addition, gene alterations of miR-210 across NSCLC samples were examined using cBioPortal. As shown in Fig. 19, 8 (0.7%) of 1144 cases had alterations (7 deep deletions and 1 amplification). Expression levels of the 6 potential target genes in GEPIA indicated that all of their mRNA levels had a reduced trend in NSCLC. The scatter point plots of the 6 target genes are presented in Fig. 20A-20F.

The protein expression levels of the 6 genes were searched for in HPA. The protein levels of 2 genes (CHRM2 and ADCY9) tended to be down-regulated in lung cancer compared to normal lung tissue (Figs. 21–22), while the remaining genes were not detected in the inspected samples of normal lung tissue and lung cancer tissue. Integrating this observation with the down-regulated mRNA levels of all 6 genes in NSCLC (Fig. 20), CHRM2 and ADCY9 are more likely to be target genes of miR-210 in lung cancer. However, due to the small

sample size provided by HPA and absence of statistical analysis, this hypothesis needs to be verified by future functional experiments.

Discussion

Lung cancer, according to estimates of the global burden of cancer, is the most common malignancy and the leading vital disease in male patients and it affects 17% of female patients with a 23% cancer-related mortality rate, which is equivalent to the burden of cervical cancer [44]. The currently used diagnostic methods of chest X-ray examination, computed tomography, magnetic resonance imaging, tumor marker detection in serum, and biopsy have limited efficacy, and therefore effective novel biomarkers of lung cancer are urgently required to improve its early diagnosis and treatment [29].

miR-210 has been explored extensively in miRNA research examining its correlation with cancers. An increasing number of studies have reported that miR-210 plays an important role in the development of various types of cancer by regulating tumorigenesis and tumor growth, and miR-210 has been shown to exert its functions under hypoxic conditions [45, 46]. A variety of hypoxic reactions that affect tumor generation are mediated by hypoxia-inducible factor (HIF).

Fig. 12. Forest plots displaying the prognostic results of miR-210 in NSCLC. A: Forest plots of OS; B: DFS; and C: PFS.

Fig. 13. A: Influence analysis. B: Funnel plot.

The promoter region of miR-210 contains a functional hypoxia-response element, to which HIF-1 α binds, thereby promoting tumor growth and cancer progression [47-50]. Kelly et al. found that glycerol-3-phosphate dehydrogenase 1-like (GPD1L), which not only reduces the stability of HIF-1 α but also is targeted directly by miR-210, has hydroxyprolinase activity [51]. miR-210 overexpression can reduce GPD1L expression under hypoxic conditions, so as to improve the stability of HIF-1 α and to enhance further the hypoxia-responsive capability of tumor cells.

miR-210 was recently shown to promote angiogenesis in hypoxic conditions [52]. Vascular endothelial growth factor (VEGF), an efficient pro-angiogenic protein, is negatively regulated by protein-tyrosine phosphatase 1B (PTP1B) through the de-phosphorylation of VEGFR2 in endothelial cells [53]. The overexpression of miR-210 can inhibit PTP1B; thus, VEGF can be expressed in hypoxic conditions to promote the regeneration of blood vessels [54, 55].

In addition, miR-210 overexpression reportedly regulates the progression of the cell cycle by inhibiting the expression of E2F3 and Myc-gene antagonistic factor Max network transcriptional repressor [48, 56, 57]. It was recently demonstrated that fibroblast growth factor receptor 1 and Homeobox protein A1, which are target proteins of miR-210, regulate cell proliferation [58, 59]. Moreover, accumulating evidence has revealed that miR-210 can obstruct the DNA repair system by targeting the enzyme RAD52, which can repair DNA double-strand and single-strand gaps. miR-210 binds directly to the 3'-untranslated region of RAD52 to repress its translation [60].

miR-210 was reported to be abnormally expressed in several kinds of human malignancy. A microarray profile and real-time qPCR analysis confirmed the outcome of patients in a large cohort study by Donnem et al., and demonstrated that miR-210 was up-regulated (fold change = 1.74) and possibly associated with angiogenesis in NSCLC [61]. A series of microarray profile-based meta-analyses, which were published in 2012, 2013, and 2017, also came to the same conclusion that miR-210, along with several other miRNAs, was overexpressed in NSCLC [62-64]. In addition, the results of the present original estimation and meta-analysis results indicate that miR-210 was elevated in NSCLC, in agreement with the abovementioned studies. Our own first-hand RT-qPCR results for 125 paired cancerous and para-cancerous clinical tissues also confirmed this.

As for the diagnostic value of miR-210 for various cancers, it was reported that miR-210 is up-regulated in the serum of patients diagnosed with diffuse large B-cell lymphoma [65] or pancreatic ductal adeno-

Fig. 14. Details of the process for target gene prediction.

Table 6. Prognostic value assessment: forest plots and publication bias analysis. OS: overall survival; DFS: disease-free survival; PFS: progression-free survival; CI: confidence interval; Fixed: fixed-effects model; Het X^2 : X^2 of heterogeneity Q test; P_{het} : P value of heterogeneity; Begg's P_{bias} : P value of Begg's test

Event type	Study number	Enrolled number	Analysis model	Overall estimate				Heterogeneity			Publication bias	
				HR	95%CI(LL-UL)	Z	P	Het X^2	I ² (%)	P_{het}	Begg P_{bias}	
OS	5	272	Fixed	0.602	0.410-0.884	2.59	0.010	4.76	16.0%	0.313	0.327	
DFS	2	124	Fixed	0.360	0.194-0.667	3.24	0.001	0.00	0.00%	1.000	1.000	
PFS	2	124	Fixed	2.020	1.366-2.988	3.52	0.000	0.01	0.00%	0.921	1.000	

carcinoma [66]. A similar study by Xing et al. reported the early diagnostic value of miR-210 for lung squamous cell carcinoma [37]. A meta-analysis conducted by Yang et al. indicated the significant diagnostic value of miR-210 (AUC = 0.80) for NSCLC by detecting its expression level in blood and sputum [67]. Likewise, our study showed the significant diagnostic capacity of miR-210 in NSCLC by detecting its expression level in serum (AUC = 0.82) and sputum (AUC = 0.81).

Foekens et al. examined the prognostic aspect of miR-210 and showed a positive correlation between elevated miR-210 expression and the progression of estrogen receptor-positive and lymph node metastasis-negative breast cancer [68]. Our findings indicate that miR-210 overexpression was associated with poor PFS in NSCLC, while it was negatively related to OS and DFS.

It is well known that miRNAs play a crucial role in posttranscriptional repression. miRNAs pair with the 3'-untranslated regions of their mRNA targets to control gene expression, the cell cycle, and development [69]. The majority of miRNAs are associated with the occurrence, progression, and prognosis of different types of cancer by repressing translation or inducing mRNA degradation [70]. It has also been demonstrated that there are different binding sites in one mRNA targeted by the same miRNA, which increases the possibility of finding real target genes and enables the experimental validation of miRNA target genes [71]. Thus, predicting the targets of miRNAs and further exploring their functions via bioinformatics analysis will help to generate a better understanding of the molecular mechanisms of miRNAs in cancer. The present study has made some contribution to the comprehensive recognition of the function of miR-210 in NSCLC, but further study is required for a complete understanding. The expression levels of the 6 potential target genes (IL-6, GNG11, CXCL12, ADRB2, ADCY9, and CHRM2) were examined using GEPIA, showing a trend for their reduced expression in NSCLC (Fig. 20A–20E). Thus, it is likely that these 6 genes are potential targets of miR-210.

IL-6, located on 7p15.3, encodes a cytokine that induces the maturation of B cells and

Fig. 15. Top 10 items. A: Biological process; B: Cellular component; C: Molecular function; and D: Kyoto Encyclopedia of Genes and Genomes.

Fig. 16. Network analyses of the prospective target genes of miR-210 in A: biological process; B: cellular component; and C: molecular function. The intensity of the color indicates P value size, node refers to pathways, and node size is representative of the number of genes included in each node. A. Network analysis of miR-210 target genes in biological process. B. Network analysis of miR-210 target genes in cellular component. C. Network analysis of miR-210 target genes in molecular function.

functions in various inflammatory processes [72]. IL-6 reportedly prevents the initiation of lung cancer by activating the transcription factor STAT3 to regulate lung macrophages and to preserve lung homeostasis [73]. However, Jiang et al. also demonstrated that IL-6 enhances the progression of lung cancer by inducing ATM phosphorylation and increasing the expression of MMP-3/MMP-13, thus enhancing cell migration and promoting the metastasis of lung tumor cells [74]. Liu et al. demonstrated that IL-6 can also boost the proliferation of lung cancer stem cells by suppressing the expression of cell cycle regulators [75]. All of these findings suggest that IL-6 might be a crucial potential therapeutic target in lung cancer. Actually, Caetano et al. found that anti-IL-6 treatment could be an alternative therapeutic strategy in lung cancer [76]. Interestingly, the reaction of patients with NSCLC to analgesics could be predicted by detecting a gene polymorphism of IL-6, which has shown significant value in improving the quality of life and prognosis of such patients [77]. However, as yet no studies have examined the relationship between IL-6 and miR-210 in NSCLC.

GNG11, located on 7q21.3, encodes G protein subunit γ 11, a member of the guanine nucleotide-binding protein (G protein) γ family [78]. Hossain et al. discovered that GNG11 was strongly related to cell senescence [79], while Ruiz-Ballesteros et al. revealed that GNG11 expression was depressed in splenic marginal zone lymphoma [80]. GNG11 might act as a tumor suppressor in malignancies; however, the relationship between GNG11 and lung cancer has not been reported. Hence, experiments are needed to probe the role of GNG11 and miR-210 in lung cancer.

CXCL12, also known as SDF-1, is located on chromosome 10 and encodes an endocrine alpha chemokine in stromal cells. The encoded protein exerts its function by binding with

Fig. 17. Protein-protein interaction network of 80 hub genes.

Fig. 18. Top 10 items of the miR-210-related disease spectrum.

Fig. 19. Mutational alterations (amplification and deletion included) of miR-210 in 1144 NSCLC cases (OncoPrint tab from cBioPortal).

chemokine receptor 4 (CXCR4), a G protein-coupled receptor, in various biological processes, including embryogenesis, inflammatory response, and tumor progress and metastasis. Pathological roles for the CXCR4/CXCL12 axis have been demonstrated in various cancers, including breast cancer [81], kidney carcinoma [82], bone sarcoma [83], and NSCLC [84,

85]. CXCR4/CXCL12 overexpression in these malignancies as well as in NSCLC may facilitate the local infiltration and dissemination of tumor cells.

ADRB2, also known as adrenoceptor β receptor (ADRB2), is located on 5q32 and encodes the β -2-adrenergic receptor, a member of the G protein-coupled receptor superfamily [86]. Gene polymorphisms, point mutations, and down-regulation of ADRB2 are closely related to obesity [87], nighttime asthma [88], and type 2 diabetes [89]. Wang et al. reported that ADRB2 has epidemiological significance in the development of lung cancer [90], and it may be a potential therapeutic target for lung cancer treatment [91]. More importantly, an agonist of ADRB2 enhanced sensitivity to cisplatin chemotherapy in an NSCLC cell line [92]. Unfortunately, no studies have examined the association between ADRB2 and miR-210 yet.

ADCY9, located on 16p13.3, is activated by the β -adrenergic receptor and catalyzes the formation of cyclic AMP from ATP [93]. It was speculated that it contributes to treatment of patients with mild to moderate asthma [94]. However, the relationship between ADCY9 and miR-210 in lung cancer has not been examined.

CHRM2, situated on chromosome 7, encodes a G protein-coupled receptor. CHRM2 interacts with acetylcholine and induces cellular biochemical processes, such as adenylate-cyclase inhibition, phosphoinositide degeneration, and potassium channel mediation. CHRM2 activation depresses cell proliferation and promotes apoptosis in glioblastoma cell lines [95]. Moreover, auto-paracrine non-neuronal acetylcholine, acting as a growth factor, stimulates tumor proliferation in NSCLC by activating CHRM2. In contrast, blocking CHRM2 arrests tumor cell growth in NSCLC [96, 97].

There are several highlights but also some limitations to this study. First, raw expression data were extracted from the GEO and TCGA databases and were subjected to estimation for the mean and SD and significance testing. In addition, RT-qPCR was conducted on 125 paired NSCLC and adjacent paired samples as a means to validate the outcome obtained from the public databases; moreover, a meta-analysis based on raw data estimation outcomes and references was conducted to give an overall conclusion. According to the individual estimation results and overall pooled estimation, miR-210 was found to be consistently elevated in solid tissues from patients with NSCLC; however, this finding was varied in easily obtained blood samples. Besides, the number of enrolled samples in the different GEO datasets ranged greatly from 12 to 224; thus, finite deviation would exist in the estimation outcomes. As the detection platforms and operating

Fig. 20. Scatter point plots of the 6 target genes.

Fig. 21. Expression levels of CHRM2 protein in normal lung tissue and lung squamous cell carcinoma. A, B: Normal lung tissue: CAB022338, female, age 49 years, lung (T-28000), bronchus (T-26000), NOS (M-00100), patient ID: 2268, pneumocyte staining: low, intensity: weak, quantity: >75%, location: cytoplasmic/membranous nuclear. C, D: Lung squamous cell carcinoma: CAB022338, female, age 47 years, lung (T-28000), NOS (M-80703), patient ID: 2100, tumor cell staining: not detected, intensity: weak, quantity: <25%, location: nuclear.

Fig. 22. Expression levels of ADCY9 protein in normal lung tissue and lung squamous cell carcinoma. A, B: Normal lung tissue: HPA044225, female, age 57 years, lung (T-28000), NOS (M-00100), patient ID: 1678, pneumocyte staining: low, intensity: weak, quantity: 75-25%, location: cytoplasmic/membranous. C, D: lung squamous cell carcinoma: HPA044225, female, age 69 years, lung (T-28000), NOS (M-80703), patient ID: 4194, tumor cell staining: not detected, intensity: weak, quantity: <25%, location: cytoplasmic/membranous.

protocols were different, this had an inevitable confounding impact on the results, causing considerable inter-study heterogeneity, so consequently, we applied a random-effects model. Regarding the relationship between miR-210 and clinical parameters, the clinical status of the patients was not provided in every GEO datasets and therefore further validation is required to draw a conclusion. The scatter plots and ROC curves for each dataset were provided as intuitive measurements, as well as the forest plots and SROC curves for overall estimation. Thus, differences between the studies could be revealed easily, which facilitated further analysis. As for the function of miR-210 in NSCLC, although several hub-genes were selected from the predicted target genes, due to budgetary constraints, we were unable to validate the target genes and communication pathways of miR-210.

Conclusion

The results of our original estimations revealed that miR-210 expression was elevated in solid NSCLC tissues from GEO microarrays, a finding that concurs with that detected from TCGA data and our own RT-qPCR results. Conversely, because the results from different GEO microarrays varied, it is unclear whether miR-210 was overexpressed in easily obtained body fluids. Combining the original estimations, a meta-analysis revealed the overall elevation of miR-210 in NSCLC. Regarding the diagnostic value of miR-210, it showed significant diagnostic capacity in NSCLC by detecting its expression in serum and sputum. Elevated miR-210 expression in serum and sputum may be a potential biological marker for NSCLC patients. The prognostic meta-analysis revealed that miR-210 overexpression was associated with poor PFS in NSCLC, while it was negatively related to OS and DFS. Thus, rigorous studies with large samples are needed to explore whether miR-210 could be used as a prognostic index for NSCLC patients. Moreover, IL-6, CXCL12, GNG11, ADCY9, ADRB2, and CHRM2 were the most likely target molecules of miR-210. However, further studies are needed to gain a more comprehensive understanding of the role of miR-210 in NSCLC.

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Disclosure Statement

No conflict of interests exists.

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