High expression of RRM2 as an independent predictive factor of poor prognosis in patients with lung adenocarcinoma

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ABSTRACT

Ribonucleotide reductase subunit M2 may play a role as a potential prognostic biomarker in several cancers. In this study, we evaluated whether RRM2 gene expression is associated with the prognosis of patients with lung adenocarcinoma (LUAD) using publicly available data from The Cancer Genome Atlas (TCGA). Wilcoxon signed-rank test and logistic regression were performed to evaluate the association between RRM2 expression and clinical features in patients with LUAD. Kaplan-Meier and Cox regression methods were used to examine the effect of RRM2 expression level in the overall survival, and a nomogram was performed to illustrate the correlation between the RRM2 gene expression and the risk of LUAD. TCGA data set was used for gene set enrichment analysis (GSEA). We also performed a further experiment in vitro to assess the effect of RRM2 expression on the proliferation and invasive abilities of LUAD cells and its key signaling pathway proteins. Our results revealed that the expression level of RRM2 in patients with LUAD was much higher than that in normal tissues ($p = 3.99e-32$). High expression of RRM2 was significantly associated with tumor stage (IV vs. I: OR = 3.02, $p = 0.012$) and TNM classification (T2 vs. T1: OR = 1.88, $p = 0.001$; N2 vs. N0: OR = 2.69, $p < 0.001$). Kaplan-Meier survival analysis showed that high expression of RRM2 was associated with a worse prognosis of LUAD compared low expression of RRM2 ($p = 7.86e-04$). Multivariate analysis showed that high RRM2 expression was an independent factor affecting overall survival (HR = 1.29, $p < 0.001$). The association between RRM2 gene expression and the risk of LUAD was presented in a nomogram. GSEA revealed that the cell cycle, p53 signaling pathway, DNA replication, small cell lung cancer, apoptosis, and pathways in cancer were differentially enriched in patients with high expression of RRM2. RRM2 over-expression promoted the proliferation and invasive abilities of LUAD cells. RRM2 over-expression increased the activation of Bcl-2 and E-cadherin signaling pathways, and reduced the activation of p53 signaling pathway. In summary, high RRM2 expression is an independent predictive factor of poor prognosis in patients with lung adenocarcinoma.

INTRODUCTION

Lung cancer is the leading cause of death globally, approximately 2.1 million new cases and 1.8 million deaths occurred in 2018 [1]. These cases were mainly non-small cell lung cancer (NSCLC), which is divided into different histological categories. Among them, lung adenocarcinoma (LUAD) is the most prevalent subtype [2]. Although multimodal treatment strategies, including immunotherapy, radiotherapy, and non-invasive surgical resection have greatly advanced in recent decades, the outcomes of curing lung cancer remain unsatisfactory and the five-year relative overall survival rate is approximately 18% [3]. Improvements in molecular pathology detection methods and targeted therapies have markedly increased the overall survival of patients with LUAD based on the emerging
concept of “precision medicine” in recent years [4]. In
precision medicine, key genes driving carcinogenesis can
be considered as therapeutic targets. Frequently reported
molecules used in pathology detection and as therapeutic
targets in adenocarcinoma include epidermal growth factor
receptor mutations, echinoderm microtubule-associated
protein-like gene, and anaplastic lymphoma kinase gene
[5–8]. Although molecularly targeted therapies have
shown good clinical results [9–11], curing patients with
LUAD remains challenging, particularly because of the
development of drug resistance [12, 13]. Therefore, it is
necessary to identify more effective indicators for
molecular pathology diagnosis and prognosis prediction in
patients with LUAD.

Ribonucleotide reductase M2 subunit (RRM2), a rate-
limiting enzyme involved in DNA synthesis and
damage repair, plays vital roles in many critical cellular
processes such as cell proliferation, invasiveness,
migration, and senescence [14]. RRM2 functions as a
tumor driver is frequently overexpressed in various malignancies [15, 16]. Lu et al. found that the
expression level of RRM2 is correlated with invasion
depth, poorer differentiation, and tumor metastasis in
patients with colorectal carcinoma [17]. RRM2
knockdown attenuated melanoma growth both in vitro
and in vivo, which was correlated with maintenance of
senescence-associated cell-cycle arrest [18]. Hsu et al.
found that RRM2 was positively correlated with tumor
grade, and patients with early stage NSCLC with
RRM2-low tumors had better outcomes [19]. These
findings indicate RRM2 may not only function as an
oncogene, but also is a promising biomarker for
molecular pathology diagnosis and prognosis prediction in
patients with LUAD.

This study was conducted to assess the prognostic
significance of RRM2 gene expression in LUAD
through bioinformatics analysis of the clinical features
and survival information from The Cancer Genome
Atlas (TCGA). We also performed in vitro experiment
to investigate the effect of RRM2 expression on cell
proliferation and invasion of LUAD cells and related
key signaling pathways. Our results demonstrated that
RRM2 is useful for predicting the prognosis of patients
with LUAD and that high expression of RRM2 is
associated with poor prognosis in these patients.

RESULTS

Baseline characteristics of patients

A total of 503 patients with the required clinical features
were acquired from TCGA data portal in April 2019. The
detailed clinical features are listed in Table 1. Among the
503 participants, 233 were male (46.3%) and 270 were
female (53.7%). The median age of all participants was 65
years. In terms of LUAD stage, 268 patients were stage I
(54.1%), 120 patients were stage II (24.2%), 81 patients
were stage III (16.4%), and 26 patients were stage IV
(5.3%). The cancer status included 105 patients with
tumors (26.0%) and 301 tumor-free patients (74.0%). The
median follow-up of the 503 patients with LUAD was
25.3 months (rang 0–227 months).

High RRM2 expression in LUAD

To assess the status of RRM2 expression in LUAD
patients, we compared the expression level to that in
normal lung tissues. The results demonstrated that RRM2
gene expression level was significantly higher in LUAD
tissues ($p = 3.99e-32$) that in normal tissues (Figure 1A).
The results were verified in LUAD tissues and paired
normal lung tissues ($p = 3.35e-18$) (Figure 1B).

Correlation between RRM2 expression and clinical
features

The association identified between RRM2 expression
and clinical features in patients with LUAD is
summarized in Table 2. High expression of RRM2 was
significantly correlated with age ($p = 0.006$), clinical
stage ($p = 1.30e-05$), T classification ($p = 7.37e-04$), M
classification ($p = 0.017$), N classification ($p = 1.15e-
05$), cancer status ($p = 5.51e-04$), new tumor event after
initial treatment ($p = 0.043$), and smoking status ($p =
1.81e-06$), as shown in Figure 2. However, high
expression of RRM2 was not significantly correlated
with other clinical features (Supplementary Figure 1A–
1G). Univariate analysis using logistic regression
demonstrated that RRM2 gene expression was a
categorical dependent variable associated with poor
prognostic clinical features (Table 2). High expression of
RRM2 was significantly associated with clinical stage
(IV vs. I: OR = 0.012, 95% confidence interval [CI] =
1.31–7.58, $p = 0.012$), T classification (T2 vs. T1: OR =
1.88, 95%CI = 1.28–2.79, $p = 0.001$), and cancer status
(OR = 1.73, 95%CI = 1.12–2.71, $p = 0.014$).

High expression of RRM2 is an independent risk
factor for overall survival

Kaplan-Meier survival analysis showed that high RRM2
expression was associated with poor prognosis ($p =
7.86e-04$), as shown in Figure 3A. Subgroup analysis by
different clinical features demonstrated that high RRM2
expression was significantly associated with poor
prognosis in LUAD cases more than 65 years old
($p = 2.287e-04$), clinical stage I/II ($p = 0.023$), M0
($p = 0.001$), N0 ($p = 0.031$), with tumor ($p = 5.617e-04$),
smoker ($p = 0.031$), and T1/T2 ($p = 0.023$), as shown in
Figure 3B–3H. Univariate Cox analysis demonstrated
Table 1. Clinical characteristics of the lung adenocarcinoma patients.

| Clinical characteristics | N   | (%) |
|--------------------------|-----|-----|
| Age (years)              |     |     |
| <65                      | 223 | 44.3|
| >=65                     | 280 | 55.7|
| Gender                   |     |     |
| Female                   | 270 | 53.7|
| Male                     | 233 | 46.3|
| Race                     |     |     |
| Asian                    | 9   | 2.0 |
| non-Asian                | 446 | 98.0|
| Stage                    |     |     |
| I                        | 268 | 54.1|
| II                       | 120 | 24.2|
| III                      | 81  | 16.4|
| IV                       | 26  | 5.3 |
| Smoking status           |     |     |
| Current smoker           | 118 | 24.1|
| Ever smoker              | 301 | 61.2|
| Never smoker             | 70  | 14.7|
| T classification         |     |     |
| T1                       | 171 | 33.2|
| T2                       | 265 | 53.0|
| T3                       | 45  | 9.0 |
| T4                       | 19  | 3.8 |
| M classification         |     |     |
| M0                       | 334 | 93.0|
| M1                       | 25  | 7.0 |
| N classification         |     |     |
| N0                       | 323 | 66.1|
| N1                       | 95  | 19.4|
| N2                       | 71  | 14.5|
| Location in lung         |     |     |
| Central                  | 64  | 36.8|
| Peripheral               | 110 | 63.2|
| Radiation therapy        |     |     |
| Yes                      | 13  | 8.3 |
| No                       | 144 | 91.7|
| New tumor event after initial treatment |     |     |
| Yes                      | 31  | 16.6|
| No                       | 156 | 83.4|
| EGFR mutation            |     |     |
| Yes                      | 80  | 29.4|
| No                       | 192 | 70.6|
| KRAS mutation            |     |     |
| Yes                      | 21  | 35.0|
| No                       | 39  | 65.0|
| EML4-ALK translocation   |     |     |
| Yes                      | 34  | 14.0|
| No                       | 208 | 86.0|
| Person neoplasm cancer status |   |     |
| With tumor               | 106 | 26.0|
| Tumor free               | 301 | 74.0|
| Primary therapy outcomes |     |     |
| SD+PD                    | 19  | 12.6|
| CR+PR                    | 132 | 87.4|

that high RRM2 expression was significantly correlated with poor overall survival (hazard ratio [HR] = 1.34, 95%CI = 1.16–1.54, p = 4.86e-05). Multivariate Cox analysis confirmed RRM2 gene expression was an independent risk factor for overall survival in patients with LUAD (HR = 1.29, 95%CI = 1.11–1.50, p < 0.001), as shown in Table 3 and Figure 4.

Diagnostic value of RRM2 expression in LUAD

We conducted ROC curve analysis of RRM2 gene expression data to evaluate the diagnostic value of this gene. The area was 0.967, which indicated high diagnostic value, as shown in Figure 5A. Subgroup analysis demonstrated the diagnostic value of RRM2 gene expression in different clinical features of LUAD, with AUC values of 0.966 for stage I/II, 0.974 for stage III/IV, 0.972 for M0, 0.961 for N0, 0.980 for N1-N3, 0.965 for T1/T2, and 0.980 for T3/T4 (Figure 5B–H). Therefore, a nomogram was constructed to predict the 1-, 3-, and 5-year survival probability of patients by combining the expression level of RRM2 with clinical variables (Figure 6).

GSEA identifies a RRM2-related signaling pathway

We performed GSEA using the low- and high-RRM2 expression data sets to identify signaling pathways that...
are differentially activated in LUAD. GSEA showed a large difference (false discovery rate < 5%, nominal p-value < 25%) MSigDB collection enrichment (c2.cp.kegg.v6.2.symbols.gmt) (Table 4). Gene sets related to cell cycle, p53 signaling pathway, DNA replication, small cell lung cancer, apoptosis, and pathway in cancer showed differential enrichment in the high RRM2 gene expression phenotype (Figure 7).

Validation using independent external database

We further validated the repeatability and portability of RRM2 expression in the prognostic effect of LUAD patients by using two other independent external datasets, including GSE30219 and GSE50081. In the validation set GSE30219, Kaplan-Meier survival analysis revealed that patients with increased RRM2 expression had shorter OS (Figure 8A–8D). The prognostic value of RRM2 was also demonstrated in patients with LUAD using GSE50081 (Figure 8E–8H). Meanwhile, the diagnostic value of RRM2 was also demonstrated in patients with LUAD using GSE30219 (Supplementary Figure 1H). Consistent with these results, RRM2 was found to be significantly over-expressed in LUAD by using five distinct LUAD datasets (Garber Lung, Hou Lung, Landi Lung, Okayama Lung, and Su Lung) via pooled analysis in the Oncomine database [20–24], and significant over-expression was also found in the TIMER database (Figure 9A–9C). Interestingly, high RRM2 gene expression is frequently detected in numerous human solid tumors, such as lung cancer, colorectal cancer, breast cancer, bladder cancer, and others (Figure 9A, 9C).

RRM2 over-expression in LUAD tissues and cell lines was shown by qRT-PCR

We examined the expression of RRM2 by qRT-PCR in 40 tumor tissue samples and corresponding non-cancerous tissue samples from LUAD patients. In result, qRT-PCR showed that RRM2 expression was significantly higher in LUAD tissues compared to adjacent non-cancerous tissues (Figure 10A). We also examined the expression of RRM2 in five lung cancer cell lines (A549, SPCA-1, 95-D, PG-49, and NCI-H292), and the results showed that the expression level of RRM2 was significantly higher than that in the normal cell lines (BEAS-2B and HaCaT) (Figure 10B). In particular, the expression level of RRM2 in A549 lung cancer cell line was higher as compared to other lung cancer cell lines, A549 cells thus were selected for the subsequent experiments.

RRM2 promoted A549 cells proliferation and invasion

To explore the role of RRM2 on cell proliferation and invasion, siRNA targeting RRM2 and non-silencing RNA sequences were transfected into A549 cell line, and the efficiency of over-expression and knockdown were validated by qRT-PCR (Figure 10C). In result, qRT-PCR showed that RRM2 expression was markedly lower after transfecting si-RRM2. CCK-8 was used to explore the role of RRM2 on cell proliferation, and the results showed that RRM2 over-expression in A549 cells promoted proliferative activity, whereas RRM2 knockdown markedly inhibited the proliferation ability of A549 cells (Figure 11A). Transwell assays were then

Figure 1. RRM2 expression in lung adenocarcinoma tissues. (A) RRM2 expression in normal and tumor tissues. (B) RRM2 expression in paired tissues.
Table 2. Logistic analysis of the association between RRM2 expression and clinical characteristics.

| Clinical characteristics                  | Total (N) | RRM2 expression OR | 95%CI       | p-Value |
|------------------------------------------|-----------|--------------------|-------------|---------|
| Age (>=65 vs. <65)                       | 503       | 0.72               | 0.50-1.03   | 0.071   |
| Gender (Male vs. Female)                 | 503       | 1.36               | 0.96-1.93   | 0.085   |
| Race (non-Asian vs. Asian)               | 455       | 0.99               | 0.23-4.26   | 0.995   |
| Stage                                    | 495       |                    |             |         |
| I                                        | Reference |                    |             |         |
| II                                       | 1.56      | 1.01-2.40          | 0.043       |
| III                                      | 2.18      | 1.33-3.63          | 0.002       |
| IV                                       | 3.02      | 1.31-7.58          | 0.012       |
| Smoking status                           | 489       |                    |             |         |
| Current smoker                           | Reference |                    |             |         |
| Ever smoker                              | 0.40      | 0.25-0.62          | 4.66e-05    |
| Never smoker                             | 0.33      | 0.18-0.60          | <0.001      |
| T (T2 vs. T1)                            | 436       | 1.88               | 1.28-2.79   | 0.001   |
| M (M1 vs. M0)                            | 359       | 2.25               | 0.97-5.65   | 0.066   |
| N classification                         | 489       |                    |             |         |
| N0                                       | Reference |                    |             |         |
| N1                                       | 1.78      | 1.12-2.83          | 0.015       |
| N2                                       | 2.69      | 1.59-4.65          | <0.001      |
| Location (central vs. peripheral)        | 174       | 1.60               | 0.87-2.97   | 0.135   |
| Radiation therapy (yes vs. no)           | 157       | 2.42               | 0.75-9.25   | 0.158   |
| New tumor event after initial treatment (yes vs. no) | 187 | 2.28               | 1.02-5.37   | 0.049   |
| EGFR mutation (yes vs. no)               | 272       | 0.76               | 0.45-1.28   | 0.306   |
| KRAS mutation (yes vs. no)               | 62        | 0.50               | 0.17-1.40   | 0.191   |
| EML4-ALK translocation (yes vs. no)      | 242       | 1.16               | 0.56-2.41   | 0.692   |
| Person neoplasm cancer status (with tumor vs tumor free) | 407 | 1.73               | 1.12-2.71   | 0.014   |
| Primary therapy outcomes (SD+PD vs. CR+PR) | 151 | 1.44               | 0.55-3.94   | 0.463   |

Abbreviations: OR, odds ratio; CI, confidence interval. Bold values indicate P<0.05.

employed to evaluate the role of RRM2 on cell invasion, and the results suggest that RRM2 over-expression markedly promoted A549 cells invasive activity (Figure 11B). Cell cycle was further determined by flow cytometry in attempt to explain the RRM2-mediated promotion of proliferative activity. A549 cells revealed G2/M cell cycle arrest after RRM2 knockdown (si-RRM2) (Figure 11C). To verify the underlying mechanism, the relevant signaling pathway proteins, p53, Bcl-2, Livin, and E-cadherin were determined by western blot assay. The results demonstrated RRM2 over-expression in A549 cells increased expression of Bcl-2/E-cadherin and decreased expression of p53, whereas RRM2 knockdown (si-RRM2) markedly decreased the expression of Bcl-2/E-cadherin and increased the expression of p53 (Figure 11D).

**DISCUSSION**

Ribonucleotide reductase, an enzyme involved in the cell cycle, has two components identified in humans, the RRM1, the regulatory subunit and RRM2, the catalytic subunit, which are vital for DNA synthesis and damage repair [14, 25]. RRM2B is one small subunit of RRM2, which is induced by p53 and plays an important role in repairing DNA damage [26]. Maintenance of mitochondrial DNA is also necessary [27]. Ribonucleotide reductase plays an important role in regulating the sizes of dNTP pools, which is required for correct DNA replication [27]. Changes in the sizes of dNTP pools or disruption in their balance will lead to increased mutation rates [27, 28]. Ribonucleotide reductase high expression is a characteristic of many solid tumors, and a series of mechanistically distinct ribonucleotide reductase inhibitors serve as effective agents for cancer treatment [29–31]. As the rate-limiting ribonucleotide reductase enzyme, the RRM2 level controls the cell cycle-dependent activity of ribonucleotide. Suppression of RRM2 expression sensitizes cancer cells to both ribonucleotide reductase inhibitors and cisplatin [29, 32]. Overexpression of RRM2 is associated with poorer prognosis in multiple
cancerous lesions [19, 33]. For instance, patients with cervical cancer with positive RRM2 expression showed a higher recurrent rate and lower survival rate than those with negative RRM2 expression [34]. Additionally, RRM2 dysregulation is related to chemoresistance during cancer treatment. RRM2 downregulation can overturn AKT-induced tamoxifen resistance and increase therapeutic efficacy in breast carcinoma [35].

Figure 2. Box plot evaluating RRM2 expression of patients with lung adenocarcinoma according to different clinical characteristics. (A) Age; (B) Clinical stage; (C) M classification; (D) N classification; (E) Cancer status; (F) New tumor event; (G) Smoking status; (H) T classification.

Figure 3. Kaplan-Meier curve for overall survival in lung adenocarcinoma. (A) Kaplan-Meier curve for RRM2 in all tumor patients; (B–H) Subgroup analysis for age greater than 65 years, stage I/II, M0, N0, with tumor, smoker, and T1/T2.
Table 3. Univariate and multivariate Cox regression analyses of clinical characteristics associated with overall survival.

| Clinical characteristics                                      | Univariate analysis | Multivariate analysis |
|---------------------------------------------------------------|---------------------|-----------------------|
|                                                               | HR                  | 95% CI                | p-Value   | HR                  | 95% CI                | p-Value   |
| Age                                                           | 1.18                | 0.86-1.63             | 0.309     | 1.36                | 0.96-1.93             | 0.083     |
| Gender                                                        | 1.04                | 0.76-1.43             | 0.789     | 1.13                | 0.80-1.59             | 0.487     |
| Race                                                          | 2.01                | 0.28-14.35            | 0.488     |                     |                      |           |
| Stage                                                         | 1.67                | 1.44-1.93             | **1.12e-11** | 1.28                | 1.01-1.64             | **0.043** |
| Smoking status                                                | 0.92                | 0.72-1.19             | 0.540     | 0.84                | 0.65-1.10             | 0.207     |
| T                                                            | 1.53                | 1.25-1.86             | **3.12e-05** | 1.16                | 0.90-1.51             | 0.245     |
| M                                                            | 0.92                | 0.77-1.11             | 0.386     | 1.13                | 0.91-1.39             | 0.259     |
| N                                                            | 1.72                | 1.44-2.06             | **1.82e-09** | 1.39                | 1.05-1.84             | **0.022** |
| Location                                                      | 0.95                | 0.57-1.58             | 0.829     |                     |                      |           |
| Radiation therapy                                             | 2.87                | 1.32-6.23             | **0.007** |                     |                      |           |
| New tumor event after initial treatment                       | 2.3                 | 1.31-4.05             | **0.003** |                     |                      |           |
| EGFR mutation                                                 | 1.33                | 0.82-2.15             | 0.242     |                     |                      |           |
| KRAS mutation                                                 | 2.21                | 0.85-5.76             | 0.103     |                     |                      |           |
| EML4-ALK translocation                                        | 1.96                | 1.06-3.63             | **0.031** |                     |                      |           |
| Primary therapy outcomes                                      | 2.44                | 0.90-6.59             | 0.078     |                     |                      |           |
| Person neoplasm cancer status                                 | 4.74                | 3.27-6.87             | **2.08e-16** | 1.69                | 1.37-2.08             | **7.20e-07** |
| RRM2 expression                                               | 1.34                | 1.16-1.54             | **4.86e-05** | 1.29                | 1.11-1.50             | <0.001    |

Abbreviations: HR, hazard ratio; CI, confidence interval. Bold values indicate P<0.05.

Figure 4. Forest plot of the multivariate Cox regression analysis in lung adenocarcinoma.
Figure 5. Diagnostic value of RRM2 expression in lung adenocarcinoma. (A) ROC curve for RRM2 in normal lung tissue and LUAD; (B–H) Subgroup analysis for stage I/II, stage III/IV, M0, T1/T2, T3/T4, N0, N1-N3.

Figure 6. Nomogram for predicting probability of patients with 1-, 3- and 5-year overall survival. For risk estimation, identify the status for each clinical factors and expression value of RRM2, draw a line straight upwards to the Points axis to see the points a single factor yields. Repeat until scores for all factors are decided. Sum the points and locate the summed point on the Total Points axis. Then 1-, 3- and 5-year related survival probabilities were obtained by draw a line straight down to the Risk axis.
Table 4. Gene sets enriched in the high RRM2 expression phenotype.

| Gene set name                          | SIZE | ES    | NES    | NOM p-Val | FDR q-Val |
|----------------------------------------|------|-------|--------|-----------|-----------|
| KEGG_CELL_CYCLE                       | 124  | 0.839 | 2.697  | <0.001    | <0.001    |
| KEGG_P53_SIGNALING_PATHWAY             | 66   | 0.666 | 2.49   | <0.001    | <0.001    |
| KEGG_DNA_REPLICATION                   | 36   | 0.942 | 2.242  | <0.001    | 1.56e-04  |
| KEGG_SMALL_CELL_LUNG_CANCER           | 84   | 0.553 | 2.077  | <0.001    | 0.003     |
| KEGG_APOPTOSIS                        | 86   | 0.454 | 1.758  | 0.013     | 0.032     |
| KEGG_PATHWAYS_IN_CANCER               | 324  | 0.44  | 1.863  | 0.004     | 0.018     |

Abbreviations: ES, enrichment score; NES, normalized enrichment score; NOM, nominal; FDR, false discovery rate. Gene sets with NOM P-value <0.05 and FDR q-value <0.25 were considered as significantly enriched.

RRM2 gene expression and functions in carcinoma have been recently reported [36–38]. However, the significance of its expression in prognosis in patients with LUAD is largely unclear. In the current study, we analyzed the RRM2 expression profile in numerous human solid tumors via using Oncomine database. The results demonstrated RRM2 gene expression was higher in lung cancer, colorectal cancer, breast cancer, bladder cancer, sarcoma cancer, liver cancer, and others than in their matched adjacent normal tissues. RRM2 gene expression and its potential prognostic impact on patients with LUAD have not been evaluated. In 2015, Mah et al. explored the expression level of RRM2 in an NSCLC stratification subgroup based on gender and smoking status to predict survival outcomes. The results revealed high RRM2 expression was associated with primarily women and non-smoking patients [39]. Toffalorio et al. investigated the expression levels of seven genes involved in gemcitabine metabolism in advanced NSCLC, including the RRM2 gene; the results revealed RRM2 gene expression was not associated with patients with advanced NSCLC treated with gemcitabine [40]. This is the first comprehensive study to evaluate RRM2 gene expression in the prognosis of patients with LUAD. In the present study, we evaluated the RRM2 gene expression profile via bioinformatic analysis using TCGA database. With respect to matched normal lung tissues, RRM2 gene expression levels were significantly higher in patients with LUAD and the result has been confirmed by qRT-PCR. Kaplan-Meier

Figure 7. Enrichment plots from GSEA. Gene set enrichment plots of (A) cell cycle, (B) p53 signaling pathway, (C) DNA replication, (D) small cell lung cancer, (E) pathways in cancer, and (F) apoptosis in lung adenocarcinoma cases with high RRM2 expression.
Figure 8. Kaplan-Meier curve for overall survival in lung adenocarcinoma in the validation datasets GSE30219 (A–D) and GSE50081 (E–H).

Figure 9. Expression analysis of RRM2 by Oncomine and TIMER databases. (A) Expression of RRM2 in different types of human cancers in the Oncomine database; (B) RRM2 is over-expression (red) in lung adenocarcinoma by Oncomine meta-analysis comparing with normal tissue; (C) Expression of RRM2 in different types of human cancers in the TIMER database.
survival analysis revealed that patients with \textit{RRM2} high expression had shorter OS ($p < 0.05$). This finding was validated in other independent external datasets (all $p < 0.05$). Multivariate Cox analysis further confirmed that \textit{RRM2} high expression was an independent risk factor for OS in patients with LUAD; other clinicopathologic features were also associated with worse prognosis in LUAD, including advanced stage, lymph nodes metastases, with tumor, as suggested by the forest plot. ROC analysis also verified the diagnostic value. At present, a predictive nomogram for LUAD by combining the expression value of \textit{RRM2} with clinical variables has not been reported. Therefore, we constructed a prognostic nomogram by integrating clinical factors and gene expression via TCGA dataset to enable clinicians to predict the risk of individual patient death and guide patient assessment and therapeutic decision-making.

We found that the high \textit{RRM2} expression phenotype was associated with the cell cycle, p53 signaling pathway, and other features such as advanced stage and lymph node metastasis.

**Figure 10.** \textit{RRM2} expression in human LUAD tissues and cell lines. (A) Expression of \textit{RRM2} in 40 LUAD tissues and corresponding non-cancerous tissues were detected by qRT-PCR; (B) \textit{RRM2} expression levels in A549, SPCA-1, 95-D, PG-49, BEAS-2B, HaCaT, and NCI-H292 by qRT-PCR; (C) \textit{RRM2} expression in A549 cells transfected with si-\textit{RRM2}, O-\textit{RRM2}, si-NC, and O-Control were confirmed by qRT-PCR. *indicates a $p$-value < 0.05, and **indicates a $p$-value < 0.01.

**Figure 11.** \textit{RRM2} promoted cell proliferation and invasion and regulated cell cycle progression of lung cancer cells. (A) CCK-8 proliferation curve of A549 cells transfected with si-\textit{RRM2}, O-\textit{RRM2}, si-NC, and O-Control; (B) Transwell assays were performed to evaluate the role of \textit{RRM2} on cell invasion; (C) The cell cycle analyzed of A549 cells by flow cytometry; (D) \textit{RRM2}-associated signaling pathways were determined by western blot assay. *indicates a $p$-value < 0.05, and **indicates a $p$-value < 0.01.
DNA replication, small cell lung cancer, apoptosis, and pathways in cancer by GSEA. DNA replication ensures that cellular genetic information is accurately copied and correctly transmitted to offspring cells [41, 42]. However, DNA replication is prone to interference and damage under various pressures in the body, leading to stagnant of DNA replication, affecting genome stability and even inducing apoptosis, necrosis, and carcinogenesis. p53 is a transcription factor that specifically binds to DNA [43]. It can regulate specific gene expression and induce cell cycle suspension, DNA repair, and apoptosis. Pathway enrichment analysis suggested that RRM2 affects the pathogenesis of proliferation and invasion in lung carcinoma through the above pathways. Han et al. found that RRM2-c2orf48 over-expression induced epithelial-mesenchymal transition in nasopharyngeal carcinoma cells results in the down-regulation of E-cadherin and promoted nasopharyngeal carcinoma cell migration and invasion [44]. Li et al. demonstrated that RRM2 is over-expressed in glioblastoma and this over-expression can promote the proliferation, migrative, and invasive abilities, whereas suppress cell apoptosis of glioblastoma cells both in vivo and in vitro [45]. Shah et al. demonstrated that nuclearfactor-kappaB (NF-kB), MAPK/JNK, and EGFR are the major signaling pathways that are affected by the over-expression of RRM2 and this overexpression increased gene expression of EGFR, Bcl-2, and MMP9 and decreased gene expression of p53, CDKN1A, and CDKN2A [46]. Furthermore, RRM2 over-expression promoted the proliferative activity, migratory and invasive capabilities of breast cancer cells. Several studies have also found RRM2 protein interact with key cell cycle genes and signaling pathway proteins, such as p53, PI3K, hypoxia inducible factor-1α, Bax, and cyclin D1, to regulate tumor cell proliferation, migratory, and invasive abilities and cell cycle progression [45–48]. Our results, partly in line with the findings in the above studies, showed that RRM2 promoted lung cancer cell proliferation and invasion and cell cycle progression. However, such mechanisms require further investigation.

In summary, we showed that RRM2 is upregulated in LUAD, and high RRM2 expression was correlated with clinical progression and considered as an independent risk factor for OS in patients with LUAD. We also found that RRM2 promotes the proliferative activity and invasive capabilities of lung cancer cells; suggesting that RRM2 play a crucial role in tumor initiation, development and malignant behavior and may be used as biomarker in the diagnosis and prognosis of patients with lung adenocarcinoma. Moreover, the p53 signaling pathway, cell cycle, DNA replication, small cell lung cancer, apoptosis, and pathways in cancer may be pivotal pathways regulated by RRM2 in LUAD. RRM2 expression may be an important prognostic factor in patients with LUAD.

MATERIALS AND METHODS

Patient data sets

mRNA expression data (535 samples, Workflow Type: HTSeq-FPKM) and clinical information were downloaded from TCGA database (https://cancergenome.nih.gov). The following samples were excluded: (1) “0” gene expression value and (2) insufficient survival information. A total of 503 patients with LUAD with the corresponding clinical features were enrolled in this study.

Gene set enrichment analysis (GSEA)

GSEA is a computational method determines whether a previously defined set of genes have significant statistical and concordant differences in two biological states [49]. GSEA was performed to identify all genes found to be correlated with RRM2 gene expression in recent studies and to examine the significant survival differences observed between the high- and low-RRM2 groups. For each analysis, gene set permutation was performed 1000 times. Gene sets with a normal p-value less than 5% and false discovery rate less than 25% were considered as significantly enriched.

Sample collection

LUAD and adjacent tissues were collected from 40 patients immediately stored in liquid nitrogen, and preserved at -80°C. The use of all samples conformed to the Declaration of Helsinki. The present study was approved by the Ethics Committee of People’s Hospital of Xinjiang Uygur Autonomous Region.

Cell culture and transfection

Human normal lung epithelial cell line (BEAS-2B), human immortalized keratinocytes cell line (HaCaT; normal control), and human lung cancer cell lines (A549, SPCA-1, 95-D, and NCI-H292) were obtained from the cell bank of the Chinese Academy of Sciences in Shanghai (Shanghai, China), and PG-49 was obtained from American Tissue Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Gran Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin; Gibco), except SPCA-1, PG49, and HaCaT, which were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco). All cells were cultured at 37°C, 5% CO2 in a humidified atmosphere incubator.

The lentiviral vector containing RRM2 small interfering RNA (siRNA) was constructed by GeneChem Co., Ltd.
(Shanghai, China). siRNA targeting RRM2 (NM_001034) sequences was 5’-GGAGGCAUUUAG CCAAGAAGU-3’. Four groups were constructed as follows: si-RRM2 transfected group (si-RRM2), RRM2 overexpressed group (O-RRM2), an empty lentiviral vector was regarded as a negative control (si-NC), and un-transfected group (O-Control). Transfection was performed as described previously [50]. The efficiency of over-expression and knockdown were validated by qRT-PCR. Finally, the transfected A549 lung cancer cells were collected for subsequent experiments.

**Quantitative real-time polymerase chain reaction (PCR) of tissues and cell lines**

Total RNA was extracted from cell lines or tissue specimens using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Shanghai, China) according to the manufacturer’s instructions, and RNA was reversely transcribed into cDNA using Transcription First Strand cDNA synthesis kit (Roche, Basel, Switzerland). Quantitative real-time PCR (qRT-PCR) analyses were quantified with SYBR® Green (Roche, Basel, Switzerland). The relative expression of RRM2 was calculated based on the 2^(-ΔΔCT) method with β-actin as an internal reference. qRT-PCR primers used in the present study were as follows: RRM2 forward primer, 5'-ACGGAGACTCTACCCAGTGG-3'; RRM2 reverse primer, 5'-GCACGACGCTGAGGATCAA-3'; β-actin forward primer, 5'-CTCCATCTCGGGCCTCGGT-3'; β-actin reverse primer, 5'-GCTGTACACTTCAC CGTTC-3'.

**Cell proliferation and invasion assay**

To evaluate the proliferative activity of lung cancer cells, a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was performed according to the manufacturer’s instructions. Briefly, A549 cells were plated in 96-well plates (Density: 3 × 10^3 cells per well) and incubated overnight. Absorbance of 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Shanghai, China) daily for four days. The experiment was independently repeated three times. Transwell chamber assays were performed to assess the invasive ability of lung cancer cells according to the manufacturer’s instructions.

**Western blot assay and antibody**

RIPA lysis buffer (Thermo Fisher Scientific, Shanghai, China) containing protease and phosphatase inhibitors was used to lyse lung cancer cells. Protein lysates were separated by SDS-PAGE gels (Thermo Fisher Scientific, Shanghai, China), blotted onto PVDF membrane (Roche, Basel, Switzerland) for analysis and incubated at 4°C for overnight with the following primary antibodies: anti-p53 antibody (1:3000 dilution; #10442-A-AP, Abcam, Cambridge, MA, USA), anti-Livin antibody (1:1000 dilution; #ab32124, Abcam, Cambridge, MA, USA), anti-E-Cadherin antibody (1:2000 dilution; #20874-1-AP, Sanying Biotechnology, Wuhan, China), anti-Bcl2 antibody (1:1000 dilution; #ab97350, Abcam, Cambridge, MA, USA), and anti-GAPDH antibody (1:5000 dilution; #HRP-60004, Sanying Biotechnology, Wuhan, China). The results of the western blot analyses were performed with Image J software.

**Cell cycle assay**

A549 cells were plated in six-well plates (Density: 2 × 10^5 cells per well) and incubated for 12 h. A549 cells were incubated to 80-90% confluency and then washed two times with PBS and fixed with 70% precooled ethanol at 4°C for 12 h. A549 cells were re-washed two times with PBS and cultured with 500 μl propidium iodide (PI)/RNase staining buffer (Cell Signaling Technology, Inc, Danvers, MA, USA) for 30 minutes away from light. The distribution of cells in G0/G1, G2/M, and S phases of the cell cycle was determined by flow cytometry (Beckman Coulter Quanta SC System).

**Statistical analysis**

The expression level of the RRM2 gene in patients with LUAD was evaluated by using box plots. The cut-off value of RRM2 expression was selected as the median method of gene expression. Wilcoxon signed-rank test and logistic regression were performed to analyze the association between clinical features and RRM2 expression in LUAD. Kaplan-Meier analysis was performed to compare the overall survival (OS) rate between the high and low RRM2 gene expression groups using the p-value determined in the log-rank test. A received operating characteristic (ROC) curve was applied to assess the diagnostic value of RRM2 gene expression, with the area under the ROC curve used as the diagnostic value. Use univariate Cox analysis to screen potential prognostic factors, and multivariate Cox analysis to verify the effect of RRM2 expression on survival along with other clinical variables. A nomogram was constructed to predict 1-, 3- and 5-year LUAD overall survival by combining the expression value of RRM2 with clinical variables. External validation was applied via using two other independent external datasets (GSE30219 and GSE50081). The expression level of RRM2 in patients with LUAD and normal individuals was further validated in the Oncomine database (https://www.oncomine.org/resource/main.html) and TIMER database (https://cistrome.shinyapps.io/timer/).
statistical analyses were performed using R statistical software (version 3.5.3), SPSS software (version 24.0), or MedCalc software (version 19.1). A p-value less than 0.05 is considered as statistically significant.

**AUTHOR CONTRIBUTIONS**

J.C.Y., and Z.Y.X designed this study and had full access to all of the data in the study; J.C.Y., and D.L. extracted the data; N.E.L.A.H., and W.X.L. analyzed and interpreted the data; Y.Y.W., and G.R. wrote the paper. All authors reviewed the manuscript.

**CONFLICTS OF INTEREST**

The authors have no conflicts of interest.

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Supplementary Figure 1. Box plot evaluating RRM2 expression of patients with lung adenocarcinoma according to different clinical characteristics. (A) Sex; (B) Race; (C) Location in lung; (D) kras mutation status; (E) EGFR mutation status; (F) EML4-ALK translocation; (G) Therapy outcomes and (H) Diagnostic value of RRM2 expression in lung adenocarcinoma in GSE30219.