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R-spondin family members as novel biomarkers and prognostic factors in lung cancer

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Abstract. The R-spondin (RSPO) family of secreted proteins consists of four members that have critical roles in embryonic development and organogenesis. However, the expression patterns and the exact roles of the individual RSPO family members in tumorigenesis and progression of lung cancer are unknown, particularly in non-small cell lung cancer, which accounts for 85% of all lung cancer cases. In the present study, data from the ONCOMINE database was used to compare the RNA expression levels of RSPOs in multiple different types of cancer with normal controls. The expression profiles of RSPOs in various types of cancer cell lines were subsequently compared based on data from the Broad Institute Cancer Cell Line Encyclopedia. Using the Kaplan-Meier plotter, the prognostic value of expression of the different RSPOs members was determined for different pathological subtypes of lung cancer. When compared with normal tissues, expression of RSPO1, RSPO2 and RSPO3 was significantly lower in patients with lung cancer. In the survival analysis, increased mRNA expression levels of RSPO1, RSPO2 and RSPO3 were associated with increased survival in patients with lung adenocarcinomas. These results suggest that RSPO1, RSPO2 and RSPO3 may serve as distinct biomarkers and prognostic factors in patients with lung cancer.

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Introduction

Lung cancer is one of the most fatal types of cancer in developed countries, and remains the leading cause of cancer-associated mortality (1), with a higher mortality rate than prostate, breast and colon cancer combined, and with >1 million deaths expected per year worldwide (2). Although treatments for lung cancer have improved in recent years, including surgery, chemotherapy, radiation therapy, local treatment and targeted therapy, >50% of patients with lung cancer present with advanced disease at the first diagnosis and therefore, no longer qualify for surgery (3).

Gene mutations serve an important role in tumorigenesis. Since the discovery of epidermal growth factor receptor (EGFR)-activating mutations and mutations of anaplastic lymphoma kinase (ALK), the potential avenues for novel or repurposed therapeutics have increased (4,5). Compared with other cancers, lung cancer has one of the highest rates of genetic alterations (6); therefore, targeted therapeutics for specific genes/proteins may lead to improvements in the outcomes of patients with lung cancer. Therefore, targeted therapies hold great promise for patients with lung cancer.

Mutant genes can be produced by chromosomal rearrangements, including the translocation, insertion, inversion and deletion of chromosomes (5). EGFR mutations and ALK fusion genes are two well-studied and common oncogenic drivers in patients with lung cancer, and small molecule inhibitors of these target genes have been utilized in the clinic to selectively inhibit the growth of gene-positive non-small cell lung cancer (7,8). Furthermore, expression of the mutant genes in cancer may additionally serve as identifying markers for lung cancer and cancer typing.

The R-spondin (RSPO) family of proteins consists of four members (RSPO1-4), all of which are secreted, and are widely expressed in vertebrate embryos and in adults (9). The four genes that encode the RSPO proteins share 40-60% pairwise amino acid sequence identity and are predicted to possess substantial structural homology (10). Furthermore, the RSPO proteins are characterized by two furin-like cysteine-rich domains, a thrombospondin-type 1 repeat domain and a basic-amino acid-rich C-terminal domain (9,11,12).

The Wnt/β-catenin signaling pathway has a vital role in cell proliferation, development and disease pathogenesis (13). RSPOs serve a regulatory role in cell progress, which include substance transport and catabolism, cell movement, cell growth and death, and cellular communication, by activating and synergizing the Wnt/β-catenin signaling pathway (14,15). For example, RSPO1 and the leucine-rich repeat-containing G-protein coupled receptor 4/5 (LGR4/5) potentiate Wnt/β-catenin signaling, increased liver size and improved liver regeneration (16). RSPO3 may promote vascular development in *Xenopus laevis* by upregulating the expression of vascular endothelial growth factor, through the activation of the Wnt/β catenin signaling pathway (17). Recently, the role of RSPOs in oncogenesis has also been investigated. The RSPO genes may facilitate tumor development in the colon by promoting Wnt signaling (18). In addition, a previous study suggested that chromosomal rearrangements of the RSPO2 and RSPO3 genes may initiate hyperplasia and tumor development *in vivo* (19). Furthermore, it has been previously demonstrated that the RSPO family of proteins may serve as tumor suppressors. RSPO1 and LGR5 co-localize and form complexes with transforming growth factor β1 (TGF-β) receptor I and II, enhancing TGF-β downstream signaling, and therefore, suppressing metastasis of colon cancer (20).

Taken together, the aforementioned studies suggest that the RSPO family of proteins may act as potential therapeutic targets or prognostic biomarkers in certain types of cancer. However, to the best of our knowledge, there are no studies demonstrating the expression or role of RSPOs in lung cancer. In the present study, the expression of the RSPO proteins in patients with different pathological types of lung cancer and in lung cancer cell lines was determined by utilizing publicly available databases and their prognostic value was assessed.

Materials and methods

ONCOMINE database analysis. ONCOMINE is an online database of tumor-related gene expression (www.oncomine.org). In the present study, ONCOMINE was used to analyze the mRNA expression differences of the RSPO family of proteins between tumor and normal tissues in common types of human cancer, including lung cancer (2,346 cases), liver cancer (524 cases) and leukemia (2,546 cases). In this database, Student's t-test was used to compare tumor and normal tissues. The thresholds of gene analysis were set as follows: P<0.01; fold-change, 2; gene rank, top 10% [where the tumor shown is within the top 10% of the tumor type of the RSPO gene, which included bladder cancer, brain and CNS cancer, breast cancer and lung cancer (Fig. 1)]; analysis type, cancer vs. normal; and data type, mRNA. The above data were obtained from studies that showed statistically differences in expression. Furthermore, to analyze the mRNA expression differences of the RSPO family of proteins between different pathological types of lung cancer, the terms 'adenocarcinoma', 'squamous cell carcinoma', 'large cell lung cancer' and 'small cell lung cancer' were used.

Cancer Cell Line Encyclopedia (CCLE) database analysis. The CCLE database (portals.broadinstitute.org/ccle/home), is an online encyclopedia of a compilation of gene expression, chromosomal copy number and massively parallel sequencing data from 947 human cancer cell lines (21). CCLE was used to analyze the mRNA expression levels of RSPOs in a range of cell lines for different types of cancer, including non-small lung cancer, liver cancer and breast cancer, and marked as 'lung_NSC', 'liver' and 'breast', respectively in Fig. 2.

Kaplan-Meier (KM) plotter database analysis. The KM plotter (kmplot.com/analysis/) is capable of assessing the effect of 54,675 genes on survival using 10,461 cancer samples, including 3,452 lung cancers samples, with a mean follow-up of 69.5/34.5/17 months for all patients with lung cancer, adenocarcinoma and squamous cell carcinoma respectively. The cancer patients were divided into high and low expression groups by the median values of mRNA expression, where expression levels above this value were classified as high expression and below as low expression. The prognostic values of the members of the RSPO family of proteins with increased expression in different pathological types of lung cancer samples were further assessed to determine overall survival (OS) using KM plotter. The log-rank test was used to compare OS between high and low expression groups. P<0.05 was used to indicate a statistically significant difference.

Results

Expression of RSPO1-3 is significantly decreased in patients with lung cancer. The ONCOMINE database was used to determine whether there was a difference in the mRNA expression levels of the members of the RSPO family of proteins between cancer and normal tissues in lung cancer. At present, four RSPO proteins have been identified in different types of cancer, including hematological malignancies and solid tumors (9). The database contained a total of 257,259,286 and 234 unique analyses for RSPO1, RSPO2, RSPO3 and RSPO4, respectively. ONCOMINE analysis revealed that RSPO1, 2 and 3 mRNA expression levels were significantly lower in lung cancer tissue compared with normal samples in a wide variety of datasets, which differed from many other types of cancer (Fig. 1). RSPO1 had a 2-fold increase in normal tissues compared with that in lung cancer tissue, while RSPO2 and RPSO3 had a 4- and 8-fold increase, respectively. In addition, analysis of the CCLE was consistent with that of ONCOMINE analysis, demonstrating that RSPO 1-3 were distinctively downregulated in lung cancer cell lines (Fig. 2). The mRNA expression of RSPO1, 2 and 3 was lower in lung adenocarcinoma compared with normal tissues (P<0.01). However, except for the expression level of RSPO2 in small cell lung cancer, which was decreased (P=0.0073), the RSPO1 and 3 expression levels were not significantly different between cancer tissue and normal tissue in other types of lung cancer, including squamous cell carcinoma, large cell lung carcinoma and small cell lung carcinoma (all P>0.05; Fig. 3A-C). However, there was no significant difference in the mRNA expression level of RSPO4 between squamous cell carcinoma, large cell lung carcinoma and small cell lung carcinoma samples and normal controls. As shown in Fig. 1, there was no statistical significant difference in the expression level of RSPO4 mRNA

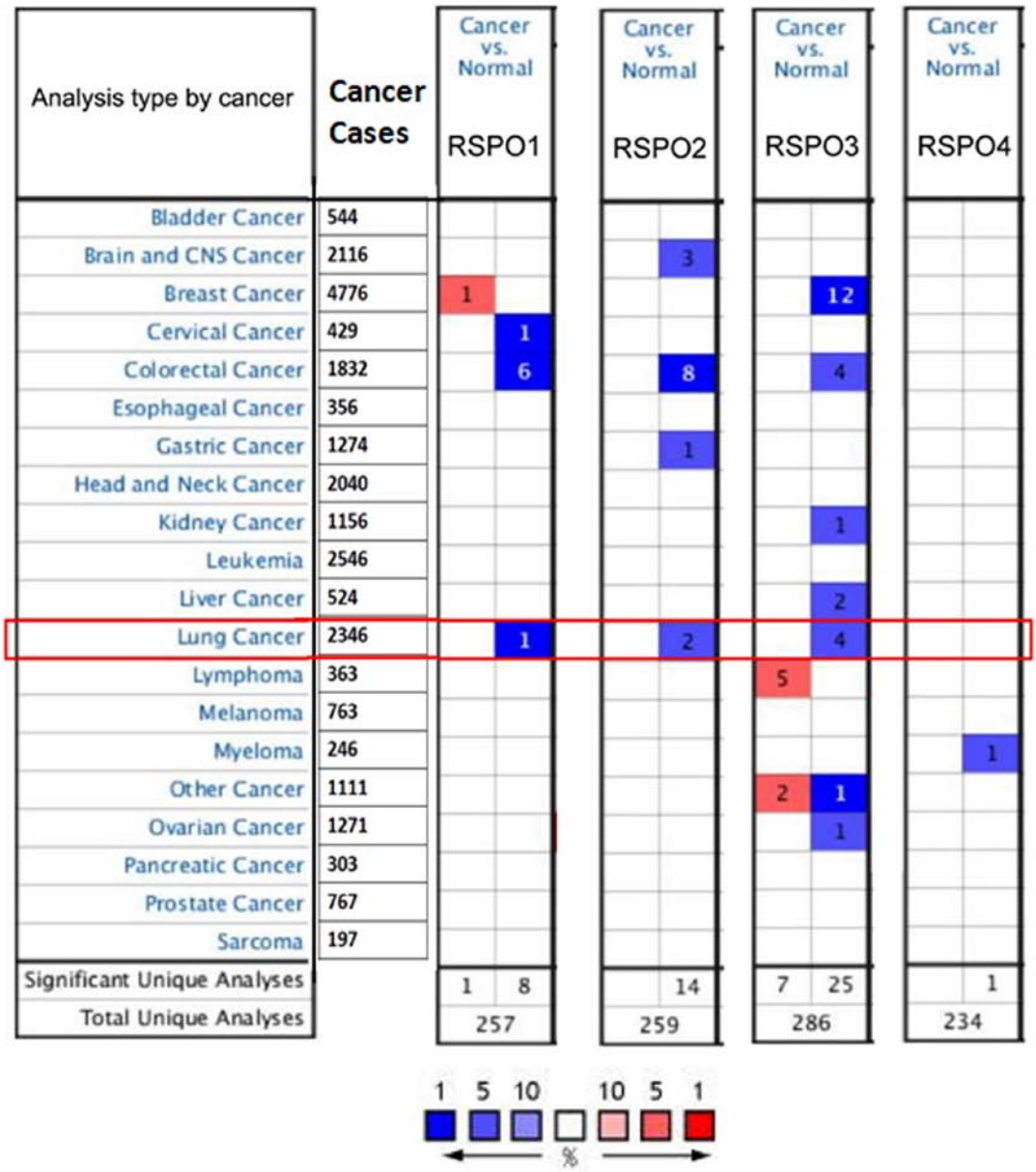

Figure 1. mRNA expression levels of R-spondin family in human types of cancer. The number in the colored cell represents the number of analyses that satisfied the threshold. Cell color was determined by gene rank. The more intense the red (high expression) or the blue (low expression) the greater the significant difference in expression. RSPO, R-spondin; CNS, central nervous system.

in lung cancer. Furthermore, except for the high expression of RSPO4 in lung adenocarcinoma (P=0.0053), there was no statistical significant difference of RSPO4 mRNA expression level between tumor tissues and normal tissues in other types of lung cancer, including squamous cell carcinoma, large cell carcinoma and small cell carcinoma (P=0.0601, P=0.7919, P=0.6257, respectively) (Fig. 3D). These results suggest that the function of RSPO1, RSPO2 and RSPO3 may differ from the function of RSPO4, and may have unique roles in tumorigenesis in lung cancer.

High RSPO 1-3 mRNA expression is associated with improved OS in patients with lung cancer. The prognostic effect of RSPO1, RSPO2 and RSPO3 in patients with lung cancer was subsequently assessed. High mRNA expression levels of RSPO2 and RSPO3 were associated with improved OS in all patients with lung cancer [RSPO2, hazard ratio (HR)=0.72, P=0.00015; RSPO3, HR=0.79, P=0.0053; Fig. 4]. In particular, analysis of lung cancer pathological sub-types revealed that high mRNA expression of RSPO1, 2 and 3 was significantly associated with improved OS in patients with lung adenocarcinoma (RSPO1, HR=0.75, P=0.022; RSPO2, HR=0.58, P=0.000018; RSPO3, HR=0.79, P=0.049); however, there was no significant association between expression of RSPO1, 2 and 3 and OS in patients with squamous cell carcinoma, which suggested that RSPO1, RSPO2 and RSPO3 may have prognostic value for patients with lung adenocarcinoma (Fig. 4). Contrary to RSPO1, 2 and 3, RSPO4 expression was associated with worse OS in patients with lung adenocarcinoma (HR=1.37; P=0.011). However, high RSPO4 mRNA expression level was independent of OS in all patients with lung cancer (HR=0.99; P=0.95; Fig. 4).

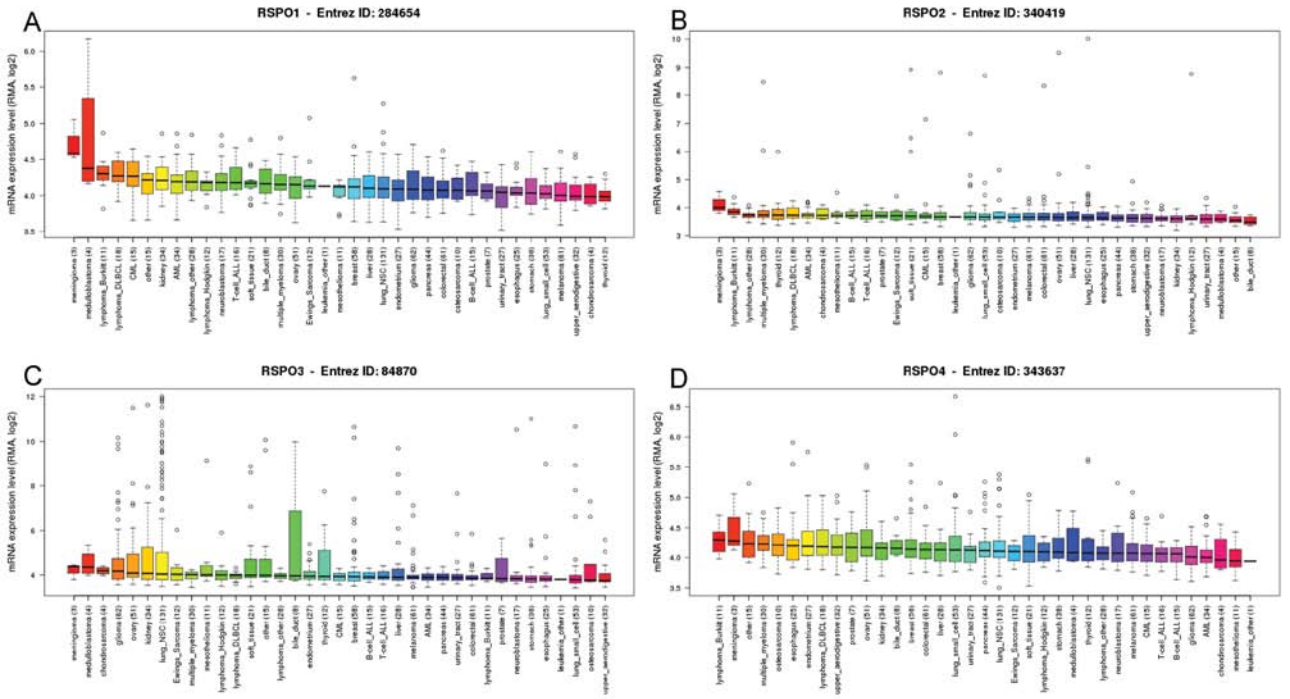

Figure 2. RSPO protein family expression across 4,103 primary tumors from Cancer Cell Line Encyclopedia database. Box-and-whisker plots showed the distribution of (A) RSPO1, (B) RSPO2, (C) RSPO3 and (D) RSPO4 mRNA expression for each subtype, ordered by the median RSPOs expression level (line), the inter-quartile range (box) and up to 1.5x the inter-quartile range (bars). RSPO, R-spondin; DLBCL, diffuse large B-cell lymphoma; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; NSC, non-small cell.

Discussion

Lung cancer presents with the highest prevalence and mortality rates among all types of cancer, with a 5-year overall survival rate of only 15%, even with conventional chemotherapy, radiotherapy and surgical interventions (22). The low overall survival rates are primarily due to resistance to chemotherapy, radiotherapy and targeted therapy, in addition to the frequent presence of distant metastases and recurrent or unresectable tumors (3). Therefore, the development of effective anticancer therapies against lung cancer is required.

The RSPO family of secreted proteins has a regulatory role in cell progress and has been demonstrated to be involved in tumorigenesis (17,19). The RSPO protein family also had lower expression in normal tissues compared with that in colorectal cancer tissue. Recently, research showed that the RSPO family fusion gene was found in superficially serrated adenoma, which is a recently proposed subtype of colorectal serrated lesion, suggesting that the deletion of RSPO protein family is closely related to the development of colorectal cancer (23). This suggests that RSPO protein family also plays an important role in the development of lung cancer. In the present study, the mRNA expression levels of the RSPO proteins in various types of cancers, and lung cancer in particular, were systematically examined using the ONCOMINE database and CCLE. In addition, the prognostic values of the four RSPO proteins were examined in patients with different subtypes of lung cancer through the KM plotter. The present study suggested that, among the four RSPO proteins, RSPO1, RSPO2 and RSPO3 expression was significantly lower in lung cancer compared to normal controls, suggesting a potential protective role in lung cancer.

Wnt signaling is a highly conserved signaling pathway that controls cell proliferation and homeostatic processes (24). Wnt signaling primarily consists of three distinct signaling pathways: Wnt/Ca^{2+} pathway; Wnt/planar cell polarity pathway; and Wnt/β-catenin pathway (24). The role of Wnt/β-catenin signaling in inflammatory responses and cell development has been extensively studied (10,12,14). The four RSPO proteins act through their cognate LGR4, LGR5 and LGR6 receptors to amplify Wnt/β-catenin signaling (10,19,20).

It has been demonstrated that RSPO1 interferes with Dickkopf-related protein 1/Kremen-mediated internalization of LRP6, therefore increasing cell surface retention of LRP6 and subsequently regulating the cellular responsiveness to Wnt ligands (25). Furthermore, studies have shown that RSPO1/LGR5 directly activates TGF-β signaling, and when RSPO1 activated LGR5, LGR5 formed complexes with TGF-β receptors, and was therefore able to suppress colon cancer progression (20). In Fig. 1, there was low expression of RSPO1-3 in colon cancer, which may be related to the progression of colon cancer. Survival analysis of RSPO1 in the present study demonstrated similar results, high expression of RSPO1 was associated with improved OS in patients with lung cancer, particularly in lung adenocarcinoma, suggesting RSPO1 tumor-suppressive role.

Recently, it was demonstrated that RSPO2 acts as a direct antagonistic ligand to E3 ubiquitin-protein ligase RNF43 and E3 ubiquitin-protein ligase ZNRF3, which together, constitute a master switch that governs limb development independent of LGR4 (26). A previous study has also indicated that induced mucosal RSPO2 expression in susceptible mice lead to the generation of a poorly differentiated epithelium and to fatal colitis, and implicated RSPO2-mediated Wnt signaling activation in intestinal morphogenesis, proliferation and

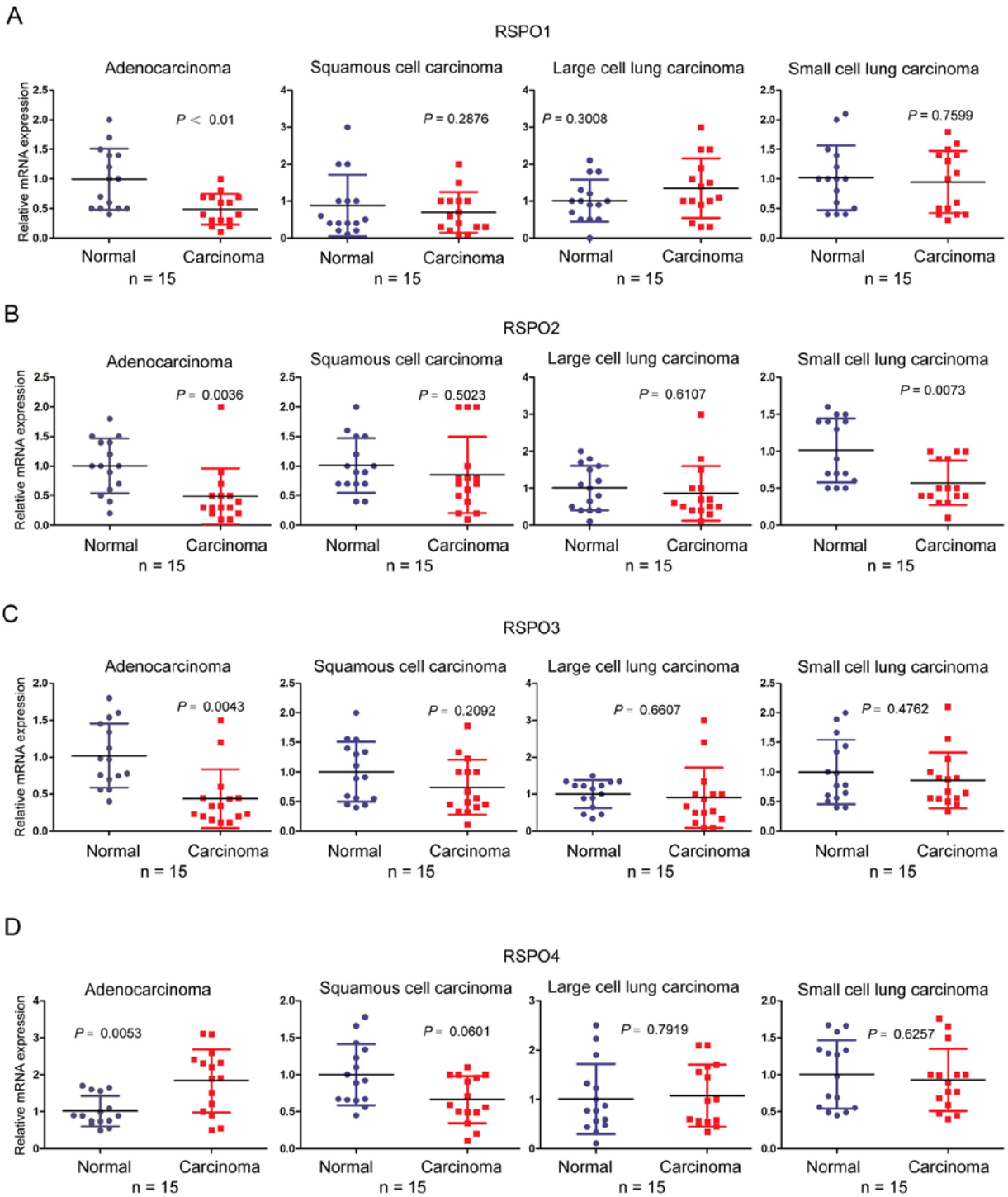

Figure 3. mRNA expression level of (A) RSPO1, (B) RSPO2, (C) RSPO3 and (D) RSPO4 in different pathological types of lung cancer from Cancer Cell Line Encyclopedia database, including adenocarcinoma, squamous cell carcinoma, large cell lung cancer and small cell lung cancer. RSPO, R-spondin. Error bars indicate mean ± SD.

carcinogenesis (27). Furthermore, in tumorigenesis, RSPO2 enhances Wnt signaling conferring stemness-associated traits to susceptible pancreatic cancer cells (28). However, its role in lung cancer progression remains largely unknown. To the best of our knowledge, the present study is the first to demonstrate that high RSPO2 expression predicted better survival in patients with lung cancer, particularly in lung adenocarcinoma, although there was no significant effect on survival in patients with squamous cell carcinoma.

RSPO3 is also associated with Wnt/β-catenin signaling. Vidal *et al* (29) suggested that deletion of RSPO3 resulted in a loss of Sonic-hedgehog signaling and therefore impaired organ growth. A previous study demonstrated that targeting protein tyrosine phosphatase receptor type K-RSPO3-fusion inhibits colorectal tumor growth and promotes differentiation (30). In the present study it was demonstrated that similar to RSPO1 and RSPO2, RSPO3 expression was significantly lower in samples from patients with lung adenocarcinoma.

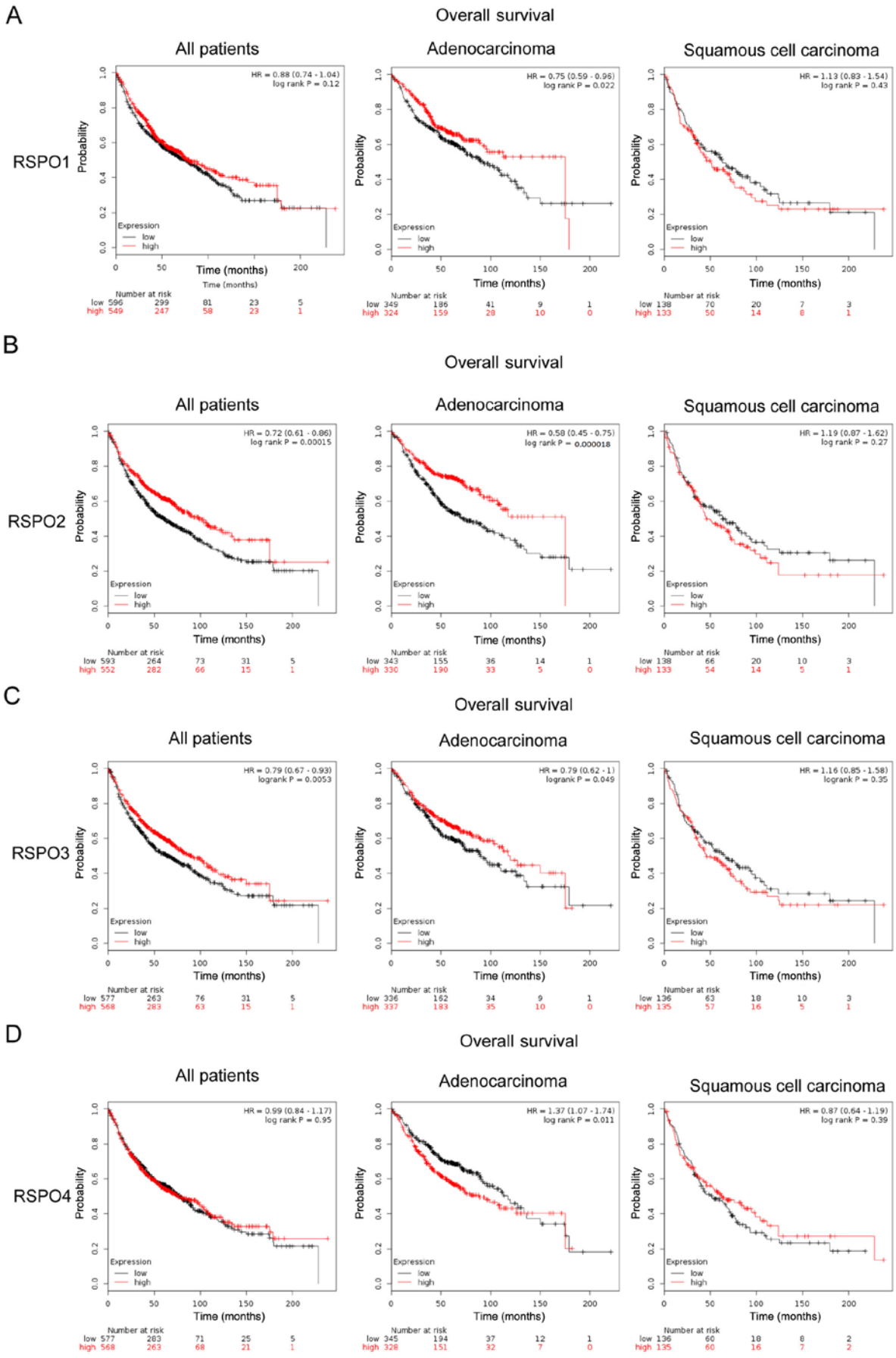

Figure 4. Survival analyses of RSPO family of proteins in lung cancer. Prognosis analysis of (A) RSPO1, (B) RSPO2, (C) RSPO3 and (D) RSPO4 in different pathological types of lung cancer. HR=1, indicated no association between exposure factors and disease. HR >1, indicated that the exposure factor is a risk factor for the disease (positive correlation). HR <1, indicated that the exposure factor is the protective factor of the disease (negative correlation). RSPO, R-spondin; HR, hazard ratio.

Furthermore, high levels of RSPO3 predicted improved OS in patients with lung cancer, particularly in lung adenocarcinoma. Taken together, RSPO1, 2 and 3 expression may be favorable prognostic predictors in patients with lung cancer, particularly lung adenocarcinoma.

There are considerably fewer studies investigating the function of RSPO4. In a recent study, it was demonstrated that an RSPO4-mutant may have been associated with isolated congenital anonychia/hyponychia (31). In the present study, RSPO4 was not detected in almost all types of cancer tissues and normal tissues. Survival analysis showed that the expression level of RSPO4 was not directly associated with OS of patients with lung cancer, suggesting a difference in the function of RSPO4 in lung cancer compared with the other family members.

In conclusion, high expression levels of RSPO1, RSPO2 and RSPO3 in lung cancer predicted improved OS, particularly in patients with lung adenocarcinoma. RSPO1, RSPO2 and RSPO3 may serve important protective roles in lung cancer, and may represent novel biomarkers and prognostic factors for patients with lung cancer. One of the limitations of this study is that validation of the expression of RSPO family proteins in lung cancer cells lines and tissues was not performed, and therefore further clinical and mechanism analysis are required to verify the results of the current study.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the author on reasonable request.

Authors' contributions

WZ and JQ conceived and designed the project. LW, WZ and JW performed data mining processes and prepared the figures. LW, LJ and CL analyzed and interpreted the data. LJ and CL wrote the manuscript. LW and WZ participated in the revision of the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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