ORIGINAL RESEARCH

AGTR1 Inhibits the Progression of Lung Adenocarcinoma

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Purpose: The occurrence and development of lung adenocarcinoma (LUAD) are related to many factors. Multiple researches showed that the renin-angiotensin system (RAS) plays an important role in lung cancer. This research mainly focuses on angiotensin II receptor 1 (AT1R) encoding gene AGTR1, an important part of the RAS.

Methods: We comprehensively evaluated the expression of AGTR1 in pan-cancer based on RNA sequencing data obtained from The Cancer Genome Atlas (TCGA). We explored the correlation of AGTR1 with clinicopathological features, prognosis and tumor microenvironment in LUAD. We also explored the mechanism through enrichment analysis and verified it with cell lines and tissue samples.

Results: We found that AGTR1 was less expressed in most tumors and related to prognosis based on the TCGA database. To further explore its mechanism, we mainly focused on LUAD. Combined with the verification results in the GEO database, AGTR1 was associated with a better prognosis in LUAD. High expression of AGTR1 was associated with less lymph node metastasis (P=0.007) and MET mutation (P=0.019). High expression of AGTR1 was related to the anti-tumor immune microenvironment with high infiltration of B cells, myeloid dendritic cells, monocytes, and low infiltration of myeloid-derived suppressor cells (all P<0.05). Enrichment analysis and in vitro verification results showed that AGTR1 was likely to play a role in LUAD through the PI3K/AKT3 pathway. Finally, we verified the above results through tissue samples and the construction of AGTR1 overexpressing cells.

Conclusion: AGTR1 inhibits the progression of lung adenocarcinoma through the PI3K/AKT3 pathway.

Keywords: AGTR1, tumor suppressor gene, TCGA, lung adenocarcinoma, AKT3

Introduction

Lung cancer is the tumor with the highest incidence and mortality in men, and its incidence rate is second only to breast cancer in women,[1] and lung adenocarcinoma is the most common tissue subtype, accounting for about 40%. Many factors are related to lung adenocarcinoma, including genetic mutations, chemical carcinogens, genetics, and hormones.[2,3] Especially, numerous researches showed great interest in the important relationship between the human hormone (for example, estrogen and angiotensin) and lung cancer.[4–6] Our previous researches mainly focused on estrogen,[7–9] and we will focus on the angiotensin receptor gene in this research.

Renin-angiotensin system (RAS) is an important basis for regulating blood volume and blood pressure homeostasis and is closely related to cardiovascular and renal diseases.[10] Recent studies have shown that in addition to being related to the cardiovascular system, the RAS system may also play an important role in the

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pathogenesis of tumors,[11] but the certain mechanism is still unclear, and studies in different tumors showed inconsistent results. A retrospective study in Scotland showed the relative risks of incident and fatal cancer among the 1559 patients treated with angiotensin converting enzyme (ACE) inhibitors were significantly reduced, and they explained that it might be related to the angiotensin II level.[12] However, based on the results of a cohort study containing 992 061 patients, the use of ACE inhibitors was associated with an increased risk of lung cancer, which was associated with the accumulation of bradykinin in the lung.[5] Moreover, the components of the RSA system are expressed in multiple cells in the tumor microenvironment (TME) and play an important regulatory role.[13] Therefore, more in-depth researches are needed to clarify the role of the RAS system in lung cancer.

Most research on the RAS system in cancer focused on RAS system inhibitors.[5,14] However, as one of the important coding genes of the RAS system, AGTR1 also plays an important role in a variety of tumors. For example, a study showed that AGTR1 mediates breast cancer metastasis by regulating CXCR4/SDF-1α.[15] Another study showed that AGTR1 promotes the invasion of breast cancer.[16] But the role of AGTR1 in lung cancer is currently unclear. Based on the pan-cancer analysis, we found that the expression of AGTR1 in most tumor tissues, including lung cancer, was significantly lower than the corresponding normal tissues and was related to prognosis. Therefore, this research mainly focused on AGTR1, explored the role of AGTR1 in lung adenocarcinoma and its mechanism through a variety of databases, and validates it in lung adenocarcinoma tissue samples and cell lines.

Materials and Methods
Gene Expression Analysis in Pan-Cancer

"Gene_DE" module in the Tumor IMmune Estimation Resource 2.0 (TIMER 2.0, http://timer.cistrome.org/) algorithm database was utilized to analyze AGTR1 gene expression levels,[17] and the expression levels of AGTR1 in different tumor and adjacent normal tissues or specific tumor subtypes of the TCGA (The Cancer Genome Atlas) project were observed. For certain tumors without normal tissues in the TIMER 2.0 database, we compared the expression levels of AGTR1 in tumors tissues and corresponding normal tissues via the Gene expression profiling interactive analysis, version 2 (GEPIA2, http://gepia2.cancer-pku.cn/#general) database.[18]

Survival Prognosis Analysis in Pan-Cancer and LUAD

The online database, Kaplan–Meier plotter were used to assess the prognostic values of AGTR1 in all TCGA tumors, and the significance maps of overall survival (OS) in different tumors were obtained.[19] Kaplan-Meier Plotter is a powerful online tool that can assess the impact of 54,000 genes in 21 cancers on survival, in which patients were divided into two clusters with the best cut-off and calculated via the Kaplan–Meier analysis and Logrank-P test. Besides, the prognosis value of AGTR1 in LUAD was verified in certain Gene Expression Omnibus (GEO) databases via the PrognoScan database[20] (http://dna00.bio.kyutech.ac.jp/PrognoScan/index.html).

Clinicopathologic Characteristics of AGTR1

The clinical phenotype of LUAD samples in TCGA was obtained via the UCSC Xena database[21] (https://xena.ucsc.edu/public/). Use the median as the node to divide the samples into high and low expression groups, and explored the relationship of AGTR1 expression and clinicopathological characteristics through the chi-square test.

Immune Cells Infiltration Analysis

The data of 24 immune cell types and infiltration score were acquired from the ImmuCellAI Database.[22] "Limma" package was used to analyze the difference in the infiltration of different immune cells in the high and low expression groups of AGTR1 with a cuff-off P value <0.05, and the relationship of these immune cells and AGTR1 expression were analyzed in GraphPad Prism 8.4.2. The data were visualized as a histogram and a scatter plot.

AGTR1-Related Gene Enrichment Analysis

We searched the AGTR1-binding proteins through the STRING website ((https://string-db.org/) with the following setting: minimum required interaction score ["Low confidence (0.150)"], meaning of network edges ("evidence"), max number of interactors to show ("no more than 50 interactors" in 1st shell) and active interaction sources ("experiments").

"Similar Gene Detection" module of GEPIA2 were used to obtain the top 100 AGTR1-correlated targeting genes based on the datasets of all TCGA tumor and normal tissues. We showed the partial correlation (cor) and

P-value of top-10 genes in the purity-adjusted Spearman's rank correlation test through heatmap obtained from TIMER2.

Finally, two sets of data were combined to perform Kyoto encyclopedia of genes and genomes (KEGG) pathway and Gene Ontology (GO) analysis, and visualized with a bubble diagram.

Tissue Specimens of the Patient and Cell Culture

This study was approved by the Ethics Review Committee of Zhongnan Hospital of Wuhan University. Tissue specimens from 140 LUAD cases and 18 benign pulmonary lesions (BPL) cases who underwent surgery from April 2014 to July 2020 were collected for tissue chips. Written informed consent was obtained from each patient. The project was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (approval number: 2021100K).

Human LUAD cell lines (Purchased from Hualianke Biological) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium with 10% fetal bovine serum in an incubator at 37°C (5% CO_2 and 95% air). Normal lung bronchial cells BEAS-2B was cultured in Dulbecco's modified Eagle's medium containing 10% FBS under the same culture conditions.

Plasmid Transfection

AGTR1 overexpression plasmid (pcDNA3.1-AGTR1) was customized to Jikai Gene. The pcDNA3.1-AGTR1 and pcDNA3.1-vector were transfected using a PolyJet transfection kit (SL100499; Signage Laboratories, Rockville, MD), following the manufacturer's instructions.

Real-Time Quantitative PCR (qPCR)

The detailed experimental methods were shown in our previously published research.[23] Primers were designed based on AGTR1, AKT3, PI3K mRNA sequence in GenBank. The primers used were as follows: AGTR1, Forward Primer: GATGATTGTCCCAAAGCTGG, Reverse Primer: TAG GTAATTGCCAAAGGGCC. AKT3, Forward Primer: ATT TGTCGAGAGAGCGGGTG, Reverse Primer: GGCT GCATCTGTGATCCCTT. PIK3CG, Forward Primer: TTA CCATCCACGGCAAGGAC, Reverse Primer: AACT GTGAGGTCGGTGTTCC. GAPDH, Forward Primer: GGTGA AGGTC GGAGT CAACG; GAPDH, Reverse Primer: CAAAG TTGTC ATGGA TGACC. Data was

analyzed using the 2-ΔΔCt method. The mRNA expression of AGTR1, PIK3CG, AKT3 were detected in the blank, empty and pcDNA3.1-AGTR1 group.

Western Blotting

Detailed methods were shown in our previously published research.[23] AT1R (ab124505) antibody was purchased from Abcam. GADPH (1E6D9) was obtained from Proteintech.

Flow Cytometry, MTT and Immunohistochemistry

The detailed steps for conducting flow cytometry, MTT and immunohistochemistry were described previously.[23] We use the MTT experiment to explore the effect of AGTR1 on the proliferation of lung adenocarcinoma cells. Flow cytometry was used to detect the apoptosis of each group of cells. AT1R (ab124505) antibody was purchased from Abcam. Immunohistochemical method to analyze the optical density was calculated by Image-Pro Plus software.

Results
Down-Regulation of AGTR1 mRNA Expression in Pan-Cancer Based on TCGA Database

In this study, the mRNA expression of AGTR1 in various cancer types of TCGA was analyzed via the TIMER 2.0 website. As shown in Figure 1A, the expression of AGTR1 in most tumor tissues is lower than the corresponding normal tissues, including Bladder Urothelial Carcinoma (BLCA), Breast invasive carcinoma (BRCA), Cervical squamous cell carcinoma (CESC), Cholangiocarcinoma (CHOL), Colon adenocarcinoma (COAD), Esophageal carcinoma (ESCA), Head and Neck squamous cell carcinoma (HNSC), Kidney chromophobe (KICH), Kidney renal clear cell carcinoma (KIRC), Kidney renal papillary cell carcinoma (KIRP), Liver hepatocellular carcinoma (LIHC), LUAD, Lung squamous cell carcinoma(LUSC), Pheochromocytoma and Paraganglioma (PCPG), Rectum adenocarcinoma (READ), Skin Cutaneous Melanoma (SKCM), Stomach adenocarcinoma (STAD), Thyroid carcinoma (THCA), Uterine Corpus Endometrial Carcinoma (UCEC). The oncomine database indicates that AGTR1 was significantly lower (blue) in almost all tumors compared with corresponding normal tissues (Figure 1B). For the tumors without corresponding normal tissues, such as Adrenocortical carcinoma (ACC), ovarian cancer (OV), Testicular Germ Cell Tumors (TGCT), Uterine Carcinosarcoma (UCS), we compared the
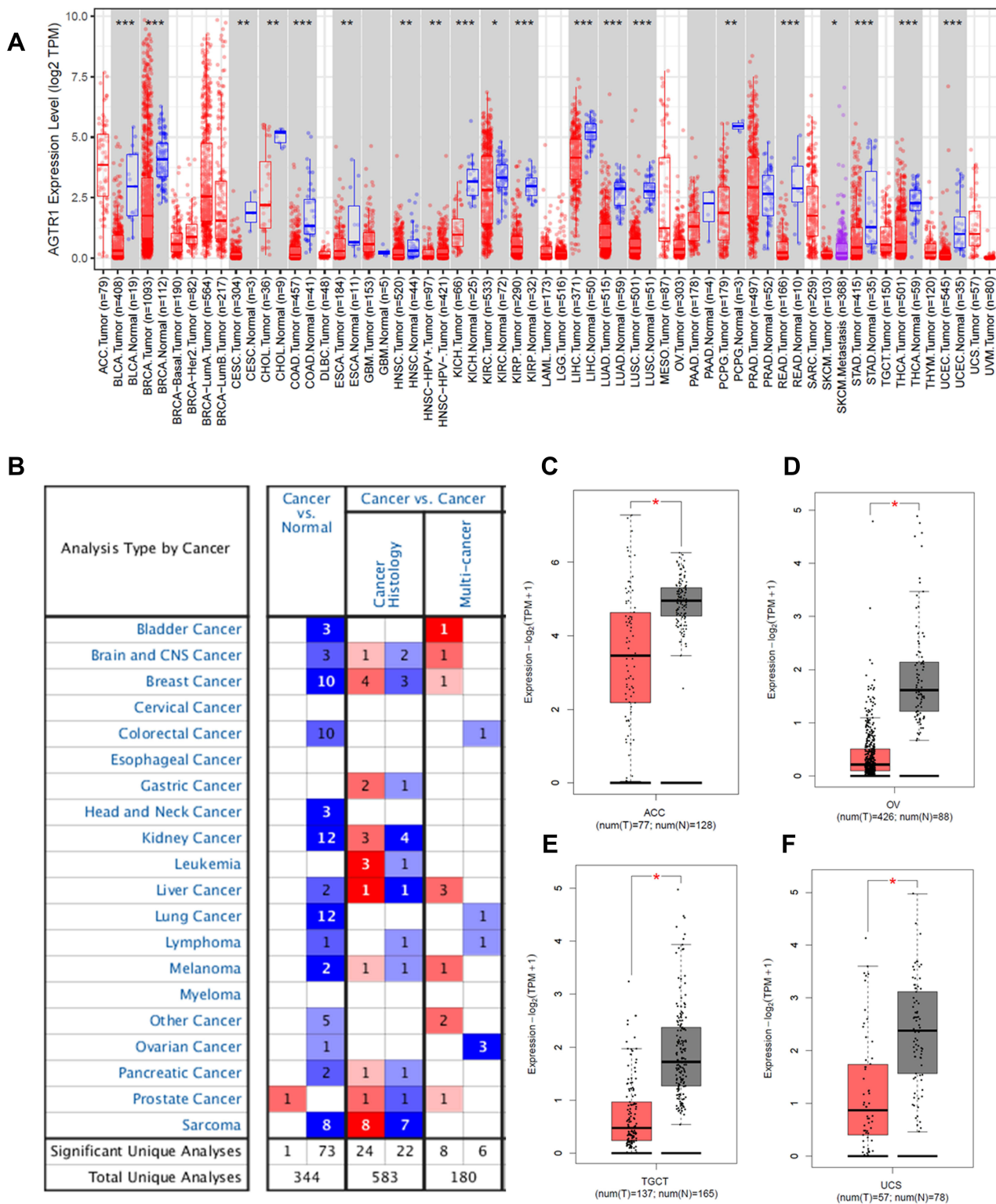
Figure 1 Expression level of AGTR1 in different tumors and corresponding adjacent normal tissues. (**A**) The expression status of the AGTR1 in different cancers or specific cancer subtypes was analyzed through TIMER2. Blue dots indicate the normal tissue and red dots for tumors. (**B**) Oncomine database indicates that AGTR1 was significantly lower (blue) in tumors compared with corresponding normal tissues. (**C**) The expression of AGTR1 in ACC and corresponding normal tissues based on the GTEx database. (**D**) The expression of AGTR1 in OV and corresponding normal tissues based on the GTEx database. (**E**) The expression of AGTR1 in TGCT and corresponding normal tissues based on the GTEx database. (**F**) The expression of AGTR1 in UCS and corresponding normal tissues based on the GTEx database. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

expression of AGTR1 in the tumor tissue with the corresponding normal tissue in GEPIA, similar results were obtained that the expression of AGTR1 in tumor tissues was significantly lower than that in normal tissues (Figure 1C–F). Only a few tumors did not show a significant difference, such as Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC), Glioblastoma multiforme (GBM), Acute Myeloid Leukemia (AML), Lower Grade Glioma (LGG), Mesothelioma (MESO), Pancreatic adenocarcinoma (PAAD), Prostate adenocarcinoma (PRAD), and Uveal Melanoma (UVM).

Prognostic Value of AGTR1 in Pan-Cancer Based on TCGA Database

Based on Affymetrix microarray information from TCGA, we analyzed the AGTR1-related overall survival through Kaplan-Meier Plotter tool. The expression of AGTR1 is associated with the survival of 12 tumors (Figure 2). The results showed that the low expression of AGTR1 is associated with a poor survival in LUAD (total number=504, HR=0.62, P value=0.0032), ESCA (total number=80, HR=0.32, P value=0.013), KIRC (total number=530, HR=0.52, P value<0.001), PAAD (total number=177, HR=0.52, P value=0.0092), THYM (total number=118, HR=0.18, P value=0.0056), SARC (total number=259, HR=0.57, P value=0.012) (Figure 2A). However, high expression of AGTR1 was linked to poor OS for BLCA (total number=404, HR=1.52, P value=0.0066), LUSC (total number=495, HR=1.61, P value=0.0032), OV (total number=373, HR=1.37, P value=0.023), STAD (total number=371, HR=1.65, P value=0.0039), READ (total number=165, HR=2.6, P value=0.012) and UCEC (total number=542, HR=2.33, P value=0.0002) (Figure 2B).

According to the above results, the expression of AGTR1 in LUAD was significantly lower than the corresponding normal tissues, and survival analysis showed low expression of AGTR1 is associated with a poor survival. We speculate that AGTR1 is an independent predictor of risk factors in lung adenocarcinoma. Therefore, we take lung adenocarcinoma as the research object to explore the role and possible mechanism of AGTR1.

Validation of the Prognostic Value of AGTR1 in LUAD Based on the GEO Database

Based on the results of Kaplan-Meier Plotter, it showed that the high expression of AGTR1 plays a protective role in lung adenocarcinoma (Figure 2A). To further verify the correlation of AGTR1 with prognosis in lung adenocarcinoma, we explored the prognostic value of AGTR1 in PrognoScan, in which the data are mainly extracted from the gene expression omnibus (GEO) database. Multiple lung adenocarcinoma databases such as GSE31210 (Figure 3A), MICHIGAN-LC (Figure 3B), Jacob-00182-MSK (Figure 3C), and Jacob-00182-CANDF (Figure 3D) all showed that high AGTR1 expression is associated with a better prognosis. The above data indicated that AGTR1 plays a role in tumor-suppressing.

The Association of AGTR1 with LUAD Clinicopathologic Characteristics and Mutation

512 LUAD samples with complete clinical data were included to analyze the association of AGTR1 expression with clinicopathologic characteristics through the chi-squared test. The results showed that the expression of AGTR1 is significantly correlated with the N stage (P=0.007), that is, patients with low expression of AGTR1 have higher lymph node metastasis analysis (Table 1). The results of exploring the correlation with mutations show that the expression of AGTR1 does not correlate with common mutation types such as EGFR and KRAS, HRAS. However, compared with patients with high expression, patients with low expression of AGTR1 have a higher risk of MET mutations (Table 1, P=0.019), with the mutation probability was as high as 13%. In order to explore whether AGTR1's prediction of lung adenocarcinoma prognosis is related to tissue molecular subtypes, we construct survival analysis based on high or low expression of AGTR1 in different molecular subtypes. The results show that AGTR1 can be used as a prognostic factor for lung adenocarcinoma in different molecular subtypes (Figure S1).

Correlation of AGTR1 Expression with Immune Cells Infiltration in LUAD

Immune cells in the tumor microenvironment play an important role in tumor progression and patient prognosis. In the study, we used the TIMER database to explore the correlation between AGTR1 expression and the level of immune cell infiltration in LUAD. As showed in Figure 4, the expression of AGTR1 was positively correlated with the infiltration of B cells, Myeloid dendritic cells (DC), monocyte, and the high infiltration of these cells is associated with a better prognosis of LUAD (Figure 4A–C). In
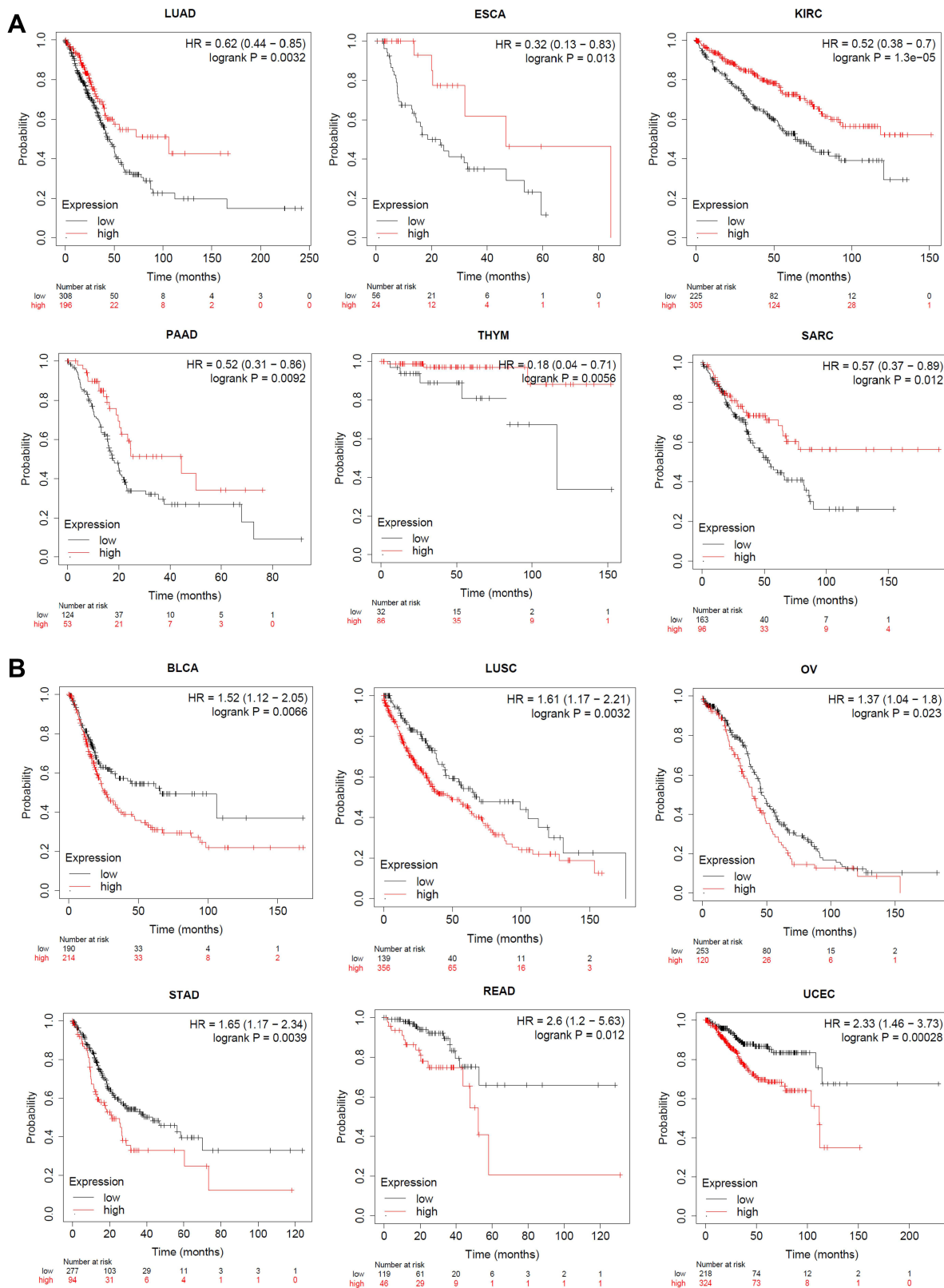
Figure 2 Correlation between AGTR1 gene expression and survival prognosis of cancers in TCGA. (**A**) Kaplan-Meier analysis results based on AGTR1 expression in LUAD, ESCA, KIRC, PAAD, THYM, SARC. (**B**) Kaplan-Meier analysis results based on AGTR1 expression in BLCA, LUSC, OV, STAD, READ, and UCEC. Red represents high AGTR1 expression, black represents low expression.
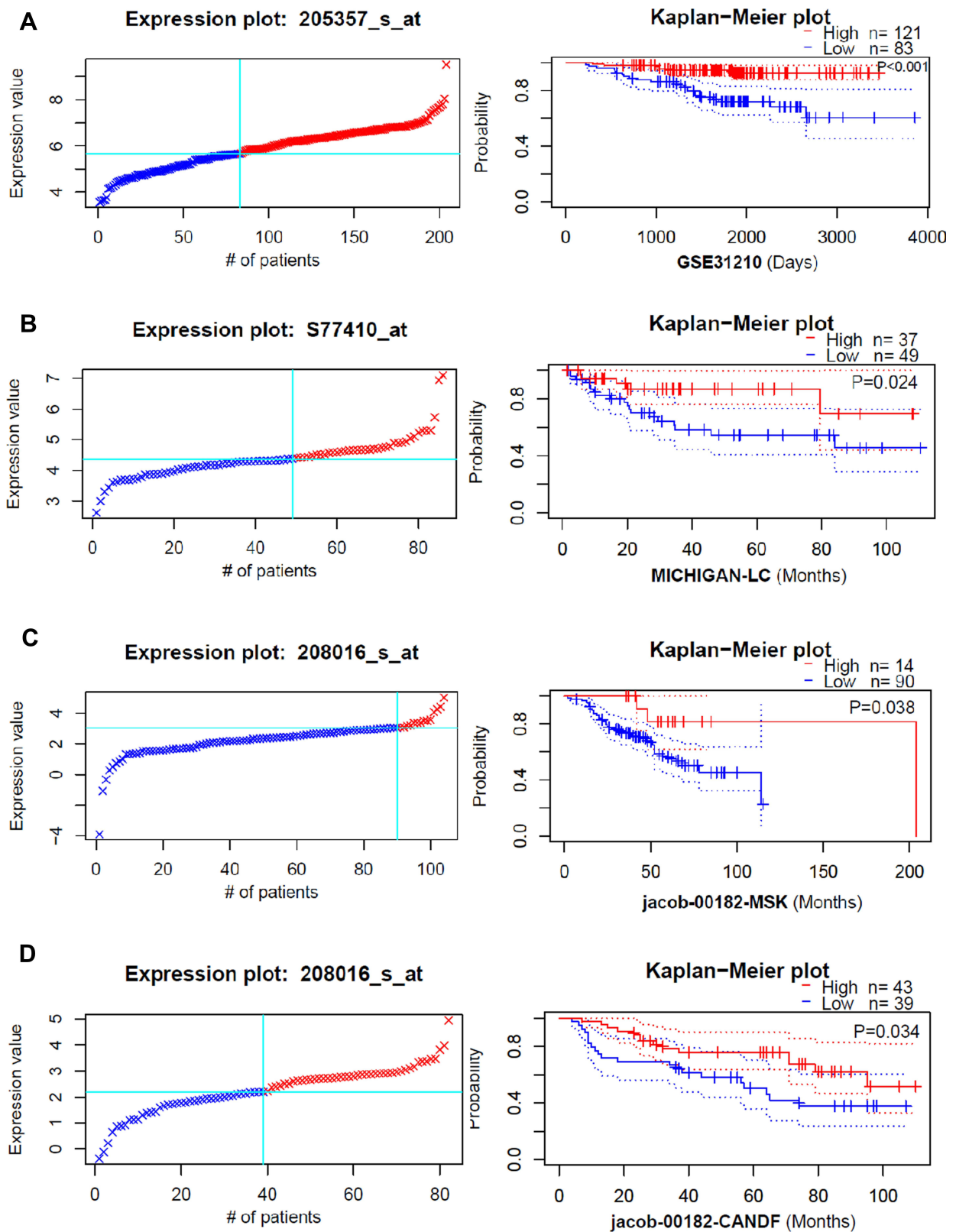
Figure 3 High expression of AGTR1 played a protective role in lung adenocarcinoma based on the GEO database. High AGTR1 expression is associated with a better prognosis in GSE31210 (**A**), Michigan-LC (**B**), Jacob-00182-MSK (**C**), and Jacob-00182-CANDF (**D**). Red represents high AGTR1 expression, blue represents low expression.

Table 1 Association Between AGTR1 and Clinicopathological Characteristics in LUAD

	Total	High Expression	Low Expression	P value
Gender				0.376
Female	274	142(27.7)	132(25.8)	
Male	238	114(22.3)	124(24.2)	
T Stage				0.792
T1–2	446	224(43.8)	222(43.4)	
T3–4	66	32(6.3)	34(6.6)	
N Stage				**0.007****
N0	339	184(35.9)	155(30.3)	
N1–3	173	72(14.1)	101(19.7)	
M Stage				0.663
M0	490	246(48.0)	244(47.7)	
M1	22	10(2.0)	12(2.3)	
Stage				0.065
Stage I-II	405	211(41.2)	194(37.9)	
Stage III-IV	107	45(8.8)	62(12.1)	
EGFR mutation				0.936
0	197	100(43.5)	97(42.2)	
1	33	17(7.4)	16(7.0)	
HRAS mutation				0.308
0	229	117(50.9)	112(48.7)	
1	1	0(0.0)	1(0.4)	
KRAS mutation				0.486
0	162	80(34.8)	82(35.7)	
1	68	37(16.1)	31(13.5)	
MAP2K1 mutation				0.296
0	226	116(50.4)	110(47.8)	
1	4	1(0.4)	3(1.3)	
MET mutation				**0.019***
0	213	113(49.1)	100(43.5)	
1	17	4(1.7)	13(5.7)	
PIK3CA mutation				0.844
0	215	109(47.4)	106(46.1)	
1	15	8(3.5)	7(3.0)	

Notes: Bold numbers in the table represent statistical significance. *P <0 0.05; **P < 0.01.

addition, a negative correlation between AGTR1 expression and the immune infiltration of myeloid-derived suppressor cell (MDSC) was observed, while high infiltration of MDSC is associated with poor prognosis of LUAD (Figure 4D).

Enrichment Analysis of AGTR1-Related Partners

To further explore the molecular mechanism of AGTR1 in LUAD, we screened out AGTR1 binding proteins and AGTR1-related genes through the STRING website and GEPIA2 tools respectively. As shown in Figure 5A, we obtained 51 experimentally verified proteins that bind to AGTR1 through the STRING website, and we constructed an interaction network. We used the GEPIA2 tool to calculate the correlation between all genes and AGTR1, and select the top 100, Figure 5B showed the heat map of the correlation between the top 10 genes and AGTR1 in pan-cancer.

The two gene sets were combined to perform KEGG and GO analyses. The results of KEGG enrichment analysis showed that AGTR1 not only participates in the angiotensin signaling pathway (RAS signaling pathway), but also involved in the pathways of cancer. And the role of AGTR1 in tumors may be related to the PI3K-AKT signaling pathway (Figure 5C). The results of GO enrichment analysis indicated that most of the genes are related to the regulation of multiple pathways or cellular biologies, such as GTP pathway, ERK1, and ERK2 pathway, cell proliferation, and adhesion, protein heterodimerization activity or binding (Figure 5D–F).

To further explore the role of AGTR1 in the PI3K-AKT and ERK signaling pathways, we explored the correlation between AGTR1 and the main genes of the two signaling pathways. As showed in Figure 6A, the expression of AGTR1 was positively correlated with AKT3, but there was no significant correlation with the expression of AKT1 and AKT2. Moreover, the high expression of AKT3 was associated with a better prognosis of lung adenocarcinoma, which was consistent with AGTR1 (Figure 6B). AGTR1 was positively correlated with most PI3K gene subtypes, of which seven subtypes were related to the prognosis of lung adenocarcinoma (PIK3CD, PIK3CG, PIK3IP1, PIK3R1, PIK3R3, PIK3R5, PIK3R6, Figure 6). Compared with normal tissues, AKT3, PIK3IP1, PIK3R1, PIK3R3, and PIK3R5 were significantly lower expressed in lung adenocarcinoma (Figure S2). Although AGTR1 was positively correlated with the expression of MAPK1, there was no correlation between MAPK1 and the prognosis of LUAD. The above results suggested that AGTR1
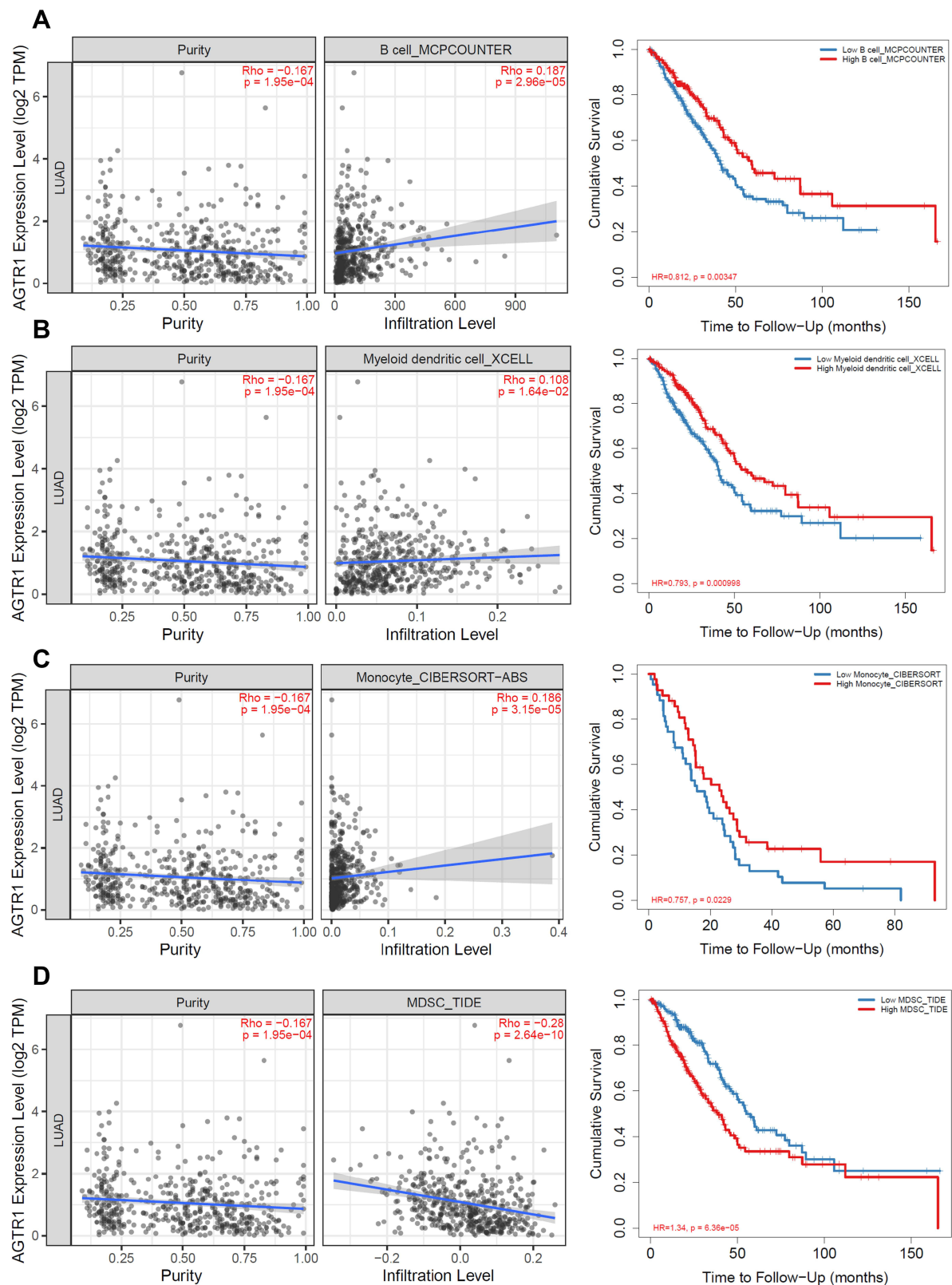
Figure 4 Correlation analysis between AGTR1 expression and immune infiltration of multiple immune cells. The correlation between AGTR1 expression and the infiltration of B cells (**A**), DC (**B**), monocyte (**C**), MDSC (**D**) in LUAD. Red represents high immune cells, blue represents low immune cells.
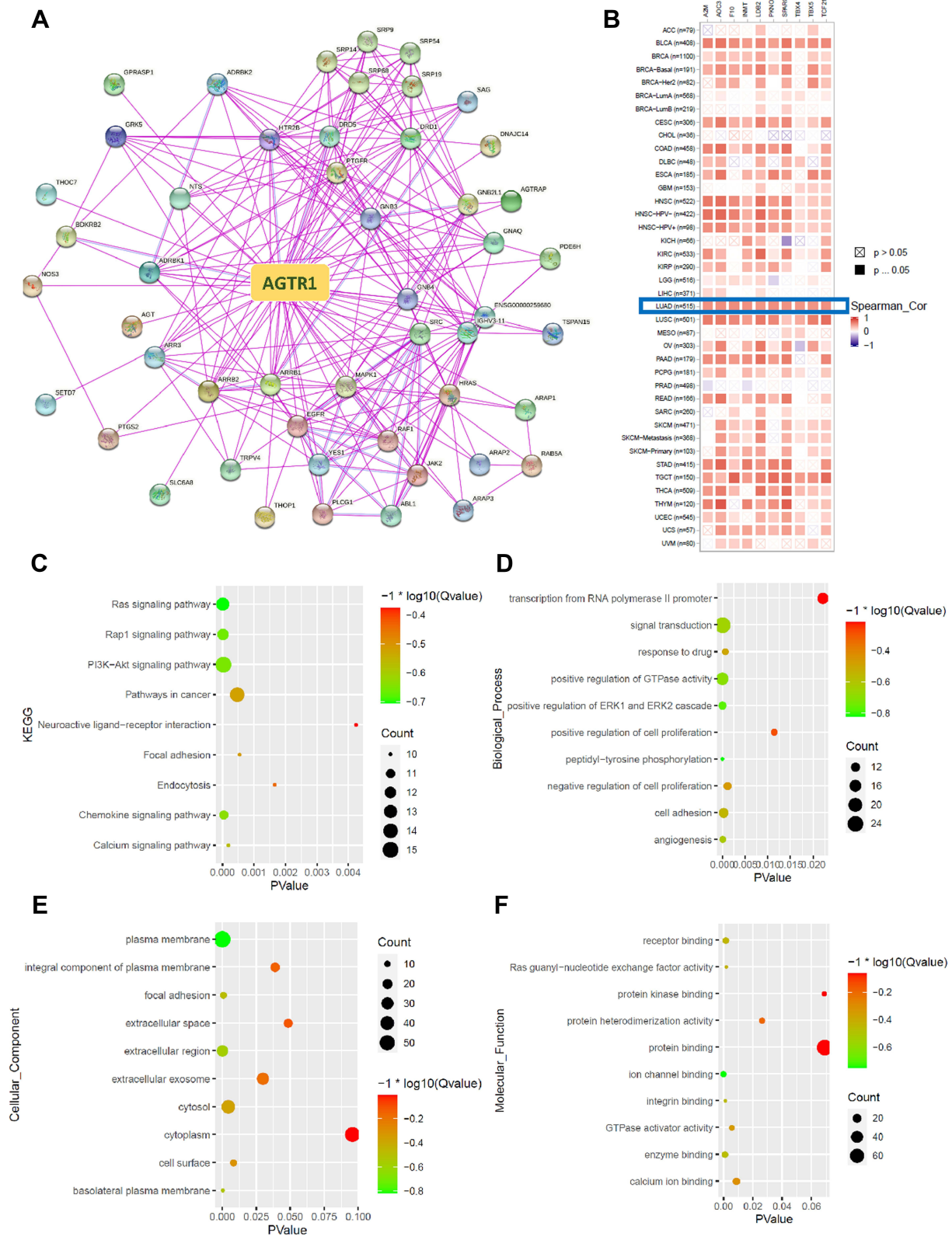
Figure 5 AGTR1-related genes enrichment analysis. (**A**) The protein-protein interaction networks of experimentally determined AGTR1-binding proteins. (**B**) Heat map of the correlation between the top 10 AGTR1-related genes and AGTR1 in pan-cancer. All ten genes were significantly positively related to AGTR1 in lung adenocarcinoma (**C**) Pathway enrichment analysis of the AGTR1-related genes. (**D–F**) The top 10 items of GO analysis: biological processes, cellular components, and molecular functions of the AGTR1-related genes.
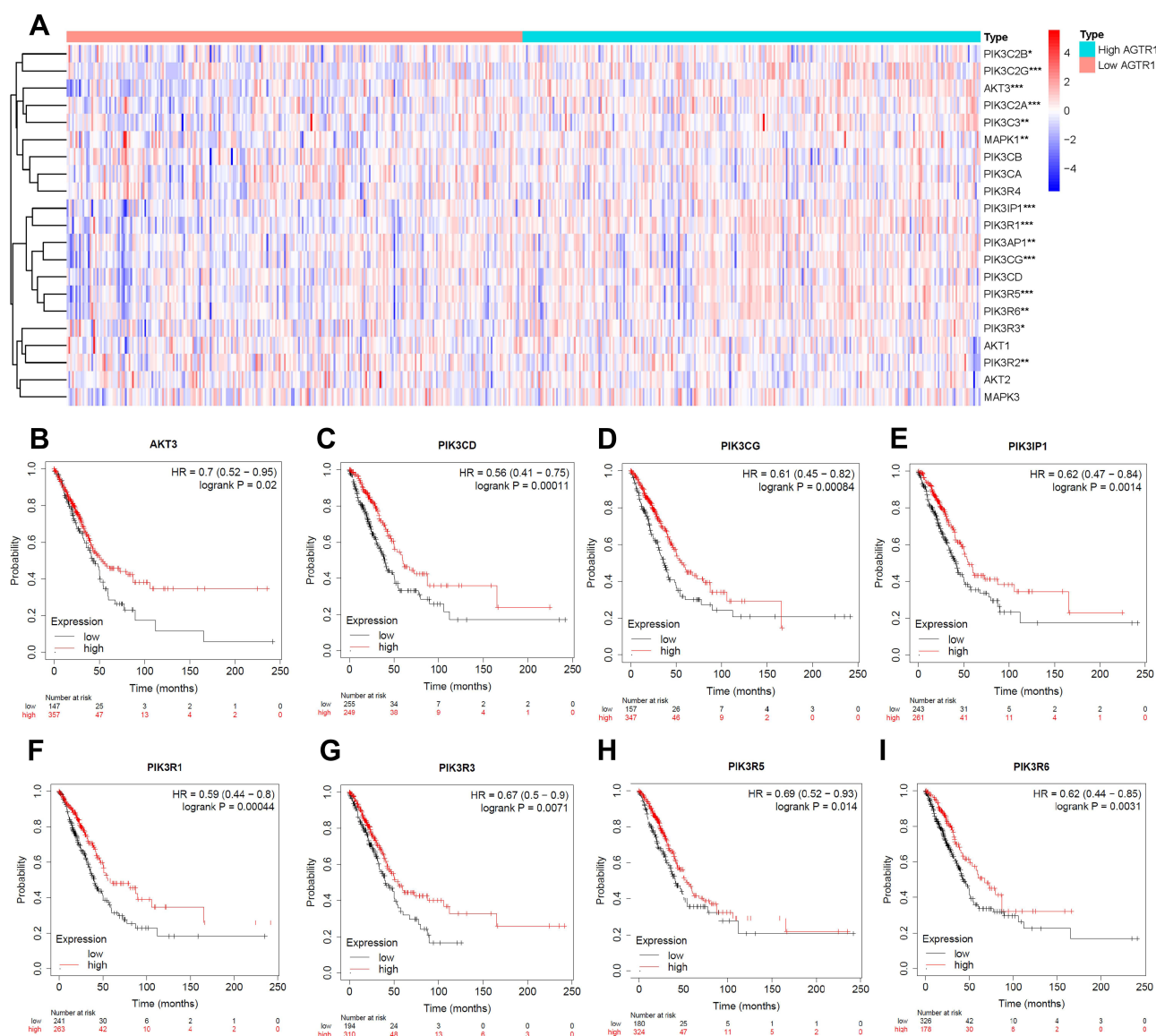
Figure 6 Correlation analysis between AGTR1 expression and PI3K/AKT, ERK1/2 pathways. (**A**) Heat map of the correlation between AGTR1 and the main molecules of PI3K/AKT, ERK1/2 signaling pathway. AGTR1 was positively correlated with AKT3 most PI3K gene subtypes. The KM analysis results of AKT3 (**B**), PIK3CD (**C**), PIK3CG (**D**), PIK3IP1 (**E**), PIK3R1 (**F**), PIK3R3 (**G**), PIK3R5 (**H**), PIK3R6 (**I**) in LUAD. Red represents high genes expression, blue represents low expression. (*P <0 0.05; **P < 0.01; ***P < 0.001).

may play important role in LUAD through the PI3K-AKT3 signaling pathway.

Validation of the Role of AGTR1 in LUAD Tissues and Cells

To verify the effect of AGTR1 in inhibiting the progression of lung adenocarcinoma, we made two tissue chips containing 140 LUAD and 18 BPL samples, and subjected to immunohistochemical analysis. The results of immunohistochemical showed that the expression of AT1R protein in BPL was significantly higher than that in LUAD (P=0.0268, Figure 7A and B). More importantly, the

survival analysis of LUAD samples in tissue chips showed that the prognosis of samples with high AT1R expression was significantly better than that of samples with low AT1R (P=0.0389, Figure 7C), which was consistent with the previous analysis results in the TCGA database. To further verify the above results in lung adenocarcinoma cell lines, we compared the expression of AGTR1 mRNA between multiple lung adenocarcinoma cell lines (H1299, H1975, PC9, A549, H460) and normal lung bronchial cell (BEAS-2B), the results showed that the mRNA expression of AGTR1 in BEAS-2B was significantly higher than that in lung adenocarcinoma cell lines (Figure 7D). Similar
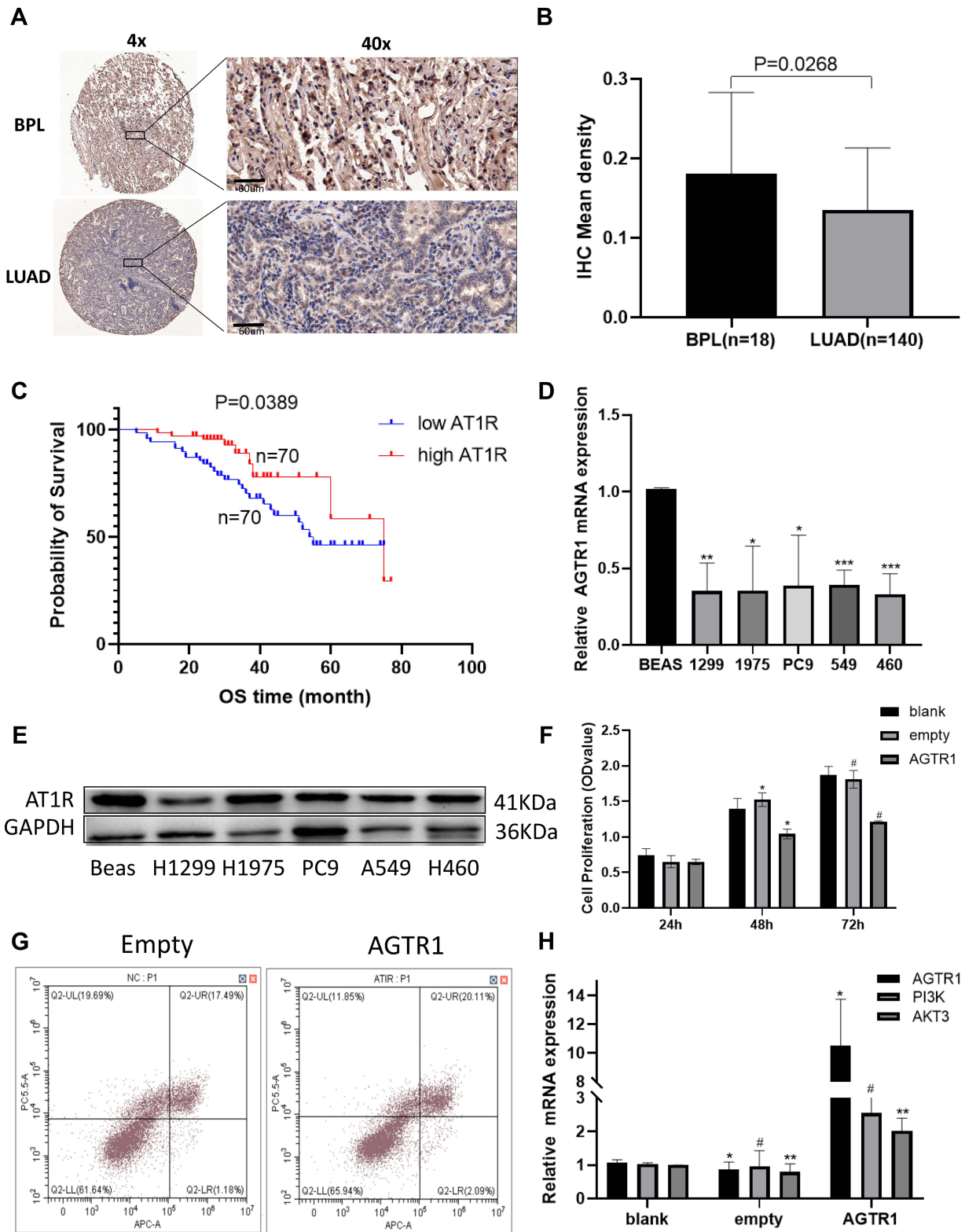
Figure 7 Validation of role of AGTR1 in LUAD tissues and cells. (**A**) Immunohistochemical analysis of the expression of AT1R in LUAD and LUAD and benign pulmonary lesions (BPL) samples. (**B**) The corresponding gray value of immunohistochemical analysis. (**C**) Survival analysis comparing high and low expression of AT1R in 140 lung adenocarcinoma samples. (**D**) The mRNA level of AGTR1 in normal lung bronchial cell (BEAS-2B) and lung adenocarcinoma cell lines (NCI-H1299, NCI-H1975, PC9, A549, NCI-H460). (**E**) The protein level of AT1R in normal lung bronchial cell and lung adenocarcinoma cell lines. (**F**) The corresponding gray value of western-blot. (**D**) The results of MTT in 24, 48, and 72 hours. *P<0.05, **P<0.01, ***P<0.001. (**G**) The percentage of H1299 cells apoptosis in empty and AGTR1 overexpressing group. (**H**) The mRNA levels of PI3K and AKT3 in a different group. *, #, **represent the comparison of AGTR1, PI3K, and AKT3 between empty and AGTR1 groups, respectively, P<0.05.

results were obtained in Western blot, the expression of AT1R protein in lung adenocarcinoma cell lines was significantly lower than that in BEAS-2B cell (Figure 7E). In order to further explore the role of AGTR1, we constructed the H1299 cell line overexpressing AGTR1 mRNA and detected its apoptosis and proliferation by flow cytometry and MTT. The results of MTT showed that AGTR1 overexpression inhibited the proliferation of lung adenocarcinoma cells (Figure 7F). Flow cytometry results show that cells overexpressing AGTR1 show more apoptosis (Figure 7G). In order to further verify the role of the PI3K/AKT3 pathway shown by the above enrichment analysis, we detected the mRNA expression of PIK3CG and AKT3 respectively. The results were consistent with the results of the enrichment analysis, the expressions of PI3K and AKT3 were up-regulated in the AGTR1 overexpression group (Figure 7H).

Discussion

Studies have shown that the dysregulation of the RAS system may be related to the occurrence and prognosis of tumors,[24] and in the researches of gastric cancer, breast cancer, ovarian cancer, etc., the expression of AGTR1 has been proved to be related to tumor staging.[15,25,26] However, the role of AGTR1 in tumors is currently unclear, and different studies have shown different results. The analysis of this study in pan-cancer also showed conflicting results. The results showed that the expression of AGTR1 in most tumors is lower than that of corresponding normal tissues (Figure 1). Low AGTR1 was associated with poor prognosis in some tumors, such as LUAD, ESCA, KIRC, etc, while high AGTR1 was associated with poor prognosis in other tumors, such as BLCA, LUSC, OV, etc (Figure 2). The expression of AGTR1 in LUAD was significantly lower than the corresponding normal tissues, and low expression was associated with poor survival and lymph node metastasis (P=0.007, Table 1). Based on the above good performance, we took LUAD as the research object and explored its possible mechanisms from multiple perspectives such as tumor microenvironment (TME), related molecules, and pathway enrichment analysis.

As a proto-oncogene, the carcinogenic effect of MET gene has been demonstrated in multiple tumors.[27] Studies revealed that mutations in the splice site of MET that result in skipping of exon 14 are important molecular drivers in NSCLC,[28] and MET inhibitors have shown therapeutic response in patients with lung adenocarcinoma.[29,30] The results of this study showed that the expression of AGTR1 was significantly related to MET mutations (P=0.019,

Table 1). Compared with the high expression of AGTR1 (3.4%), samples with low expression of AGTR1 have a higher MET mutation rate (11.5%), which indicates that the presence of AGTR1 may inhibit the occurrence of MET mutations, and then play a tumor suppressor effect in lung adenocarcinoma. However, due to the lack of relevant research at present, more in-depth research is needed to clarify the relationship between the two. TME plays an important role in tumor progression, as the important part of TME, the immune components have been confirmed to be related to tumor prognosis.[31] A recent study showed that CD40-activated B cell was a vigorous antigen-presenting cell and was able to induce the effect of antitumor immunity.[32] Our analysis based on TCGA-LUAD data showed that samples with high infiltration of B cell were significantly better than low infiltration samples in survival (P=0.0152), and the expression of AGTR1 is positively correlated with B cell infiltration (P<0.001, Figure 4A). DC cells are important antigen presenting cells and can trigger T cell-mediated immune responses, which play important role in the anti-tumor response.[33] Similar results have been observed that the infiltration of DC cells was positively correlated with the prognosis of patients with lung adenocarcinoma in the study (P<0.001), that is, patients with high expression of AGTR1 have high DC cell infiltration and better prognosis (Figure 4B). There is a strong heterogeneity of monocytes, and currently, 3 different subsets have been established.[34] Monocytes display different or even opposite functions at different stages of tumor. Monocytes can not only be recruited into the tumor microenvironment and promote tumor progression,[35] but also mediate tumor cell death through cytokines or directly phagocytosis.[36,37] The results of this study showed that LUAD samples with high monocytes cell infiltration have a longer survival time (Figure 4C). MDSCs can contribute to the formation of an immunosuppressive environment by interacting with other immune components and promote tumor progression.[38] The samples with high DMSCs infiltration showed a worse prognosis in LUAD. The above results suggested that the expression of AGTR1 may be related to the infiltration of certain immune cells and affect the prognosis of lung adenocarcinoma.

Our enrichment analysis results showed that AGTR1-related genes were significantly related to the PI3K/AKT pathway (Figure 5). Previous researches illustrated that the PI3K/AKT pathway was involved in multiple cellular processes, such as proliferation and survival, and associated with a poor prognosis.[39] However, there are three isoforms

of AKT (AKT1, AKT2, AKT3), and different isoforms are responsible for different biological outcomes.[40,41] According to the results of this study, the expression of AGTR1 was not significantly correlated with AKT1 and AKT2, but positively related to AKT3 (Figure 6). There is no clear conclusion on the role of AKT3 in lung cancer, and different studies have shown inconsistent results. Most studies have shown that inhibiting the PI3K/AKT3 pathway can inhibit the progression of lung cancer.[42,43] A research suggested that polymorphisms of the AKT3 promoter regions do not contribute to the risk of Korean lung cancer.[44] However, an animal study showed that knocking out AKT3 promoted the proliferation and reduced apoptosis of tumor cell,[45] similar results were obtained in this study, the results in lung adenocarcinoma cells showed that overexpression of AGTR1 leads to increased levels of PI3K/AKT3, while overexpression of AGTR1 leads to lower proliferation and higher apoptosis of lung adenocarcinoma cells (Figure 7D). And a high expression of AKT3 was associated with better survival of LUAD (Figure 6B). Similarly, compared with normal tissues, multiple PI3K gene subtypes (PIK3IP1, PIK3R1, PIK3R3, PIK3R5) were lowly expressed in lung adenocarcinoma, and LUAD samples with high expression have a worse prognosis (Figure 6C–H). The results of this study show that AGTR1 inhibits the progression of lung adenocarcinoma by promoting the PI3K/AKT3 pathway.

In conclusion, our study indicated that AGTR1 is a potential tumor suppressor gene in pan-cancer. High expression of AGTR1 in lung adenocarcinoma promoted the formation of the anti-tumor microenvironment and was associated with low MET mutations. Especially, our results showed that AGTR1 plays a role in lung adenocarcinoma through the PI3K/AKT3 pathway.

Data Sharing Statement

The data that support the findings of this study are available from TCGA database (https://cancergenome.nih.Gov/publications/publicationguidelines).

Ethics Approval

The project was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (approval number: 2021100K).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests.

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