Clinical Complementary Medicine and Pharmacology 1 (2021) 100005

Contents lists available at ScienceDirect

Clinical Complementary Medicine and Pharmacology

journal homepage: www.elsevier.com/locate/ccmp

Correlation of ARNTL2 with Immune Infiltration and Its Role as a Potential Prognostic Biomarker in Lung Adenocarcinoma

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ARTICLE INFO

Keywords:
ARNTL2
Lung adenocarcinoma
Prognosis
Cell cycle
Immune infiltration

ABSTRACT

Background: ARNTL2 is a core component of the circadian clock genes and plays regulatory roles in the cell cycle and immune infiltration, but its mechanism in lung cancer (LC) remains unclear.
Objective: To investigate the clinical and therapeutic value of ARNTL2 in LC.
Methods: The Oncomine and Cancer Cell Line Encyclopedia (CCLE) databases were adopted for assessing the ARNTL2 expression, after which the Kaplan-Meier plotter and Gene Expression Profiling Interactive Analysis 2 (GEPIA2) databases were used to assess the correlation of ARNTL2 with prognosis. The univariate and multivariate Cox regression analyses were utilized to identify independent prognostic factors. Also, we explored how ARNTL2 expression is related to immune infiltration, and immunomodulators in non-small lung cancer (NSCLC) using the Tumor Immune Estimation Resource (TIMER) database, TISIDB database and Gene Set Enrichment Analysis (GSEA). Finally, coexpression of ARNTL2 and PD-L1 in lung adenocarcinoma (LUAD) was verified via immunofluorescence staining and COXPRESdb v7 database.
Results: Our study demonstrated a remarkable upregulated expression of ARNTL2 in multiple cell lines and cancers, including NSCLC. Prognostic analysis displayed a remarkable correlation between high ARNTL2 expression and unfavorable overall survival (OS) and first progressive (FP) survival among patients ailing from LUAD, and ARNTL2 was an independent predictor of prognosis for LUAD patients. GSEA analysis showed that overexpression of ARNTL2 was significantly linked with cell cycle and immunity. Furthermore, we reported a correlation of ARNTL2 expression with immunomodulators and lymphocytes. Immunofluorescence staining revealed that ARNTL2 and PD-L1 were elevated relative to normal tissue for LUAD, and colocalization of them was observed.
Conclusion: Elevated ARNTL2 expression in LUAD revealed the prognostic values and its prospective role as a target for cell cycle and immune therapy.

1. Introduction

Lung cancer (LC), particularly small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), has become the deadliest among the highly prevalent malignancies currently (Sung et al., 2021). NSCLC is a major histological type of LC in China. The higher morbidity is attributable to risk factors such as tobacco smoking and air pollution, while lower diagnosis rate at early stage may be linked to the higher mortality(Feng et al., 2019). Although application of chest computed tomography (CT) in LC screening has improved the diagnosis, approximately 16.6% of patients in clinical stage I have lymphatic metastasis due to lacking of special symptoms at an early stage (Chen et al., 2019). Furthermore, poor prognosis is linked to lymph node metastasis as an independent risk factor (Goldstraw et al., 2016). LC patients with early diagnosis, timely surgical treatment followed by radiotherapy or adjuvant chemotherapy have better clinical outcomes (Duma et al., 2019). Thus, revealing efficacious biomarkers for timely diagnosis and prognosis prediction is critical for lowering mortality.

The tumor microenvironment (TME), primarily comprised of a heterogeneous cell population including numerous infiltrating immune cells and tumor cells, is closely correlated with the prognosis of NSCLC patients (Hinshaw and Shevde, 2019). Tumor cells take advantage of immune checkpoint molecules to restrain immunity and evade immune surveillance, particularly in cytotoxic T lymphocytes (CTLs) that require tumor-specific antigen activation (Choi et al., 2020). CTLs play a crucial role in cell immune, and cytotoxic activity depends on the balance between inhibition and activation signals via various receptors and ligands (Ai et al., 2020). Programmed cell death-1 (PD-1) is mainly ex-

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https://doi.org/10.1016/j.ccmp.2021.100005
Received 22 May 2021; Received in revised form 2 September 2021; Accepted 6 September 2021

pressed on activated T cells and functions as a brake of T-cell activation through binding to the PD-1 ligand programmed cell death ligand-1 (PD-L1/CD274) (Fransen et al., 2018, Poggio et al., 2019). Recent reports show that PD-1/PD-L1 inhibitors exhibit an attractive therapeutic prospect in multiple solid tumors, including NSCLC (Xia et al., 2019). Hence, identifying novel immune therapy gene targets must be geared towards exploring how immune molecular phenotype correlates with tumor infiltrating lymphocytes (TILs) in NSCLC.

ARNTL2 (Aryl hydrocarbon receptor nuclear translocator-like 2) is a core component of the circadian clock genes within the PAS (PER, ARNT, SIM) superfamily (Lebailly et al., 2017). The helix-loop-helix, which it encodes, is a transcriptional activator and participates in regulated immune infiltration and cell cycle progression (Gaucher et al., 2018, Wu et al., 2019). Abnormal expression of circadian clock genes are closely linked to tumor initiation and progression (Shafi and Knudsen, 2019). At present, ARNTL2 overexpression has been reported in several solid tumors, for example, colorectal cancer, lung adenocarcinoma (LUAD) and breast cancer (Mocellin et al., 2018, Mazzoccoli et al., 2012, Ha et al., 2016). However, the underlying mechanism of how ARNTL2 affects the prognosis of patients with tumors needs to be further elucidated urgently from the perspective of the cell cycle, immune infiltration, and immune molecules.

Herein, we purposed to identify the clinical value of ARNTL2 and provide theoretical insights for timely diagnosis, prognostic prediction, and developing immune therapies for NSCLC.

2. Materials and Methods

2.1. Oncomine analysis

The ARNTL2 mRNA expression level in LC was determined via analysis in the Oncomine database (http://www.oncomine.org), with cancer microarray database for the public, comprisong 715 datasets and 86733 cancer and normal tissue samples (Rhodes et al., 2007).

2.2. Cancer cell line encyclopedia (CCLE) analysis

The ARNTL2 mRNA level in NSCLC cell line was explored via the CCLE database (https://portals.broadinstitute.org/ccle), an online human cancer cell lines platform, including 1475 cell lines and 84434 genes.

2.3. Kaplan-Meier plotter analysis

The Kaplan-Meier plotter (http://kmplot.com) is adopted to evaluate the function of 54675 genes and 10188 samples from cancers of ovarian, breast, lung, liver, etc (Győrffy et al., 2013). Here, it was utilized in exploring the prognostic value of ARNTL2 in LC. Overall survival (OS) and first progressive (FP) of low and high expression of ARNTL2 in LC, LUAD and lung squamous cell carcinoma (LUSC) were assessed following 95% confidence interval (CI), log-rank *P*-values, and the hazard ratio (HR). Statistical data showing significant differences were considered at $P<0.05$.

2.4. The univariate and multivariate Cox regression analyses

Transcriptome (n=551) and clinical data (n=487) for LUAD patients were initially downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). Cases with incomplete clinicopathological information were excluded from the clinical profile (n=325). Then, merging clinical profile with gene expression profile by Strawberry Perl programming language, a matrix text file was obtained (n=316). Finally, the univariate and multivariate Cox regression analyses were performed by R packages of survival and survminer.

2.5. Gene expression profiling interactive analysis 2 (GEPIA2) analysis

GEPIA2 (http://gepia2.cancer-pku.cn/#index) is an upgrade GEPIA version for RNA expression data analysis of 9,736 tumors in addition to 8,587 normal samples derived from the GTEx TCGA projects (Tang et al., 2019). In our study, it was adopted to explore the association of cell cycle-related genes with ARNTL2 expression and prognosis.

2.6. Tumor immune estimation resource (TIMER) analysis

TIMER (https://cistrome.shinyapps.io/timer/) is an online server for comprehensively exploring tumor-infiltrating immune cells (Taiwen et al., 2017). Levels of $CD4^+$ T cells, B cells, $CD8^+$ T cells, macrophage, dendritic cells, and neutrophils are calculated for 10,897 tumors derived from 32 distinct cancers. Firstly, the differential expression level of ARNTL2 in particular tumor types was evaluated by the TIMER database. Secondly, the correlation of ARNTL2 expression with the level of TILs subsets in LUAD and LUSC was investigated. Thirdly, the “survival” module of TIMER was used to explore six tumor immune subsets in LUAD and LUSC, which were flexible in terms of correcting multiple covariates (age, race, gender, stage, and tumor purity) in a multivariable Cox proportional hazard model. Lastly, Kaplan-Meier curves were drawn to explore how the six types of TILs are associated with LUAD as well as LUSC prognosis.

2.7. TISIDB analysis

TISIDB (http://cis.hku.hk/TISIDB) is an online web portal that integrates 988 genes reported to promote or inhibit anti-tumor immunity, high-throughput screening and genomic analyses, multi-omics of pan-cancer data, and molecular patient profiles after immunotherapy derived from various public resources databases (Ru et al., 2019). We adopted the TISIDB database to determine how ARNTL2 expression correlates with lymphocytes as well as immunomodulators in LUAD and LUSC.

2.8. Kyoto encyclopedia of genes and genomes (KEGG) analysis

The duplicates in the transcriptome data were averaged to obtain a duplicate-free gene expression dataset. The cls and gct text files of ARNTL2 were extracted by Strawberry Perl programming language, which were used to perform KEGG enrichment analysis via Gene Set Enrichment Analysis (GSEA, https://www.gsea-msigdb.org/gsea/index.jsp).

2.9. COXPRESdb v7 analysis

COXPRESdb v7 (http://coxpresdb.jp) is a gene coexpression database for 11 animal species supported by 23 coexpression platforms (Obayashi et al., 2019) and it was used to explore the coexpression relationship between ARNTL2 and CD274.

2.10. Double immunofluorescence (IF) staining

Formalin-fixed, paraffin-embedded histologic sections of pulmonary nodules biopsy samples, including 5 well differential (WD) adenocarcinoma tissues, 5 poorly differential (PD) adenocarcinoma tissues and 5 normal lung (NL) tissues, were obtained from Hangzhou Hospital of Traditional Chinese Medicine from April 1, 2020 to January 31, 2021. Paraffin sections were dewaxed, hydrated and then heated in pH 6.0 citric acid buffer for antigen repair, which was subjected to incubation with polyclonal rabbit anti-ARNTL2 (1:100, Abcam, ab221557) and monoclonal mouse anti-PD-L1 (1:100, PTG, 66248-1-Ig) antibody at 4°C, overnight. Afterwards, 0.1% twain-80 were utilized to wash the red blood cell fragments repeatedly. Lastly, we incubated the sections with

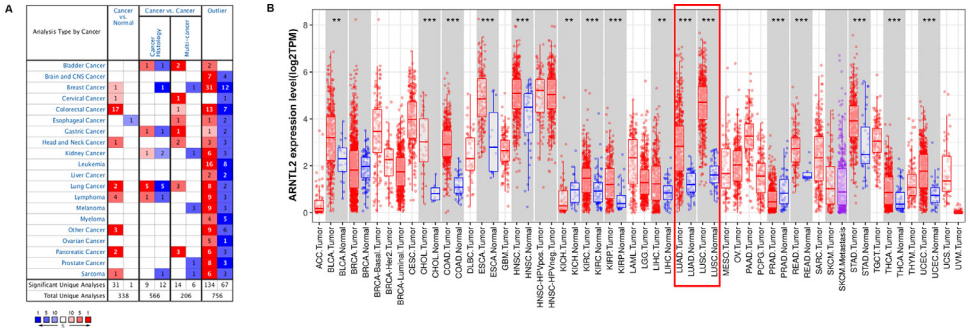

Fig. 1. The mRNA expression level of ARNTL2 in various cancer and normal tissues. ARNTL2 expression levels in various cancer and normal tissues in (A) Oncomine (fold change=2, *P*-value=1E-4, gene rank=top10%) and (B) TIMER database (*P*-value Significant Codes: 0 ≤ *** < 0.001 ≤ ** < 0.01).

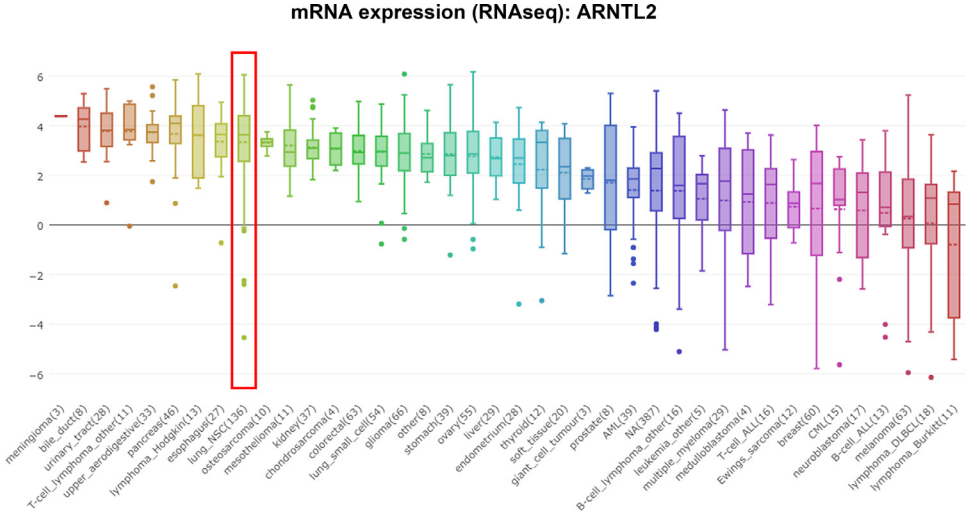

Fig. 2. mRNA expression level of ARNTL2 is ninth highest in NSCLC across multiple cancer cell lines (shown in red frame).

FITC-labeled donkey anti-mouse (1:1000, PTG, SA00003-8) and TRITC-labeled goat anti-rabbit (1:1000, PTG, SA00009-2) antibody for 30min. Specimens were visualized with a laser confocal microscopy (Olympus). IF density and Manders coefficient M1 were calculated by ImageJ.

3. Results and Discussion

3.1. Assessing ARNTL2 expression in various cancer and normal tissues

Here, we initially applied the Oncomine database to evaluate the ARNTL2 mRNA expression in multiple cancer tissues and normal tissues. Results indicated that expression of ARNTL2 was high relative to normal tissues for cancers of the breast, cervix, pancreas, head and neck, colorectum, lung, lymphoma and sarcoma (Fig. 1A). We further found that ARNTL2 was markedly elevated both in LUAD and LUSC than in normal lung tissue via the TIMER database (Fig. 1B).

Furthermore, CCLE analysis results concurred with that of Oncomine and TIMER, indicating that ARNTL2 was elevated in NSCLC cell lines (Fig. 2). Brandy et al. discovered that ARNTL2 was highly expressed in LUAD cell lines of H1792 and H2009. Moreover, ARNTL2 was necessary for the metastasis and clonal growth of LUAD cells in vivo, and could promote the secretion of Smoc2 by combining with the circadian clock gene of CLOCK to achieve self-sufficiency in metastasis of LUAD (Brady et al., 2016). These results implied that ARNTL2 may be served as a cancer-promoting gene and is high specificity and sensitivity in distinguishing patients with NSCLC from healthy individuals.

3.2. High ARNTL2 expression predicts poor prognosis in LC patients, particularly in the LUAD

Subsequently, correlations between ARNTL2 expression and OS, FP in LC patients were evaluated via the Kaplan-Meier plotter database (Fig. 3). The findings displayed that elevated expression of ARNTL2 was linked with unsatisfactory OS and FP in all LC (Fig. 3A, 3D) and LUAD patients (Fig. 3B, 3E), but not LUSC (Fig. 3C, 3F).

Table 1
The univariate Cox regression analysis of the correlation of ARNTL2 expression with OS among LUAD patients.

Clinicopathological Factors	HR	HR.95L	HR.95H	P-Value
Age	1.00	0.98	1.02	>0.05
Gender	1.09	0.78	1.54	>0.05
Stage	1.59	1.36	1.86	***
Tumor (T)	1.62	1.33	1.97	***
Metastasis (M)	1.90	1.07	3.38	*
Node (N)	1.71	1.41	2.08	***
ARNTL2	1.05	1.03	1.07	***

* *P*<0.05; ** *P*<0.01; *** *P*<0.001.

Accordingly, we focused on the predictive value of prognosis of clinicopathological factors (Table S1) and ARNTL2 in patients with LUAD. The univariate Cox regression analysis showed that the risk factors linked with the prognosis of LUAD were stage, T/M/N stage and ARNTL2 (Table 1). Moreover, the HR and 95% CI of T stage and ARNTL2 in the multivariate Cox regression analysis were 1.3 (1.02-1.60, *P*<0.05) and 1.1 (1.03-1.10, *P*<0.001), separately (Fig. 4). These results suggested that ARNTL2 and T stage can be regarded as independent predictors of prognosis in LUAD patients.

3.3. Correlations between cell cycle-related genes and ARNTL2 expression as well as NSCLC patients prognosis

ARNTL2, a member of circadian clock genes, regulates the cell cycle. G1/S and G2/M phases are two key checkpoints and require cyclin-

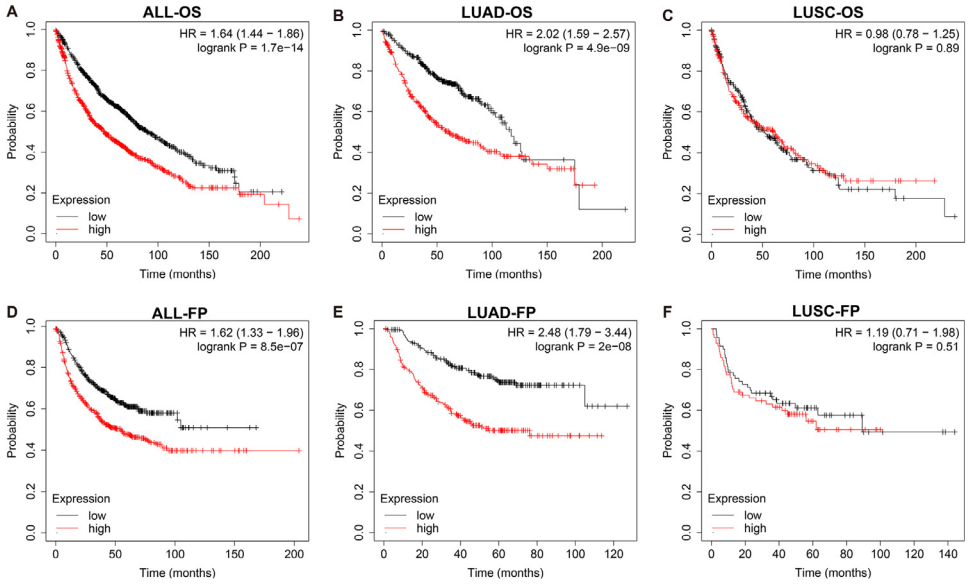

Fig. 3. A Kaplan-Meier curve, showing the comparison of LC patients with low and high expression of ARNTL2 using (A-C) OS curves of all LC patients (n=1925), LUAD patients (n=719) and LUSC patients (n=524). (D-F) FP curves of all LC patients (n=982), LUAD patients (n=461) and LUSC patients (n=141).

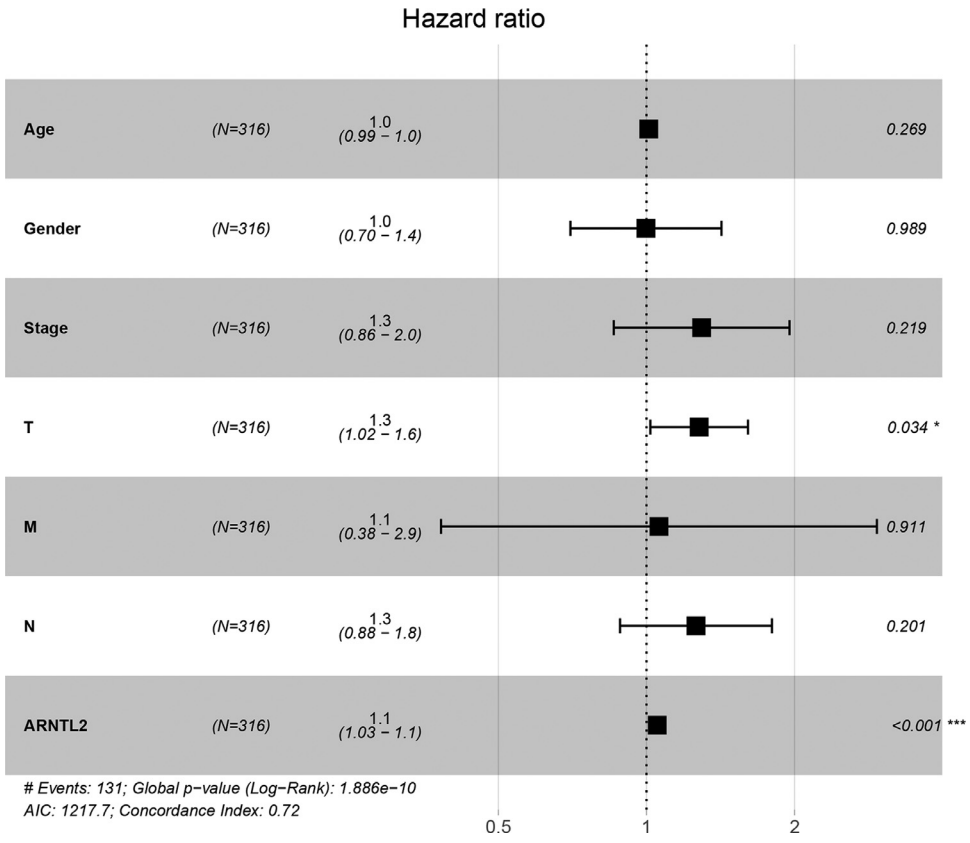

Fig. 4. The multivariate Cox regression analysis of the correlation of ARNTL2 expression with OS among LUAD patients. * P<0.05; *** P<0.001

dependent kinases (CDKs) phosphorylate target proteins to drive cell cycle progression. CDK inhibitors (CKIs) bind CDKs to inhibit CDKs activity and cause cell cycle arrest, including INK4 family (p15, p16, p18, p19) and Cip/Kip family (p21, p27, p57). Transition through G1 to S phase is regulated by the CDK6-cyclinD, CDK2-cyclinE and CDK2-cyclinA complexes, while G2 to M phase is controlled via the CDK1-cyclin B complex (Gaucher et al., 2018, Roskoski et al., 2019). Therefore, we initial assessed the correlation between ARNTL2 expression and G1/S as well as G2/M phases related genes (Fig. 5). In LUAD, ARNTL2 expression was moderately positively correlated with cyclin A2/B1/B2/E1, CDK1/2/6 and CDC25A/C while a slight positive correlated with cyclin A1/D1/D2/E2. INK4 and Cip/Kip family members were marginally positive correlated with ARNTL2 expression, except with p57. Meanwhile, a slight positive correlation between ARNTL2 expression and cyclin A1/A2/D2/E2, CDK1/2/4/6, p15/21/27, wee1 as well as CDC25A while negatively correlated with p57 were observed in LUSC.

Then, GEPIA2 was adopted to explore how CDKs and the prognosis of NSCLC patients are related. The Kaplan-meier curves demonstrated that patients exhibiting CDK1 and CDK6 expression exhibited poor prognosis, but not CDK2 and CDK4 (Fig. 6A-D). There was no correlation between CDK1/2/4/6 expression and prognosis in LUSC (Fig. 6E-H). Furthermore, the KEGG enrichment analysis displayed that high expression

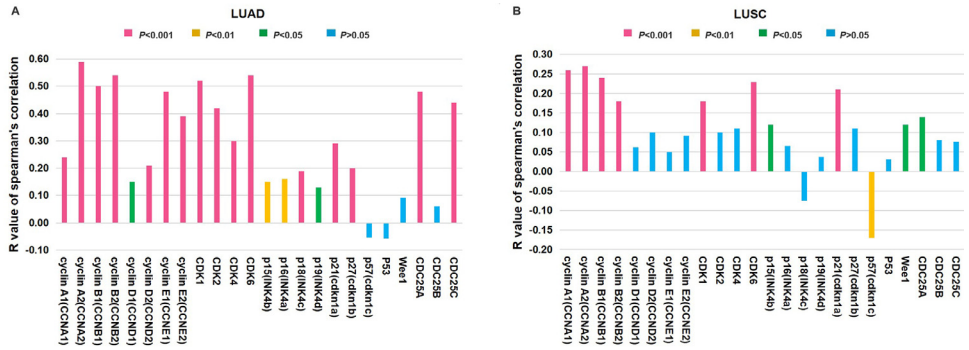

Fig. 5. Correlation analysis of ARNTL2 with related genes of G1/S and G2/M phases in GEPIA2.

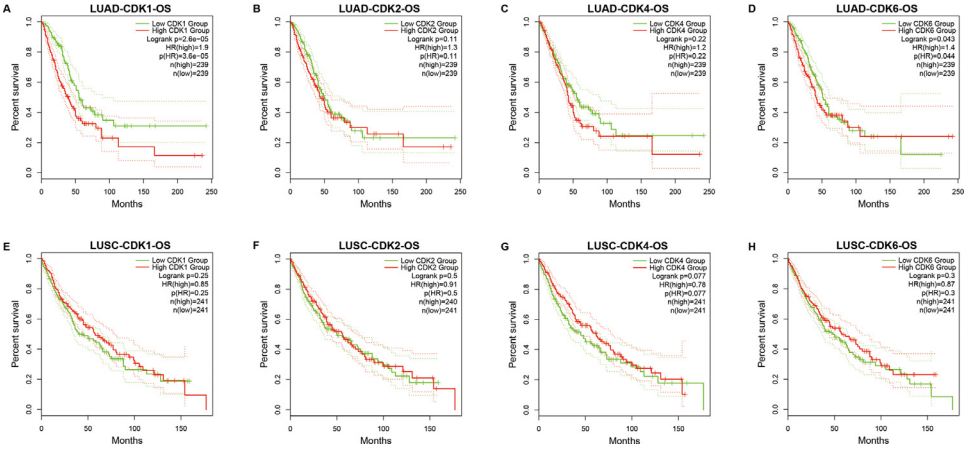

Fig. 6. Kaplan-Meier curves comparing (A-D) LUAD and (E-H) LUSC patients with low and high expression of CDK1/2/4/6.

of ARNTL2 was closely related to cell cycle and P53 signaling pathway (Fig. 8A, 8F). A study demonstrated that the p53 protein was employed as a transcriptional activator of p53-regulated genes and participated in regulation of cell cycle (Abd El-Hafeez et al., 2018). These results suggested that ARNTL2 may drive cell cycle progression by promoting CDK1 and CDK6 overexpression and accelerate tumor cell proliferation, which leads to a poor prognosis of LUAD.

3.4. *ARNTL2 expression is related to immune infiltration*

Following recent reports, immunotherapy is regarded as an effective strategy for tumor treatment that has generated a worldwide upsurge of immunotherapy (Steven et al., 2016). Tumor cell proliferation and metastasis are not only influenced by cell cycle, but also regulated by immune system (Wu et al., 2019, Liu et al., 2018). The same histology type of NSCLC may show distinct immune infiltration degrees, resulting in different clinical outcomes (Chen et al., 2019). Hence, assessment of the correlation between ARNTL2 and immune infiltration degrees may uncover the underlying mechanism by which ARNTL2 influences the NSCLC prognosis in patients.

Firstly, we determined the relationship of ARNTL2 expression with tumor purity and immune infiltration via TIMER. A slight inverse correlation was observed between ARNTL2 expression and tumor purity in LUSC, which showed a higher negative association with immune infiltration and tumor purity degrees in LUAD. The ARNTL2 expression demonstrated a positive correlation with $CD8^+$ T (R=0.216, *P*=1.51e-06), macrophages (R=0.171, *P*=1.58e-04), neutrophils (R=0.397, *P*=1.03e-19) and dendritic cells (DC) (R=0.199, *P*=9.79e-06) in LUAD, but had a negative association with B cells (R=-0.167, *P*= 2.21e-04) (Fig. 7A). In LUSC, ARNTL2 expression exhibited a positive correlation with neutrophils (R=0.092, *P*=4.50e-02), but negatively correlated with B cells (R=-0.208, *P*=5.38e-06), $CD4^+$ T (R=-0.207, *P*=5.30e-06) and macrophages (R=-0.126, *P*=5.79e-03) (Fig. 7B).

Next, Kaplan-meier curves were generated via the TIMER in exploring the association between immune infiltration and cumulative survival in LUAD and LUSC. B cell (*P*=0.000) and DC (*P*=0.048) infiltration positively correlated with favorable prognosis in LUAD (Fig. 7C), whereas we did not report any correlation of prognosis with immune cells infiltration (*P*>0.05) was observed in LUSC (Fig. 7D). Researchers have demonstrated that the degree of B-cell infiltration in NSCLC is positively associated with favorable outcomes (Wang et al., 2019). Taken together, the above results displayed the relationship between ARNTL2 and immune infiltration in LUAD and LUSC, but only B cells and DC have a positive effect on cumulative survival in LUAD.

Afterwards, the associations between ARNTL2 expression and specific immune cell markers in LUAD and LUSC was explored by TIMER (Table S2). In LUAD, ARNTL2 expression exhibited a negative correlation with few specific markers of B and DC, while positively correlated with most markers of T cell exhaustion, tumor-associated macrophage, M1/2 macrophage, monocyte, neutrophil, natural killer cells, $CD8^+$ T and Th1 after adjustment in purity. Meanwhile, ARNTL2 expression was negatively associated with most immune cell markers after adjusting for purity in LUSC. Notably, markers of Tregs (IL2RA, Cor=0.422, *P*<0.0001; CCR8, Cor=0.230, *P*<0.0001; FOXP3, Cor=0.170, *P*<0.001) and T cell exhaustion (PD-1, Cor=0.250, *P*<0.0001; CTLA-4, Cor=0.231, *P*<0.0001; LAG3, Cor=0.264, *P*<0.0001; TIM-3, Cor=0.340, *P*<0.0001; GZMB, Cor=0.451, *P*<0.0001) revealed a positive linear correlation with ARNTL2 expressions in LUAD. Tregs and immunosuppressive molecules are the key to trigger T cell exhaustion in TME (Sasidharan Nair and Elkord, 2018, , Tang et al., 2019, Rotte, 2019, Datar et al., 2019). From these observations, we hypothesize that ARNTL2 may activate Tregs and inhibit T cell activation.

Besides, the KEGG analysis manifested that higher expression of ARNTL2 was significantly linked with immune system, including primary immunodeficiency, B/T cell receptor signaling pathway, natural killer cell mediated cytotoxicity and Toll like/NOD like signaling path-

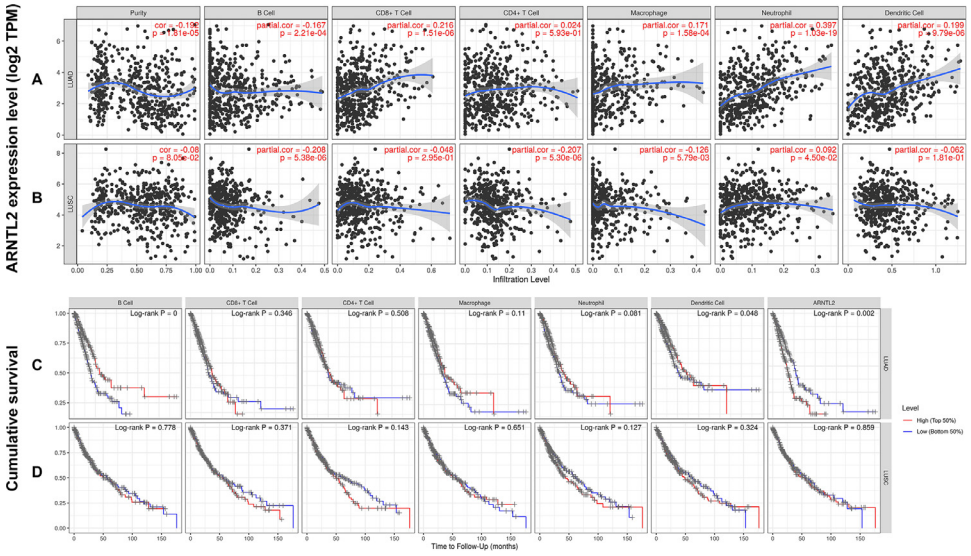

Fig. 7. Correlation of immune infiltration with (A-B) ARNTL2 expression level and (C-D) cumulative survival in LUAD and LUSC.

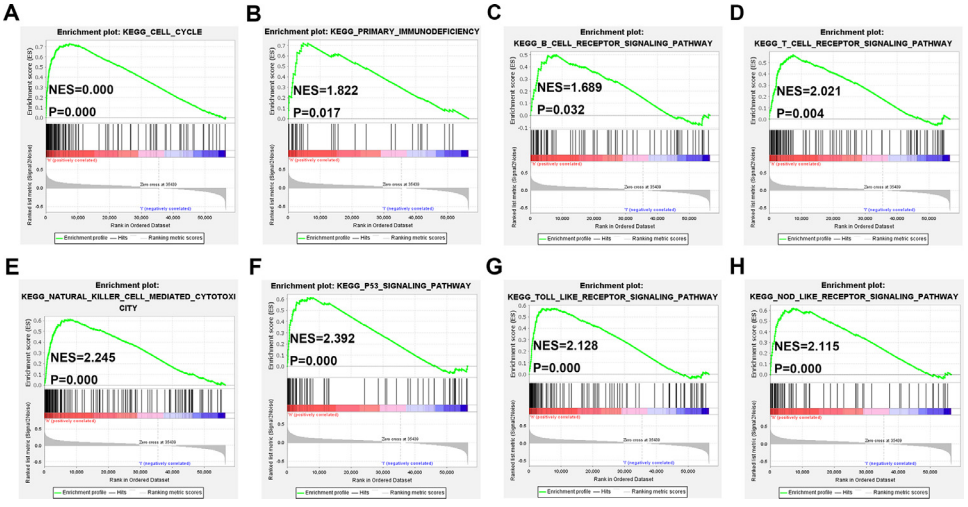

Fig. 8. The KEGG enrichment analysis of the high expression group of ARNTL2 by GSEA. NES, normalized enrichment score.

way (Fig. 8B-H). Some studies demonstrated that the Toll and NOD like receptors were involved in regulating innate immune response (Bauer et al., 2017, Shen et al., 2021). The foregoing results may give insights into the varying prognosis of ARNTL2 in LUAD.

3.5. Regulation of lymphocytes and immune molecules by ARNTL2

The relationship between ARNTL2 expression and TILs and immunomodulators was explored by the TISIDB database (Fig. 9). Fig. 9A displays the correlation of ARNTL2 expression with TILs in multiple cancers. The top two lymphocytes, including activated CD4 T cell (Act CD4, rho=0.542, P<2.2e-16) and type 2 T helper cell (Th2, rho=0.418, P<2.2e-16), were positive correlated with ARNTL2 in LUAD (Fig. 9b1-2). Central memory CD8 T cell (Tcm CD8, rho=0.237, P=8.61e-08) and CD56^{bright} natural killer cell (CD56^{bright}, rho=0.161, P=3.06e-04) were the greatest positive correlation with ARNTL2 in LUSC. (Fig. 9b3-4).

Immunomodulators include immunostimulators, major histocompatibility complexes (MHC) molecules, and immunoinhibitors. In Fig. 9C, we described the correlation of ARNTL2 expression with immunoinhibitors. CD274 (rho=0.416, P<2.2e-16) and PDCD1LG2 (rho=0.44, P<2.2e-16) or CD274 (rho=0.297, P=1.48e-11) and TGFB1 (rho=0.213, P=1.65e-06) are the top two immunoinhibitors positive correlated with ARNTL2 in LUAD and LUSC, respectively (Fig. 9D). Fig. 9E depicts the relationship between ARNTL2 expression and immunostimulators. IL2RA (rho=0.41, P<2.2e-16) and KLRC1 (rho=0.405, P<2.2e-16) or CD276 (rho=0.25, P=1.57e-08) and TNFRSF18 (rho=0.134, P=2.68e-03) are the top two immunostimulators positive linked with ARNTL2 in LUAD and LUSC, separately (Fig. 9F). Fig. 9G indicates the association between ARNTL2 expression and MHC. TAP1/2 (rho=0.475, P<2.2e-16) or TAP1 (rho=0.125, P=5.16e-03) and TAP2 (rho=0.116, P=9.44e-03) are the top two MHC positive associated with ARNTL2 in LUAD and LUSC, severally (Fig. 9H).

3.6. IF staining validation of ARNTL2 and PD-L1 colocalization in LUAD

In LUAD, green fluorescence (FITC) represented PD-L1 and mainly localized on cell membrane, while red fluorescence (TRITC) showed ARNTL2 and expressed in the cell membrane and cytoplasm. Yellow fluorescence indicated the colocalization relationship between PD-L1 and ARNTL2 (Fig. 10A-I). The results displayed that ARNTL2 and PD-L1 in LUAD were significantly enhanced relative to NL tissue, and the expression of ARNTL2 in PD group was stronger than WD group (Fig. 10J-K, P<0.01). Moreover, high colocalization relationship between ARNTL2 and PD-L1 was observed in each group ($M1_{PD}$=0.941, $M1_{WD}$=0.957, $M1_{NL}$=0.962) (Fig. 10L). In addition, COXPRESdb v7 database was used to verify the coexpression correlation between ARNTL2 and PD-L1. The results indicated that PD-L1 ranked ninth among the top 50 genes co-expressed with ARNTL2, and the coexpression supportability of CD274

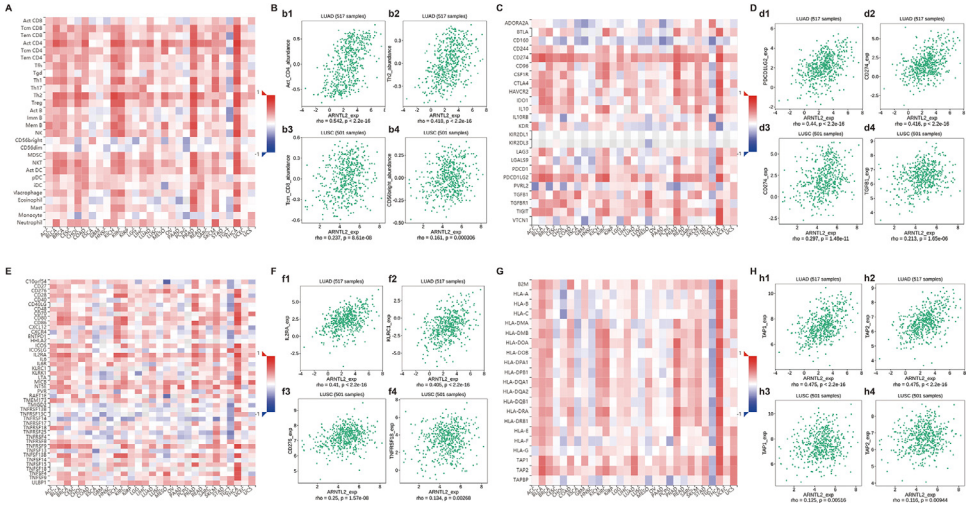

Fig. 9. Spearman's relationship of ARNTL2 with immunomodulators (TISIDB) and lymphocytes. Association of the abundance of (A) TILs, (C) immunoinhibitors, (E) immunostimulators, (G) MHC molecules with ARNTL2 expression. Top 2 (B) TILs, (D) immunoinhibitors, (F) immunostimulators and (H) MHC molecules showing the greatest correlation with ARNTL2 expression in LUAD or LUSC, respectively. Cells labelled in red and blue depict positive and negative correlations, separately. The intensity of color and the correlation strength are directly proportional.

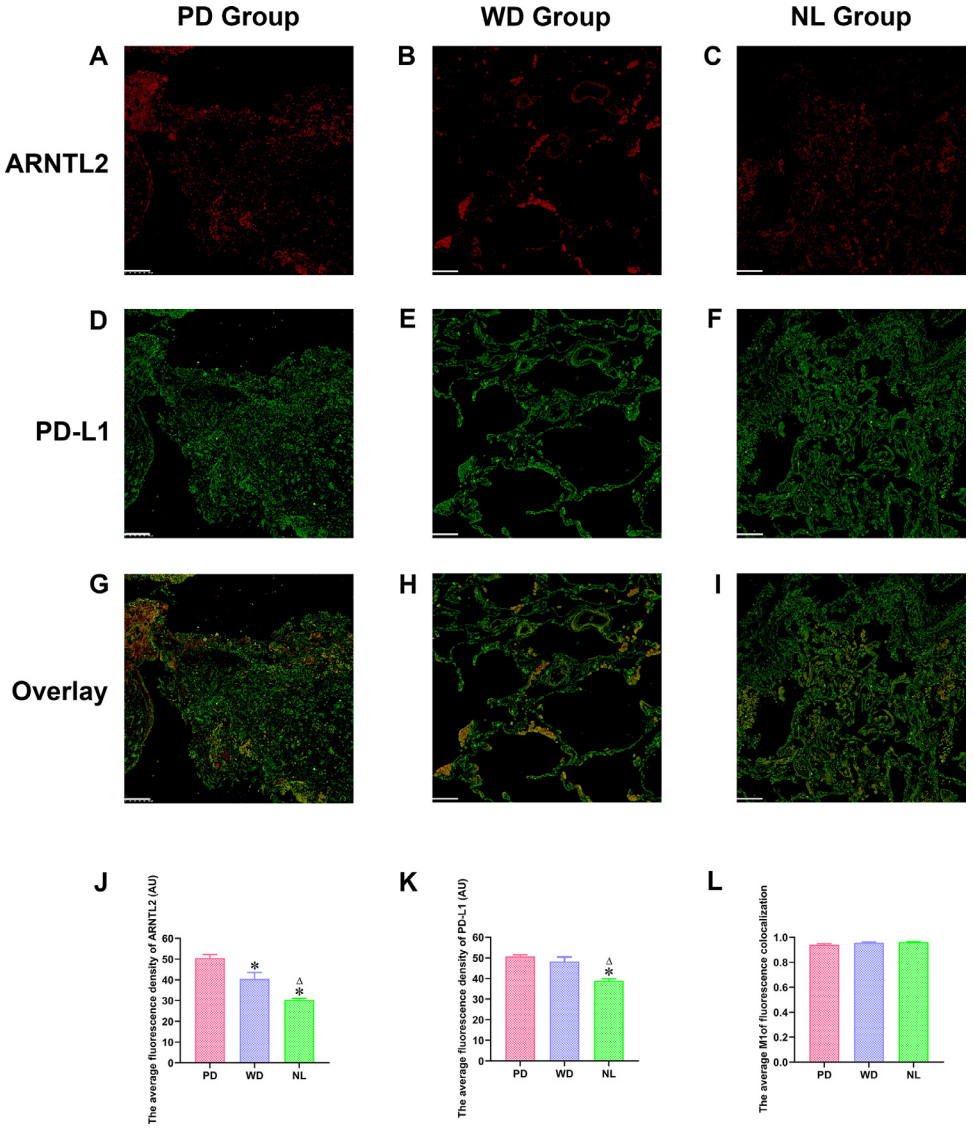

Fig. 10. (A-C) ARNTL2 and (D-F) PD-L1 immunofluorescence density and (G-I) colocalization in LUAD. Comparison of the mean values of (J) ARNTL2, (K) PD-L1 and (L) Manders coefficient M1 among different groups. compare with PD Group, $^{*}P<0.01$; compare with WD Group, $^{\triangle}P<0.01$. PD Group, poorly differentiation group (n=5); WD Group, well differentiation group (n=5); NL Group, normal lung group (n=5); AU, Arbitrary Units; white scale bar=100μm.

and ARNTL2 was strong (Table S3). Obviously, the results of our study are consistent with the analysis of the COXPRESdb v7. PD-L1 and PD-L2 are recognized to suppress T-lymphocyte function as it binds to its PD-1 receptor (Ohaegbulam et al., 2015). PD-1/PD-L1 inhibitors have become the standard second-line management strategy for advanced NSCLC due to their remarkable efficacy in some patients (Topalian et al., 2020). Thus, the correlation between ARNTL2 and PD-L1 provides a new target for the study of immune escape in TME, and may be a candidate therapy target for LUAD.

4. Conclusion

In this study, the multiple databases and clinical samples verification results uncovered that ARNTL2 was an oncogenic gene and could be served as an independent predictor of prognosis in patients with LUAD. Meanwhile, it was found that ARNTL2 was closely related to the underlying mechanisms of cell cycle and immune infiltration, such as cell cycle, B/T cell receptor signaling pathway, natural killer cell mediated cytotoxicity and Toll like/NOD like signaling pathway. Furthermore, the colocalization of ARNTL2 and PD-L1 was observed and verified. Taken together, ARNTL2 may be a vital regulator of cell cycle progression and immune infiltration and a promising gene for predicting the prognosis as well as developing targeted drugs of LUAD.

Ethical Approval

This research has approved by the ethics committee of Hangzhou Hospital of Traditional Chinese Medicine and indicating that the secondary use of pathological samples is free from the patient's informed consent (No. 2020KY132).

Data Availability

All the original data on the findings our study were obtained from the cancer genome atlas (TCGA) at [https://portal.gdc.cancer.gov] and gene expression omnibus (GEO) at [https://www.ncbi.nlm.nih.gov/gds/].

Funding

We got support through grants from the Zhejiang Provincial Natural Science Foundation of China (LQ20H290004) and Zhejiang Provincial Chinese Medicine Research Program of China (2020ZQ039).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Junjie Pan: Conceptualization, Writing – original draft. **Hongkuan Yang:** Formal analysis. **Lihong Zhu:** Data curation, Writing – review & editing. **Yafang Lou:** Writing – review & editing.

Acknowledgements

We appreciate Jingwei Wang for the guidance in the immunofluorescence staining experiment and the Public Platform of Medical Research Center, Academy of Chinese Medical Science, Zhejiang Chinese Medical University.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ccmp.2021.100005.

References

Ai, L., Xu, A., Xu, J., 2020. Roles of PD-1/PD-L1 Pathway: Signaling, Cancer, and Beyond. Adv. Exp. Med. Biol. 1248, 33–59. doi:10.1007/978-981-15-3266-5_3.

Abd El-Hafeez, AA., Fujimura, T., Kamei, R., et al., 2018. A methoxyflavanone derivative from the Asian medicinal herb (Perilla frutescens) induces p53-mediated G2/M cell cycle arrest and apoptosis in A549 human lung adenocarcinoma. Cytotechnology 70 (3), 899–912. doi:10.1007/s10616-017-0116-1.

Brady, JJ., Chuang, CH., Greenside, PG., et al., 2016. An Arntl2-Driven Secretome Enables Lung Adenocarcinoma Metastatic Self-Sufficiency. Cancer Cell 29 (5), 697–710. doi:10.1016/j.ccell.2016.03.003.

Bauer, AK., Upham, BL., Rondini, EA., Tennis, MA., Velmuragan, K., Wiese, D., 2017. Toll-like receptor expression in human non-small cell lung carcinoma: potential prognostic indicators of disease. Oncotarget 8 (54), 91860–91875. doi:10.18632/oncotarget.19463.

Chen, B., Wang, X., Yu, X., et al., 2019. Lymph node metastasis in Chinese patients with clinical T1 non-small cell lung cancer: A multicenter real-world observational study. Thorac. Cancer 10 (3), 533–542. doi:10.1111/1759-7714.12970.

Choi, Y., Shi, Y., Haymaker, CL., Naing, A., Ciliberto, G., Hajjar, J., 2020. T-cell agonists in cancer immunotherapy. J. Immunother. Cancer 8 (2), e000966. doi:10.1136/jitc-2020-000966.

Chen, F., Yang, Y., Zhao, Y., Pei, L., Yan, H., 2019. Immune Infiltration Profiling in Nonsmall Cell Lung Cancer and Their Clinical Significance: Study Based on Gene Expression Measurements. DNA Cell Biol. 38 (11), 1387–1401. doi:10.1089/dna.2019.4899.

Duma, N., Santana-Davila, R., Molina, JR., 2019. Non-Small Cell Lung Cancer: Epidemiology, Screening, Diagnosis, and Treatment. Mayo Clin. Proc. 94 (8), 1623–1640. doi:10.1016/j.mayocp.2019.01.013.

Datar, I., Sanmamed, MF., Wang, J., et al., 2019. Expression Analysis and Significance of PD-1, LAG-3, and TIM-3 in Human Non-Small Cell Lung Cancer Using Spatially Resolved and Multiparametric Single-Cell Analysis. Clin. Cancer Res. 25 (15), 4663–4673. doi:10.1158/1078-0432.CCR-18-4142.

Feng, RM., Zong, YN., Cao, SM., Xu, RH., 2019. Current cancer situation in China: good or bad news from the 2018 Global Cancer Statistics? Cancer Commun. (Lond.) 39 (1), 22. doi:10.1186/s40880-019-0368-6.

Fransen, MF., Schoonderwoerd, M., Knopf, P., et al., 2018. Tumor-draining lymph nodes are pivotal in PD-1/PD-L1 checkpoint therapy. JCI Insight 3 (23), e124507. doi:10.1172/jci.insight.124507.

Goldstraw, P., Chansky, K., Crowley, J., et al., 2016. The IASLC Lung Cancer Staging Project: Proposals for Revision of the TNM Stage Groupings in the Forthcoming (Eighth) Edition of the TNM Classification for Lung Cancer. J. Thorac. Oncol. 11 (1), 39–51. doi:10.1016/j.jtho.2015.09.009.

Gaucher, J., Montellier, E., Sassone-Corsi, P., 2018. Molecular Cogs: Interplay between Circadian Clock and Cell Cycle. Trends Cell Biol. 28 (5), 368–379. doi:10.1016/j.tcb.2018.01.006.

Győrffy, B., Surowiak, P., Budczies, J., Lánczky, A., 2013. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small–cell lung cancer. PLoS One 8 (12), e82241 10.1371/journal.pone.0082241.

Hinshaw, DC., Shevde, LA., 2019. The Tumor Microenvironment Innately Modulates Cancer Progression. Cancer Res. 79 (18), 4557–4566. doi:10.1158/0008-5472.CAN-18-3962.

Ha, NH., Long, J., Cai, Q., Shu, XO., Hunter, KW., 2016. The Circadian Rhythm Gene Arntl2 Is a Metastasis Susceptibility Gene for Estrogen Receptor-Negative Breast Cancer. PLos Genet. 12 (9), e1006267. doi:10.1371/journal.pgen.1006267.

Lebailly, B., Langa, F., Boitard, C., Avner, P., Rogner, UC., 2017. The circadian gene Arntl2 on distal mouse chromosome 6 controls thymocyte apoptosis. Mamm. Genome 28 (1–2), 1–12. doi:10.1007/s00335-016-9665-4.

Liu, M., Liu, H., Chen, J., 2018. Mechanisms of the CDK4/6 inhibitor palbociclib (PD 0332991) and its future application in cancer treatment (Review). Oncol. Rep. 39 (3), 901–911. doi:10.3892/or.2018.6221.

Mocellin, S., Tropea, S., Benna, C., Rossi, CR., 2018. Circadian pathway genetic variation and cancer risk: evidence from genome-wide association studies. BMC Med. 16 (1), 20. doi:10.1186/s12916-018-1010-1.

Mazzoccoli, G., Pazienza, V., Panza, A., et al., 2012. ARNTL2 and SERPINE1: potential biomarkers for tumor aggressiveness in colorectal cancer. J. Cancer Res. Clin. Oncol. 138 (3), 501–511. doi:10.1007/s00432-011-1126-6.

Obayashi, T., Kagaya, Y., Aoki, Y., Tadaka, S., Kinoshita, K., 2019. COXPRESdb v7: a gene coexpression database for 11 animal species supported by 23 coexpression platforms for technical evaluation and evolutionary inference. Nucleic. Acids. Res. 47 (D1), D55–D62. doi:10.1093/nar/gky1155.

Ohaegbulam, KC., Assal, A., Lazar-Molnar, E., Yao, Y., Zang, X., 2015. Human cancer immunotherapy with antibodies to the PD-1 and PD-L1 pathway. Trends Mol. Med. 21 (1), 24–33. doi:10.1016/j.molmed.2014.10.009.

Poggio, M., Hu, T., Pai, CC., et al., 2019. Suppression of Exosomal PD-L1 Induces Systemic Anti-tumor Immunity and Memory. Cell 177 (2), 414–427. doi:10.1016/j.cell.2019.02.016, e13.

Rhodes, DR., Kalyana-Sundaram, S., Mahavisno, V., et al., 2007. Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. Neoplasia 9 (2), 166–180. doi:10.1593/neo.07112.

Ru, B., Wong, CN., Tong, Y., et al., 2019. TISIDB: an integrated repository portal for tumor-immune system interactions. Bioinformatics 35 (20), 4200–4202. doi:10.1093/bioinformatics/btz210.

Roskoski Jr., R, 2019. Cyclin-dependent protein serine/threonine kinase inhibitors as anticancer drugs. Pharmacol. Res. 139, 471–488. doi:10.1016/j.phrs.2018.11.035.

Rotte, A., 2019. Combination of CTLA-4 and PD-1 blockers for treatment of cancer. J. Exp. Clin. Cancer Res. 38 (1), 255. doi:10.1186/s13046-019-1259-z.

Sung, H., Ferlay, J., Siegel, RL., et al., 2021. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J. Clin. 71 (3), 209–249. doi:10.3322/caac.21660.

Shafi, AA., Knudsen, KE., 2019. Cancer and the Circadian Clock. Cancer Res. 79 (15), 3806–3814. doi:10.1158/0008-5472.CAN-19-0566, Epub 2019 Jul 12.

Steven, A., Fisher, SA., Robinson, BW., 2016. Immunotherapy for lung cancer. Respirology 21 (5), 821–833. doi:10.1111/resp.12789.

Sasidharan Nair, V., Elkord, E., 2018. Immune checkpoint inhibitors in cancer therapy: a focus on T-regulatory cells. Immunol. Cell Biol. 96 (1), 21–33. doi:10.1111/imcb.1003.

Shen, E., Han, Y., Cai, C., et al., 2021. Low expression of NLRP1 is associated with a poor prognosis and immune infiltration in lung adenocarcinoma patients. Aging (Albany NY) 13 (5), 7570–7588. doi:10.18632/aging.202620.

Tang, Z., Kang, B., Li, C., Chen, T., Zhang, Z., 2019. GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. Nucleic. Acids. Res. 47 (W1), W556–W560. doi:10.1093/nar/gkz430.

Taiwen, Li., Jingyu, Fan., Binbin, Wang., et al., 2017. TIMER: A web server for comprehensive analysis of tumor-infiltrating immune cells. Cancer Res. 77 (21), e108–e110. doi:10.1158/0008-5472.CAN-17-0307.

Tang, R., Rangachari, M., Kuchroo, VK., 2019. Tim-3: A co-receptor with diverse roles in T cell exhaustion and tolerance. Semin. Immunol. 42, 101302. doi:10.1016/j.smim.2019.101302.

Topalian, SL., Taube, JM., Pardoll, DM., 2020. Neoadjuvant checkpoint blockade for cancer immunotherapy. Science 367 (6477). doi:10.1126/science.aax0182, eaax0182.

Wu, Y., Tao, B., Zhang, T., Fan, Y., Mao, R., 2019. Pan-Cancer Analysis Reveals Disrupted Circadian Clock Associates With T Cell Exhaustion. Front. Immunol. 10, 2451. doi:10.3389/fimmu.2019.02451.

Wang, SS., Liu, W., Ly, D., Xu, H., Qu, L., Zhang, L., 2019. Tumor-infiltrating B cells: their role and application in anti-tumor immunity in lung cancer. Cell. Mol. Immunol. 16 (1), 6–18. doi:10.1038/s41423-018-0027-x.

Xia, L., Liu, Y., Wang, Y., 2019. PD-1/PD-L1 Blockade Therapy in Advanced Non-Small-Cell Lung Cancer: Current Status and Future Directions. Oncologist 24 (1), S31–S41. doi:10.1634/theoncologist.2019-IO-S1-s05, Suppl.