Immunological Role And Clinical Prognostic Significance of Risk Marker P2RY6 In Lung Adenocarcinoma

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Abstract

There is increasing evidence that P2RY6 may be involved in inflammatory responses. However, the role of P2RY6 in a variety of cancers remains unknown. In this study, we comprehensively analyzed P2RY6 expression and its association with different types of malignancies using different multiple databases, and further explored the relationship between P2RY6 expression and prognosis in LUAD, and finally validated it using immunohistochemistry in LUAD. We found that P2RY6 was expressed at significantly higher levels in a variety of cancer tissues, and that poor patient prognosis was strongly associated with high P2RY6 expression. Further risk scoring revealed that P2RY6 is closely associated with the immune microenvironment of LUAD, which would suggest that P2RY6 would be a new target for immunotherapy in LUAD

Introduction

Lung cancer is currently the most common malignancy in the world, with the highest prevalence and death rate^[1]. Lung cancer is classified into two tissue subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), and NSCLC accounted for 85% of all^[2]. Lung Adenocarcinoma (LUAD) is one kind of the NSCLC and is the most prevalent histological type of primary tumor of the lung^[3]. Current treatments for lung cancer include targeted therapy and immunotherapy combined with traditional surgery, radiotherapy and chemotherapy, which have contributed greatly to improving the survival rate of patients. However, due to diagnostic limitations, most patients with LUAD are found to have advanced cancer with concomitant tumor metastasis suggesting poor prognosis^[4]. Despite the many previous studies on the pathogenesis of this disease and new approaches to its treatment, LUAD unfortunately remains one of the most aggressive and lethal tumor types, with a 5-year survival rate of approximately 23%^[5].

In recent years, immune-related targeted therapies such as cytotoxic T-lymphocyte-associated antigen 4, PD-1 and PD-L1 have shown good efficacy in many malignancies and also act as a role in the treatment of advanced non-small cell lung cancer, suggesting that immune-related targeted therapies may have a possible significance in the treatment of lung cancer^[6, 7]. The tumor microenvironment of the lung is infiltrated by a variety of immune cells^[8, 9], including natural killer cells B cells, macrophages, dendritic cells, and T lymphocytes. It has been suggested that LUAD may be a "hot" tumor type, suggesting sensitivity to immunotherapy^[10].

P2RY6, which is also known as P2Y6, belongs to a family of P2 receptors that are activated by extracellular nucleotides and variably spliced leading to multiple transcript variants encoding different protein isoforms. New evidence suggests a role for P2RY6 signaling in the regulation of immune functions associated with inflammation^[11, 12]. Under inflammatory conditions, P2RY6 regulates neutrophil migration through^[13, 14]. It has been found that host defense against bacterial infection can be promoted through the release and activation of P2RY6 receptors, suggesting that P2RY6 may act as a

novel mediator to enhance the innate immune response to invading pathogens^[15]. Previous articles have investigated the role of P2RY6 in pancreatic^[16] and colorectal cancer^[17], but no corresponding article has examined its role in LUAD.

This is the first study time that comprehensively analyze the expression of P2RY6 and its association with different types of malignancies. Then we further explored the relationship between the expression of P2RY6 and the prognosis of LUAD and validated using immunohistochemistry in LUAD. Our study provides new ideas for studying the role of P2RY6 in LUAD, highlighting the intrinsic mechanisms by which P2RY6 affects the tumor microenvironment and its potential for application in tumor immunotherapy.

Methods

Clinical specimens

Paired cancer tissue and normal paracancer tissue samples were collected from twenty patients with LUAD. All patients were pathologically diagnosed as LUAD before tissue collection, and had not received radiotherapy or chemotherapy before. We obtained all patients' informed consent. The study was approved by the Ethics and Anthropology Committee. All experiments and methods are carried out in accordance with relevant standards and regulations.

Data Collection

We downloaded The Cancer Genome Atlas (TCGA)^[18], genotypic tissue expression (GTEx)^[19] RNA expression information and clinical data from the UCSC Xena database^[20] (<https://xenabrowser.net/datapages/>), which included more than 1000 tumor samples from 33 human cancers. We downloaded DNA copy number and methylation data from the cBioPortal database^[21] (<https://www.cbioportal.org/>), and we ensured that the acquisition and application methods were consistent with relevant guidelines and regulations.

Differential Expression Analysis Of P2ry6

The Oncomine database^[22] has the most complete gene expression data, cancer mutation profile, and related clinical information, and is known as the world's largest cancer gene chip database and integrated data mining platform at present. Through this database, we revealed the differences in P2RY6 mRNA expression levels between tumors and normal tissues of different types of cancer. We used SangerBox software to compare the difference between P2RY6 in cancer tissues and normal tissues base on TCGA data, and the normal samples from the GTEx database were added to compare the differences of P2RY6 expression in cancer samples and normal paracancerous samples.

Analysis Of Genetic Alterations

Gene alteration characteristics of P2RY6 were queried by using the cBioPortal network(<https://www.cbioportal.org/>). After selecting the "Quick Select" as well as "TCGA Pan-Cancer Atlas Studies" options, information about the frequency of alterations, copy number alteration and their mutation type were described in the "Cancer Types Summary" section of the TCGA database.

Immunomodulators

To elucidate tumor-immune system interactions, we searched the online comprehensive database TISIDB (<http://cis.hku.hk/TISIDB/>) for immunosuppressants and immunostimulants significantly associated with P2RY6-related gene expression (Spearman correlation test, $P < 0.05$). And then P2RY6-related immunomodulators were uploaded by us to cBioPortal for Cancer Genomics (www.cbioportal.org). On the basis of RNA-seq data from cancer samples, we were able to capture 50 simultaneously altered genes through a web-based module for the queried genes. The obtained protein networks were subjected to GO annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway using two web-based tools (<https://string-db.org/>) and the WEB-based GENE SeT AnaLysis Toolkit (<http://www.webgestalt.org/>) enrichment analysis.

Survival Analysis

Kaplan-Meier analysis was performed to show the survival of patients in the high and low P2RY6 expression groups and to assess the potential of P2RY6 as a prognostic. Survivor, glmnet^[23], survivorROC and survminer were the R packages used in the operations. To explore the relationship between P2RY6 expression and Disease-specific Survival (DSS), Disease-Free Survival (DFS), Overall Survival (OS) and Progression-free Survival (PFS) in patients with each cancer type, we performed Cox regression analysis using TCGA in an R environment.

Immune Infiltration Analysis

We explored the correlation between P2RY6 expression and immune infiltration by using the TIMER2 database and the CIBERSOFT method. P2RY6 expression was also analyzed at the following cellular abundances, including B cells, CD4+ T cells, CD8+ T cells, neutrophils, dendritic cells (DCs) and macrophages. We compared their expression with P2RY6 after obtaining stromal score, ESTIMATE score and immune score for each tumor type sample in TCGA by using ESTIMATE and summarized their relationship.

Genome Enrichment Analysis

We identified the pathways associated with P2RY6 by gene set enrichment analysis (GSEA) by the cluster Profiler R package. Fold changes in mean gene expression between patients with high and low P2RY6 expression were sorted, and the sorted gene list was then represented in the input file. And assessed biological processes by using HALLMARK pathways, Genomes (KEGG) pathways and Kyoto Encyclopedia of Genes.

Construction Of Nomogram

After combining the clinical characteristics and risk scores of the patients, we made a nomogram of the cancer prognosis, which was created by the rms package of the R software. In order to predict the deviation of the probability from the actual situation, we display it by applying the bootstrap method (1,000 repetitions) and using a calibration curve. We also use the coordination index (C-index) to measure the prediction accuracy of the nomination map.

Statistical analysis

P2RY6 high and low expression groups were defined according to TPM expression values. We used Spearman correlation test to mine the relationship between P2RY6 expression and targets such as tumor mutation burden and microsatellite instability. We calculated log-rank P values and HR values in survival analysis by COX regression analysis. P values were considered statistically significant when they were less than 0.05.

Immunohistochemical Staining

Tumor tissue samples and normal tissue samples next to the cancer were embedded in formalin. The tissue was cut to a thickness of 4 mm and fixed on a glass slide. The tissue was left at room temperature for 60 minutes and then dewaxed by immersion in xylene for 10 minutes and then replaced with fresh xylene for another 10 minutes. Soaking in 5% bovine serum albumin for thirty minutes at 37°C inhibited and blocked endogenous peroxidase activity. The slices were allowed to spend the entire night at 4°C incubated with anti-P2RY6 (1:300, Signalway Antibody), followed by three consecutive 5-minute rinses with PBS buffer solution and another 30-minute incubation with secondary antibodies to hydrogen peroxide at 37°C. Finally, the PBS rinse just mentioned was repeated three times and the slices were visualized using diaminobenzidine.

Results

Increased expression of P2RY6 in pan-cancer

Using the data from the pan-cancer database, we evaluated the mRNA expression level of P2RY6. By comparing the expression level of P2RY6 in cancer and paracancerous tissues, it was observed that there

was a significant difference between cancer and paracancerous tissues. As shown by Figure 1A (all $P < 0.01$), according to the data obtained from TCGA database, it could be found that the expression of P2RY6 mRNA in most tumor tissues was significantly higher than that in normal tissues, including BLCA(bladder urothelial carcinoma), ESCA(esophageal carcinoma), CHOL(cholangiocarcinoma), BRCA(breast invasive carcinoma), LIHC(liver hepatocellular carcinoma), HNSC(head and neck squamous cell carcinoma), KIRC(kidney renal clear cell carcinoma), LUAD(lung adenocarcinoma), LUSC(lung squamous cell carcinoma), READ(rectum adenocarcinoma), THCA(thyroid carcinoma), STAD(stomach adenocarcinoma), UCEC(uterine corpus endometrial carcinoma). Its expression only showed a significantly decreased state in KICH. Considering the small number of normal samples in TCGA, in order to further evaluate the expression level of P2RY6 in different types of cancers, We decided to use TCGA's tumor tissue data and the normal tissue data in the GTEx database to analyze the expression of 27 kinds of tumors. The results showed that in addition to the above tumors, P2RY6 was also highly expressed in CESC(cervical squamous cell carcinoma and endocervical adenocarcinoma), GBM(glioblastoma multiforme), COAD(colon adenocarcinoma), LAML(acute myeloid leukemia), PAAD(pancreatic adenocarcinoma), OV(ovarian serous cystadenocarcinoma), LGG(brain lower grade glioma), PRAD(prostate adenocarcinoma), SKCM(skin cutaneous melanoma) and TGCT(testicular germ cell tumors) (Figure 1B, all $P < 0.01$). These results suggested that the differential expression of P2RY6 might have a connection with the occurrence and development of many kinds of tumors, and it might be a kind of oncogene, which played an important role in promoting the growth of tumors.

The expression of P2RY6 is proportional to the stage of tumor

Through the "pathological staging" module of the World Health Organization, we analyzed the correlation between the expression of P2RY6 and the pathological stage of cancer, and found that the expression level of P2RY6 in III-IV stage of BLCA and THCA was significantly increased (Figures 2A-B, all $P < 0.01$). And interestingly, although the expression of P2RY6 had an upward trend in the high staging of most tumors, including KICH(kidney chromophobe), KIRC, LUSC, KIRP(kidney renal papillary cell carcinoma), MESO(mesothelioma), LUAD, SKCM, TGCT, UVM(uveal melanoma), STAD and so on, the difference was not statistically significant (Additional file 1). Therefore, it could be speculated that P2RY6 might be a risk factor for the human body, so in tumor staging, the later the expression level was, the higher the expression level was.

P2ry6 Has Clinical Prognostic Significance

We used the Kaplan-Meier plotter database and univariate Cox regression analysis to investigate the impact of P2RY6 expression on the survival of cancer patients. From the results of Kaplan-Meier analysis, it could be seen that the high expression of P2RY6 was significantly correlated with the deterioration of OS in CESC, BRCA, GBM, COAD, ESCA, KIRC, HNSC, KIRP, LIHC, LGG, LUAD, PAAD, PCPG(pheochromocytoma and paraganglioma), TGCT, THYM(thymoma), STAD, UCEC and UVM(Figure 3, all $P < 0.05$). In the meanwhile, the decrease of PFI was significantly correlated with the high expression

of P2RY6 in BLCA, DLBC, ESCA, GBM, HNSC, KIRC, LUSC, LGG, LUAD, PAAD, PRAD, TYM, STAD, UCEC and UVM.(Figure 4, all $P < 0.05$). Moreover, the high expression of P2RY6 also predicted that it was significantly associated with DSS malfunction in BRCA, BLCA, COAD, HNSC, GBM, ESCA, KIRC, LUSC, KIRP, LGG, LIHC, LUAD, MESO, PAAD, TCGT, STAD, THYM, UCEC and UVM(Figure 5, all $P < 0.05$). The results of Cox regression analysis showed that poor OS, PFI and DSS were closely related to the high expression of P2RY6, and the high expression of P2RY6 might be a risk factor for most tumor patients. (Figure 6, all $P < 0.05$). Combined with the above two data, we speculated that the expression of P2RY6 was an important factor affecting the survival of cancer patients, and the poor prognosis of various cancers might be closely related to the high expression of P2RY6.

Gene Set Enrichment Analysis Of P2ry6

To further study the potential molecular functions of P2RY6 in various cancers, we did the gene cluster enrichment analysis of P2RY6. From the results of the enrichment analysis of the KEGG signaling pathway, it could be understood that IL-17 signaling pathway, cytokine-cytokine receptor interaction, inflammatory bowel disease, toll-like receptor signaling pathway and NF-kappa B signaling pathway were significantly enriched in the high expression group of P2RY6(Figure 7A-B, all $P < 0.01$), while bile secretion, drug metabolism-cytochrome P450, cholesterol metabolism, steroid hormone biosynthesis and retinol metabolism were significantly enriched in the low expression group. It could be seen that the high expression of P2RY6 was significantly associated with a variety of immune-related signaling pathways. At the same time, the data were verified by HALLMARK pathway, it was observed that the high expression group of P2RY6 was significantly enriched in allograft rejection, ,tnfa signaling via nfkb, interferon-alpha response, inflammatory response, IL6/JAK/STAT3 signaling and other signal pathways, while the low expression group was significantly enriched in adipogenesis, fatty acid metabolism, androgen response, xenobiotic metabolism, bile acid metabolism and other signal pathways(Figure 7C-D, all $P < 0.01$). These results further confirmed that P2RY6 could regulate immune-related signaling pathways, suggesting that P2RY6 might be involved in the immune response in the occurrence and development of different cancers.

Analysis of immune cell infiltration and related immune regulatory genes and chemokines in P2RY6

In view of the increasing correlation between tumor microenvironment, especially related immunological characteristics, and the prognosis of various cancers, we chose "CIBERSOFT" method to analyze the correlation of 22 immune cells, and we found that the expression of P2RY6 in most tumors was significantly related to the infiltration level of CD4 memory resting T cells, Treg cells and M2 macrophages in these immune cells (Figure 8, all $P < 0.01$). Thus, these evidences suggested that P2RY6 might be involved in the immune response of different cancers by increasing the infiltration of related immune cells, which could also explain its possible carcinogenic effect in most tumor types.

We also studied the immune effect of P2RY6 in cancer from other aspects, and analyzed the relationship between P2RY6 and immunosuppressive genes, immune activating genes, chemokines and chemokine

receptors. It was found that the expression of P2RY6 was positively related to immunosuppressive genes such as CD86, CD80, IL2RA, TGFB1, LGALS9 and other immunosuppressive genes in pan-cancer (Figure 9A-B, all $P < 0.01$). At the same time, it was positively related to chemokines and chemokine receptors such as CCL5, CCL8 and their receptors CCR5 and CCR8 (Figure 9C-D, all $P < 0.01$). Furthermore, we found that the expression of P2RY6 was strongly correlated with StromalScore, ImmuneScore and ESTIMATEScore of most tumors, and showed a significant positive correlation. Details could be found in Additional file 2-4 (all $P < 0.05$). Therefore, we speculated that P2RY6 might be involved in the chemotactic recruitment of related immune cells and the regulation of related immune signal pathways in tumor immune response.

Correlation between P2RY6 and immune checkpoint genes, TMB and MSI in human cancer

Immune checkpoint gene plays a very important role in tumor immunotherapy. Therefore, we analyzed the correlation between P2RY6 and immune checkpoint gene to explore the possibility of P2RY6 in clinical immunotherapy. Results as shown in Figure 13, among the 47 common ICP genes we collected, it was observed that the expression of P2RY6 was closely correlated with many types of cancers, while in these tumors, the expression of P2RY6 was positively related to most of the immune checkpoint genes (Figure 10, all $P < 0.05$). To sum up, the high expression of P2RY6 suggested that the activity of these immune checkpoint genes might be coordinated by P2RY6 in different signal transduction pathways, indicating that P2RY6 might be an ideal target for immunotherapy.

Moreover, we analyzed the connection between P2RY6, TMB and MSI by Spearman correlation test, and explored the role of P2RY6 in the immune response and immune mechanism of tumor microenvironment. The results showed that P2RY6 was positively related to TMB in BLCA, BRCA, GBM, ESCA, KIRP, LGG, LUAD, SARC and THYM, and positively related to MSI in BLCA, PAAD, COAD, BRCA, DLBC, KIRC, READ, HNSC, SKCM, STAD and TGCT (Figure 11, all $P < 0.05$).

Expression level of P2RY6 in LUAD and its relationship with immune cells

It was worth noting that from the previous data, it could be found that the expression level of P2RY6 in LUAD was significantly higher than that in other cancer tissues. The high expression of P2RY6 in LUAD suggested that it might promote the occurrence of LUAD and was a related oncogene. We observed a similar phenomenon from Kaplan-Meier analysis and univariate Cox regression analysis. The high expression of P2RY6 was significantly related to the deterioration of OS, PFI and DSS in patients with LUAD. Therefore, we further analyzed the role of P2RY6 in LUAD.

Similarly, we first studied the protein expression level of P2RY6 in LUAD. Immunohistochemical staining of LUAD and its adjacent normal tissues was performed by immunohistochemical method. The results showed that the staining intensity of P2RY6 in LUAD was higher than that in normal paracancerous tissues (Figure 12A). According to the corresponding immunohistochemical score we also found that the expression level of P2RY6 in LUAD was significantly higher than that in the corresponding paracancerous normal tissues (Figure 12B, $P < 0.01$). It could be concluded that the protein expression of P2RY6 was

closely related to the expression of mRNA. The high protein expression of LUAD was often accompanied by the high expression of mRNA.

Then, through the analysis of the signal pathways that P2RY6 might regulate LUAD immune response, we identified a variety of immunomodulators, including IDO1, LGALS9, IL10, TIGIT, IL10RB, TNFRSF18, CD40, IL2RA, ULBP1 and so on, which were significantly positively correlated with P2RY6 in LUAD. In addition, we used the TCGA database to determine the level of immune cell infiltration in LUAD with low and high P2RY6 expression. It was worth noting that in the group with high P2RY6 expression, we found that the level of resting T cell infiltration in CD4 was lower (Figure 13A, $P < 0.01$), which was related to the poor OS in LUAD. In addition, with the occurrence and development of the tumor, the number of Treg cells increased, which was very consistent with the high infiltration level in the figure (Figure 13B, $P < 0.05$). This series of results showed that P2RY6 could regulate the immune response of LUAD through related pathways, play an important role in the immune cell infiltration of LUAD, and affect the occurrence and development of tumor in varying degrees.

Prognostic value and risk model of P2RY6-related immunomodulators in LUAD

Univariate Cox regression analysis was used for studying the prognostic significance of P2RY6-related immunomodulators in LUAD. Several genes significantly associated with P2RY6 were found, including PVRL2, TGFBR1, CD276, CD70, MICB, NT5E, RAET1E (Figure 14A, all $P < 0.05$). Then, we performed multivariate COX regression analysis on the genes with statistical significance in univariate COX regression analysis. From this, we identified some genes that were closely related to poor prognosis in LUAD, including TGFBR1, CD70, CD276, NT5E, MICB, RAET1E (Figure 14B, all $P < 0.05$). Based on these, we constructed the related prognostic risk model, calculated the gene expression value and coefficient, and calculated the risk score of patients with LUAD. After that, the correlation between these genes and OS was evaluated by Kaplan-Meier plotter database, and it was found that the survival time of patients with high risk score was significantly lower and had worse OS (Figure 14C, $P < 0.01$). From the corresponding subject working curve (ROC curve), we could also see that the area under the curve (AUC) of the risk score was 0.807, while the AUC of the staging was 0.683. The AUC value obtained by the combination of the two was as high as 0.83 (Figure 14D). It could be concluded that P2RY6-based prognostic model had better discrimination than TNM staging.

Prognostic Value Of P2ry6-related Risk Models In Luad Cohort

After studying the risk score, we decided to explore the accuracy and prognostic value of the risk model through correlation heat map and Cox regression analysis. The risk score distribution, survival time and gene expression profile of LUAD were shown in Figure 15A. It could be seen from the chart that the higher the risk score, the shorter the survival time and the higher the mortality. Most importantly, we could clearly observe from the figure that the expression of P2RY6 gene increased with the increase of risk score. In the

univariate and multivariate Cox regression analysis of Figure 15B(all $P < 0.05$), it was found that the risk score was significantly related to the survival rate of LUAD patients, it might be an independent prognostic factor, and its effect on survival rate was much greater than that of age and stage. Therefore, the risk score of P2RY6 indicated that the gene had good prognostic performance and might represent an independent prognostic index of LUAD. To sum up, our P2RY6 risk model had a more accurate prognostic value for the prognosis of LUAD, and could well show that the prognosis of high and low risk population had significant statistical significance.

Construction Of P2ry6-related Nomogram

Finally, we constructed a prognostic nomogram about LUAD (Figure 16A), which could be used to predict the 3-year and 5-year survival probability of related individuals. From the calibration curve, it could be seen that the predicted survival probability of the nomogram for 3 and 5 years was in good agreement with the ideal reference line, which was very close to the ideal 45 degree (Figure 16B-C).

Discussion

As we mentioned in the introduction, LUAD is a highly malignant cancer, and due to the limitations of diagnosis and treatment at this stage, often lead to poor prognosis, so the clinical mortality rate of LUAD is on the high side. We reviewed the published articles and found more and more evidence that P2RY6 is closely related to a range of cancers such as colorectal cancer^[12], pancreatic cancer^[16], melanoma^[24], gastric cancer^[25], astrocytoma^[26] and so on. Some studies have also shown that P2RY6 has a certain therapeutic significance in tumor progression^[27]. These studies suggest that P2RY6 may play an extraordinary role in the occurrence and development of tumors. However, there are no corresponding articles pointing out the expression pattern and role of P2RY6 in LUAD, which greatly hinders the development of related clinical work. Therefore, there is an urgent need for further work to explore the specific characteristics of P2RY6 in LUAD, and actively look for new and possible treatments.

In our study, we first analyzed the expression level of P2RY6 from the perspective of pan-cancer, and found that compared with normal tissues, the expression level of P2RY6 in a variety of cancer tissues was significantly higher, and the results of Kaplan-Meier analysis and univariate Cox regression analysis showed that the poor prognosis of patients was closely correlated with the high expression of P2RY6, so we concluded that the high expression of P2RY6 in most tumors may not be conducive to the survival of patients with related tumors. Previous studies have pointed out that the progression of the disease is closely correlated with the expression of P2RY6, and there is a strong correlation between different levels of P2RY6 expression and different prognosis. For example, Hanxing Wan et al pointed out that P2RY6 is closely correlated with the prognosis and treatment of gastric cancer^[25]. LinHe Qing et al pointed out that the high expression of P2RY6 can be used as a prognostic indicator of pancreatic cancer^[28]. Therefore, after analyzing several databases including GEPIA, Timer, Kaplan-Meier plotter and TCGA, we speculate

that P2RY6 may be a carcinogenic gene, and its expression may be a valuable prognostic biomarker in cancer.

To further study the potential mechanism of P2RY6 in various cancers, we used TCGA database to analyze the enrichment of P2RY6-related signaling pathways and found that it was significantly correlated with many types of immune-related pathways. Our work also shows that the expression of P2RY6 gene is closely correlated with the degree of immune cell infiltration. Previous studies have pointed out that tumor infiltrating lymphocytes can be used as a new prognostic index^[29] and can be used to treat some solid tumors^[30]. Interestingly, we found that P2RY6 is closely related to the immunity of many cancers, especially LUAD. Combined with the previously observed low infiltration of CD4 memory resting T cells and high infiltration of Treg cells in LUAD, we speculate that immune cell infiltration may play an extraordinary role in the formation and treatment of LUAD. It has been proved that these cells play an important role in innate immunity and participate in the function of immune cells^[31–33]. Richard Kennedy et al pointed out that CD4+T memory cells can enhance anti-tumor response by enhancing clonal expansion of tumor site, preventing activation-induced cell death, and giving priority to the generation of immune memory cells by CTL as antigen presenting cells^[34], which is consistent with the low infiltration level of CD4 memory resting T cells caused by high expression of P2RY6 in our study. In the process of tumorigenesis and development, Treg cells can inhibit the anti-tumor response, so a large number of Treg cells infiltrate into tumor tissues, which is often closely related to poor prognosis^[32]. From these we can also explain why the increased level of Treg cell infiltration in tumor microenvironment often leads to worse prognosis of patients with high expression of P2RY6. In addition, it is worth noting that when we explored the potential relationship between P2RY6 and immunomodulators, we found that the expression of this gene was positively related to a variety of immune activators and immunosuppressants, which means that P2RY6 may have a synergistic effect with other checkpoint members, thus playing a specific role in the regulation of tumor immune microenvironment.

Therefore, we speculate that the expression of P2RY6 is inseparable from the infiltration of related immune cells and the role of related immunomodulators, and they are closely related to the tumor microenvironment. Tumor microenvironment is an important part of tumor tissue, and its composition varies with tumor types, but its specific characteristics include blood vessels, stromal cells, immune cells and extracellular matrix^[35–38]. With the rapid development of tumor immunotherapy, there is a lot of evidence that tumor microenvironment has high application value in clinical treatment^[38–41]. and now immunotherapy has been clinically selected for the treatment of a variety of tumors (such as renal cell carcinoma^[42], melanoma^[43]). After analyzing the 47 immune checkpoint genes collected, we thought that P2RY6 may be a new target for immunotherapy, and after analyzing the relationship between P2RY6, TMB and MSI, we understand that P2RY6 was positively related to TMB and MSI in most tumors. These results may provide a theoretical basis for combined molecular targeted immunotherapy in the future. Our understanding of the immunological characteristics of P2RY6 microenvironment makes us confident to infer that LUAD may affect tumor immunity and thus affect the prognosis of LUAD patients. Some

genes of the same family with P2RY6 have developed corresponding immunotherapy^[44]. Inspired by this, we think that P2RY6 may have a good prospect in the immune regulation of tumor microenvironment.

Before our study, many researchers have used gene expression-based modeling as a prognostic factor for non-small cell lung cancer^[45–48], but there is no P2RY6-related LUAD risk model. Therefore, based on the immunomodulators related to P2RY6, we successfully constructed the relevant risk model by using Cox regression analysis. Finally, the predicted value of the risk model is verified by the nomogram. Our results show that the risk score is closely correlated with the survival rate of patients with LUAD, and may become an independent prognostic factor with high prognostic value. As a result, patients with early LUAD can be divided into high-risk and low-risk groups with significantly different overall survival rates. The risk scores obtained from P2RY6-related immunomodulators can distinguish the risk groups defined by different gene differential expression, which may be helpful for the further study of LUAD-related prognostic signals. Combining these results, we can find that the risk score is closely related to the immune microenvironment of LUAD, so we are more confident that P2RY6 will be a new target of LUAD immunotherapy and bring results different from traditional treatments in clinical treatment.

It is undeniable that our experimental data come from a wide range of sources and have adopted a variety of research methods, but our research still has some limitations. First of all, even if there are strict standards, the individual differences between different cancer patients make our information biased to varying degrees. Secondly, we use Cox regression to analyze P2RY6, and it is inevitable that there are potential prognostic factors such as patients' economic status and treatment plan, which are also the disadvantages of database research. Most importantly, this study is based on bioinformatics. Due to the limitations of public databases, it is inevitable that there is a lack of analysis of clinical data of LUAD patients. Therefore, before applying this model to clinical practice, more in vivo and in vitro studies are needed to confirm the interaction between P2RY6 and LUAD tumor microenvironment.

Conclusions

In summary, our results suggest that P2RY6 may be a new prognostic marker of LUAD, and we also discussed the potential role of P2RY6 in tumor immunology and its prognostic value in LUAD. Finally, we combined independent clinical factors to construct a nomogram which can accurately predict the prognosis, and concluded that P2RY6 is expected to become a target for individualized immunotherapy in patients with LUAD. It is hoped that these findings can provide more reference value for the laboratory research and clinical application of P2RY6 in cancer prognosis and immunotherapy in the future.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics and Human Subject Committee of Yiwu Central Hospital. Ethics Approval and Consent to Participate

Consent for publication

Written consent was obtained from all study participants for this study.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article. Xena database[20] (<https://xenabrowser.net/datapages/>), cBioPortal database[21] (<https://www.cbioportal.org/>), cBioPortal network(<https://www.cbioportal.org/>). TISIDB (<http://cis.hku.hk/TISIDB/>). Cancer Genomics (www.cbioportal.org)

Competing interest

The author reports no conflicts of interest in this work.

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None

Author Contributions

Conceived and designed the experiments: HW, DX, LH, JS, XB; Performed the data collection: HW, LH, JS, DX; Analyzed the data: HW, LH, JS; Contributed reagents/materials/analysis tools: LH, DX, JS; Contributed to the writing of the manuscript: JS, XB, LH; All authors reviewed the manuscript.

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None

Data Availability Statement

The data used to support the findings of this study are deposited in TCGA and GEO database.

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Figures

Figure 1

P2RY6 expression level in different human cancers. (A) The pan-cancer expression level of P2RY6 from TCGA database. Tumor tissues are represented by yellow spindles, and normal tissues are represented by blue spindles (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$). (B) The pan-cancer expression level of P2RY6 from TCGA and GTEx databases. Tumor tissues are represented by yellow spindles, and normal tissues are represented by blue spindles (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$).

Figure 2

The expression level of P2RY6 in different stages of tumor. (A–B). The logging scale uses Log2 (TPM+1), and analyzes the expression of P2RY6 gene through the TCGA database according to the main pathological stages (I-II, III-IV). Only tumor types with logarithmic grade $p < 0.05$ are shown here.

Figure 3

Analysis of the protein expression level of P2RY6. (A) Representative immunohistochemical staining of P2RY6 in LUAD and adjacent tissues. (B) Box plot of immunohistochemical scores related to LUAD and adjacent tissues.

Figure 4

Kaplan-Meier survival curve of OS difference of various tumor types drawn by Kaplan-Meier plotter. Only tumor types with logarithmic grade $p < 0.05$ were shown here.

Figure 5

Kaplan-Meier survival curve of PFI difference of various tumor types drawn by Kaplan-Meier plotter. Only tumor types with logarithmic grade $p < 0.05$ were shown here.

Figure 6

Kaplan-Meier survival curve of DSS difference of various tumor types drawn by Kaplan-Meier plotter. Only tumor types with logarithmic grade $p < 0.05$ were shown here.

Figure 7

Correlation forest plot for univariate Cox regression analysis of P2RY6. The univariate cox regression analysis results of P2RY6 on OS of TGCA pan-cancer patients are shown in (A). The univariate cox regression analysis results of P2RY6 on the PFI of TGCA pan-cancer patients are shown in (B). The results of univariate cox regression analysis of P2RY6 on DSS of TGCA pan-cancer patients are shown in Figure (C). Red represents a significant result.

Figure 8

The results of gene set enrichment analysis of P2RY6 high expression group and low expression group through different ways. (A) The enrichment analysis results of the P2RY6 high expression group in the KEGG pathway. (B) The enrichment analysis results of the P2RY6 low expression group in the KEGG pathway. (C) The enrichment analysis results of the P2RY6 high expression group in the HALLMARK pathway. (D) The enrichment analysis results of the P2RY6 low expression group in the HALLMARK pathway. $P < 0.05$ was considered significant.

Figure 9

The results of correlation analysis between the expression of P2RY6 in pan-cancer and the impact of immune microenvironment are related to the level of immune cell infiltration. Positive correlation is represented by yellow, and negative correlation is represented by blue, and the darker the color, the stronger the correlation. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$.

Figure 10

The results of correlation analysis between P2RY6 expression and immune regulation-related genes. (A) Heat map of the correlation between P2RY6 expression in pan-cancer and immune activation genes. (B) The heat map of the correlation between the expression of P2RY6 in pan-cancer and the immunosuppressive state-related genes. (C) Heat map of the correlation between the expression of P2RY6 in pan-cancer and chemokine genes. (D) Heat map of the correlation between the expression of P2RY6 in pan-carcinoma and chemokine receptor genes. Positive correlation is represented by red, and negative correlation is represented by blue, and the darker the color, the stronger the correlation. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$.

Figure 11

Results of correlation analysis between P2RY6 expression and TCGA pan-cancer immune marker set. *P < 0.05; **P < 0.01; *** P < 0.001.

A

B

Figure 12

The relationship between P2RY6 expression and TMB (A), MSI (B) in pan-cancer. TMB, tumor mutation burden; MSI, microsatellite instability. The radar chart shows Spearman's correlation coefficient and p-value.

A

T_cells_CD4_memory_resting

B

Tregs

Figure 13

Correlation analysis between the expression of P2RY6 and the level of immune cell infiltration in LUAD.

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

Figure 14

The construction of P2RY6 related risk model. (A) Forest plot results of univariate Cox regression analysis for patients with LUAD. (B) Forest plot results of multivariate Cox regression analysis for patients with

LUAD. (C) Kaplan-Meier curve for predicting OS in patients with LUAD based on the TCGA data set. (D) Receiver operating characteristic curve (ROC) analysis results of patients with LUAD.

Figure 15

Analysis of the prognostic value of the risk scoring model in LUAD patients. (A) The risk score distribution, patient's survival status and related heat map of gene expression profile for LUAD. (B) The results of univariate and multivariate Cox regression analysis on the overall survival risk score of LUAD.

B

C

Figure 16

The establishment of a nomogram related to the risk score is used for the prognosis of LUAD patients. (A) A nomogram that can be used to predict the 3-year and 5-year survival rates of LUAD patients. (B) A calibration curve used to calibrate the 3-year survival rate of the nomogram related to LUAD patients. (C) A calibration curve used to calibrate the 5-year survival rate of the nomogram related to LUAD patients.

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