



PLXNC1: A Novel Potential Immune-Related Target for Stomach Adenocarcinoma

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Background: Gastric cancer is associated with tumor microenvironment and chronic inflammation, but the underlying tumor-promoting mechanisms still remain unknown.

Methods: The ATAC-seq was used to identify genes with chromatin accessibilities in promoter regions. The RNA-seq datasets were performed to identify differentially expressed genes (DEGs). Pearson correlation analysis with the mRNA expression of three families of tumor-related inflammation TFs was used to filter downstream DEGs. Cox univariate survival analysis was performed to identify the prognostic value. The ImmPort database and CIBERSORTx algorithm were used to investigate the regulatory relationship between hub DEGs and immune cells. Immunohistochemistry (IHC) and multidimensional database were performed to verification.

Results: In this case, we require 2,454 genes with chromatin accessibility in promoter regions by ATAC-seq. Based on the gene expression profiles (RNA-seq), we identified 365 genes with chromatin accessibility and differential expression. Combined with the Cox univariate survival analysis, we identified 32 survival-related DEGs with chromatin accessibility. According to ImmPort database, *CXCL3*, *PLXNC1*, and *EDN2* were identified as immune-related genes in STAD. By applying the CIBERSORTx algorithm and Pearson correlation, *PLXNC1* was the only gene correlated with various immune cells, significantly associated with M2 macrophages. Furthermore, gene set variation analysis (GSVA) suggests the “hallmark_interferon_gamma_response” pathway was most significantly correlated with *PLXNC1*. Immunohistochemistry results revealed that *PLXNC1* protein level was significantly higher in STAD tissues than in normal tissues ($p < 0.001$).

Conclusion: *PLXNC1*, regulated by IRF5, is an immune-related gene that was significantly associated with M2 macrophages and poor outcome in stomach adenocarcinoma.

Keywords: gastric cancer, plexin C1, tumor microenvironment, macrophage, stomach adenocarcinoma, chromatin accessibility

INTRODUCTION

Gastric cancer (GC), the third leading cause of malignancy-related deaths, remains a considerable health problem worldwide (Siegel et al., 2019). Stomach adenocarcinoma (STAD) accounts for approximately 95% of all GC cases (Ajani et al., 2017). Although there have been great advances in diagnostic technology and therapeutic methods in recent decades, the prognosis of STAD patients remains poor.

Chronic inflammation due to infection with *Helicobacter pylori* is associated with GC (Balkwill and Mantovani, 2001). Much attention has been paid to the inflammatory response during initiation, promotion, and progression of GC (Mantovani et al., 2008; Bernard et al., 2019). Not only regarded as a model of inflammation-related cancer, the tumor immune microenvironment plays a vital role in tumor progression, especially tumor-associated macrophages (Li et al., 2019). Accumulating evidence indicates that a high rate of macrophage infiltration is strongly associated with poor prognosis of STAD (Su et al., 2018). Among the variety of molecules involved in tumor-related inflammation, signal transducers and activators of transcription (STATs), nuclear factor κ B (NF- κ B), and interferon regulatory factors (IRFs) are three crucial families of transcription factors (TFs) that control the inflammatory response (Yu et al., 2009; Yamashita et al., 2010; O'Reilly et al., 2018; Platanitis and Decker, 2018).

Chromatin accessibility maps are vital for the functional interpretation of DNA sequences. ATAC-seq (assay for transposase-accessible chromatin using sequencing), DNase I-seq (DNase I hypersensitive sites sequencing), and NOMe-seq (nucleosome occupancy and methylome sequencing) are the three main experimental methods employed to analyze chromatin accessibility at a genome-wide level. ATAC-seq identifies accessible chromatin regions (Nordstrom et al., 2019), through which enhancers, promoters, insulators, and chromatin-binding factors cooperatively regulate gene expression. ATAC-seq is increasingly used because of its relative technical simplicity and high sensitivity (Picelli et al., 2014; Corces et al., 2016).

Therefore, the goal of the current study was to identify genes with chromatin accessibility in promoter regions that may be directly downstream of TFs regulating STAT, NF- κ B, and IRF proteins, using ATAC-seq data downloaded from The Cancer Genome Atlas (TCGA). After identifying differentially expressed genes (DEGs) by RNA-seq, we constructed a univariate cox regression model to explore genes with prognostic value and definite immune-related genes in the ImmPort database. We then performed correlation analysis with tumor infiltrating immune-cell types using CIBERSORTx. The study identified an immune-related gene that interacts with macrophages, providing a novel potential target for STAD treatment.

MATERIALS AND METHODS

Ethics Statement

The studies involving human participants were reviewed and approved by Shanghai Tongji Hospital (2018-LCYJ-005). The

patients/participants provided their written informed consent to participate in this study.

Data Collection

RNA-seq data and corresponding survival information of patients with STAD were downloaded from TCGA¹, comprising 375 STAD and 32 normal tissue samples. Data from patients who were followed up < 90 days were deleted to minimize the impact of patient death due to non-tumor-related reasons. ATAC-seq profiles of 21 STAD samples were downloaded from TCGA². The list of STAT, NF- κ B, and IRF families of TFs was downloaded from The Human Transcription Factors³ database. The list of immune-related genes was downloaded from ImmPort database⁴ (Bhattacharya et al., 2018).

Screening for DEGs

The R package “limma” was used to analyze DEGs between tumor and normal samples in the RNA-seq dataset. For DEG identification, a false discovery rate (FDR)-adjusted $p < 0.05$ and $\log_2 |\text{fold-change (FC)}| > 1$ were considered cut-off criteria for genes with significantly altered expression.

Chromatin Accessibility Analysis

To explore genes with chromatin accessibility, the “karyoploteR,” “ChIPseeker,” and “TxDb. Hsapiens. UCSC. hg38. knownGene” packages in R software were used on the ATAC-seq profile dataset (Huang et al., 2021). Pearson correlation analysis with the mRNA expression of 21 TFs was used to filter downstream DEGs. Then, the lists acquired from Pearson correlation analysis were merged, and duplicated genes were removed.

Cox and Kaplan-Meier Survival Analysis

Cox univariate survival analysis of DEGs was performed using “survival” package in R with $p < 0.05$ as the threshold. Survival-related DEGs were identified and those with chromatin accessibility were considered hub DEGs. Kaplan-Meier survival analysis was used for further survival analysis.

Enumerating Tumor-Infiltrating Immune Cells

Hub DEGs were screened from the list of immune-related genes downloaded from the ImmPort database. CIBERSORTx algorithm was used to enumerate the proportions of immune cells. Normalized gene expression data were uploaded to the CIBERSORTx web portal, and the algorithm was run using the LM22 signature for 100 permutations. Cases with a CIBERSORTx output of $p < 0.05$ were considered eligible for further analysis. The Wilcoxon test was used to analyze differences in the composition of immune cells between STAD and normal tissues.

¹<https://tcga-data.nci.nih.gov/tcga/>

²<https://gdc.cancer.gov/about-data/publications/ATACseq-AW>

³<http://humantfs.ccb.utoronto.ca/>

⁴<https://www.immport.org/shared/home>

Correlation Between Gene Expression and Hallmark Pathways

Hallmark gene sets “h.all.v7.2.symbols.gmt,” downloaded from the GSEA database⁵, were used to find signaling pathways involved in STAD occurrence *via* the “GSVA” package in R. Significantly enriched pathways in the hallmark gene sets were determined applying a threshold of $p < 0.05$ and $\log_2 |FC| \geq 0.15$. Pearson correlation analysis was performed to identify gene co-expression and significantly altered hallmark pathways. The GeneMANIA database⁶ was employed to analyze the gene-gene interaction network and assess the potential interactive functional-association network of *PLXNC1* (Warde-Farley et al., 2010).

Multiple Database Validation

A variety of online databases were employed to validate our hypothesis. Most methods used to enumerate immune cells were based on a deconvolution algorithm, such as TIMER (Li et al., 2017)⁷, quanTIseq (Nordstrom et al., 2019), and EPIC (Racle et al., 2017)⁸. The GEPIA online database (Tang et al., 2017)⁹ was employed to validate the differential expression of *PLXNC1* and co-expression analysis of *PLXNC1* and macrophage surface markers. The Kaplan-Meier Plotter online database (Nagy et al., 2018)¹⁰ was used to validate prognostic value. The LinkedOmics database (Vasaikar et al., 2018)¹¹ was used to verify the correlation between clinicopathological features and *PLXNC1* expression.

Quantitative Real-Time Polymerase Chain Reaction Validation

Clinical samples of STAD and paired non-tumor tissues were acquired from 6 patients from Tongji Hospital, Shanghai, China. The quantitative real-time polymerase chain reaction (qPCR) was carried out using SyberGreen (Yeasen, 11200ES03), cDNA and the human-*PLXNC1* primers (Forward: AGAGTCCAACCAATCGCATCA, Reverse: AGTCCTGTTTACCACGGT). Total RNA was extracted from STAD or non-tumor tissues or cells using the TRIzol reagent. GAPDH served as an internal control. Every sample in each group was detected in triplicate. Paired *t*-test was applied for paired samples in qPCR results.

Tissue Microarray and Immunohistochemistry

Immunohistochemistry (IHC) was performed on tissue microarray slides (Cancer-cell Tumor Sample Center, Soochow University, Changzhou, China, #20191022R20), comprising 56 tumor and normal tissues. Patient information was provided

by the supplier. The slides were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed using citrate buffer solution (pH = 6.0) with a pressure cooker. Endogenous peroxidases were quenched using 3% H₂O₂ for 25 min. Slides were blocked with 3% bovine serum albumin for 30 min at room temperature and then incubated overnight at 4°C in primary antibody anti-*PLXNC1* (1:100; Bioss Antibodies, Beijing, China). After the slides were incubated with secondary antibody and stained, hematoxylin counterstaining was used to visualize cell nuclei. Image Pro Plus software (Media Cybernetics, Inc., Rockville, MD, United States) was used to quantify IHC-positive stained cells.

Statistical Analysis

All statistical analysis were performed with R software version 4.0.2. *P* value < 0.05 and $\log_2 |fold-change (FC)| > 1$ were considered cut-off criteria for genes with significantly altered expression. Significantly enriched pathways in the hallmark gene sets were determined applying a threshold of $p < 0.05$ and $\log_2 |FC| \geq 0.15$. Paired *t*-test was used for paired samples in qPCR and immunohistochemical analysis.

RESULTS

Examining the TF Catalog

A literature search was performed to identify proteins belonging to the STAT, NF- κ B, and IRF families of TFs. Then we examined the TF catalog downloaded from The Human Transcription Factors database, comprising 1,639 TF genes (Supplementary Table 1). Among them, the following 21 TFs were examined: STATs 1-4, 5a, 5b, and 6, IRF1-9, NF- κ B 1-2, RelA, RelB, and c-Rel. The 21 TFs were used for ATAC-seq analyze.

Identifying Genes With Chromatin Accessibility

Figure 1 depicts the study workflow. From the ATAC-seq profile dataset, we identified 4,287 genes with chromatin accessibility after removing duplicate genes (Supplementary Table 2). TFs binding to TF-binding sites, especially specific DNA tracts in gene promoter regions, is a central step toward transcriptional repression or activation. Therefore, we searched for genes with chromatin accessibility in these regions, identifying 2,454 genes (Supplementary Table 3). The coverage of chromatin accessibility over the whole genome is shown in Figure 2A while Figure 2B depicts the genomic features.

Gene Expression and Survival Analyses

A total of 6,739 DEGs were identified in STAD tissues compared to normal adjacent tissues, including 5,593 upregulated and 1,146 downregulated genes. After intersection of the DEGs with chromatin accessibility in promoter regions, we obtained a group of 365 DEGs with chromatin accessibility (Supplementary Table 4). Cox univariate survival analysis was performed, identifying 32 hub DEGs that were significantly related to overall survival of STAD patients (Figure 2C). Then, Kaplan-Meier survival analysis was performed on the hub DEGs

⁵<https://www.gsea-msigdb.org/gsea/downloads.jsp>

⁶<http://genemania.org>

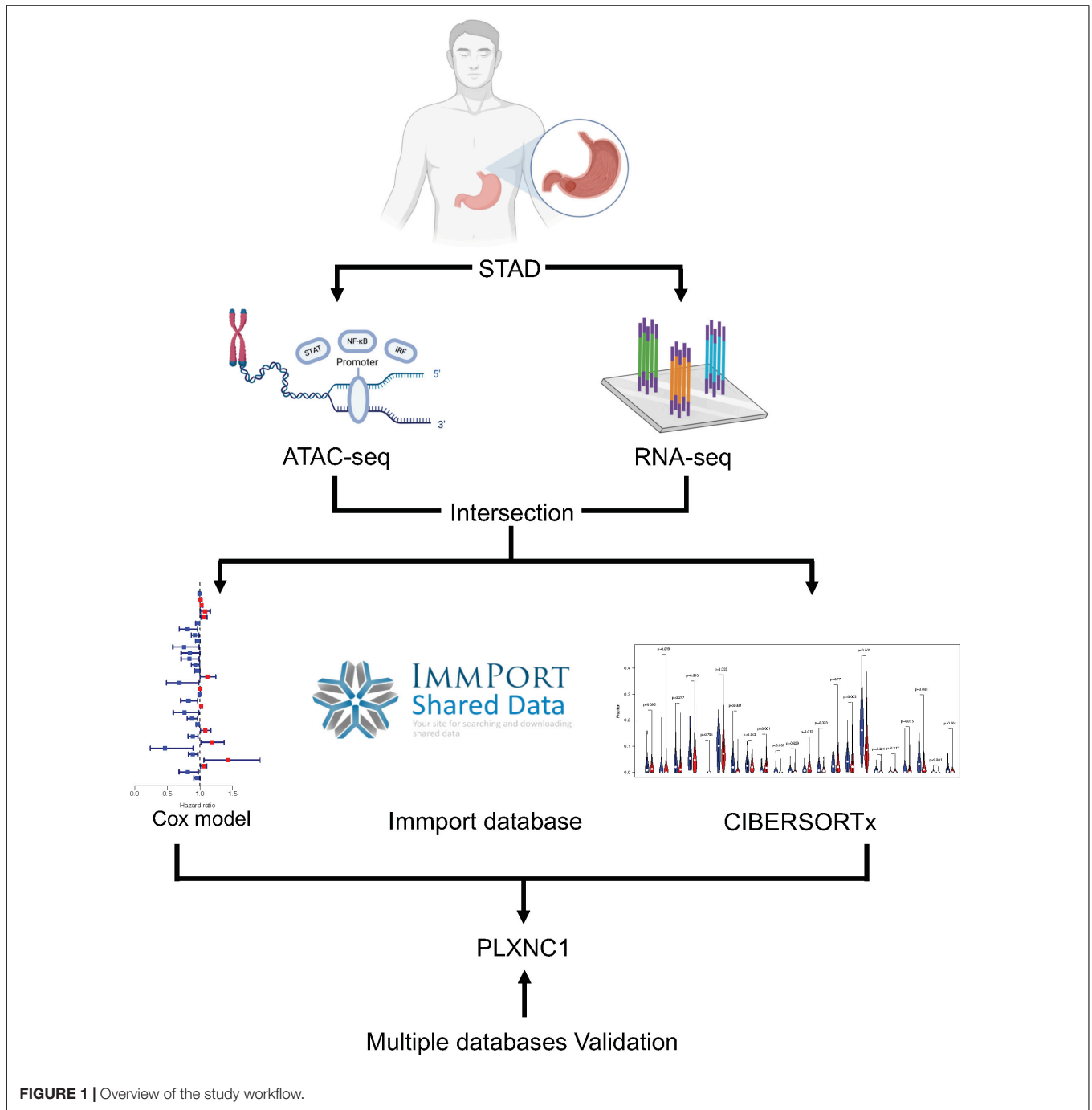
⁷<http://timer.comp-genomics.org/>

⁸https://gfellerlab.shinyapps.io/EPIC_1-1/

⁹<http://gepia.cancer-pku.cn/>

¹⁰<https://kmplot.com/analysis/>

¹¹<http://www.linkedomics.org/login.php>

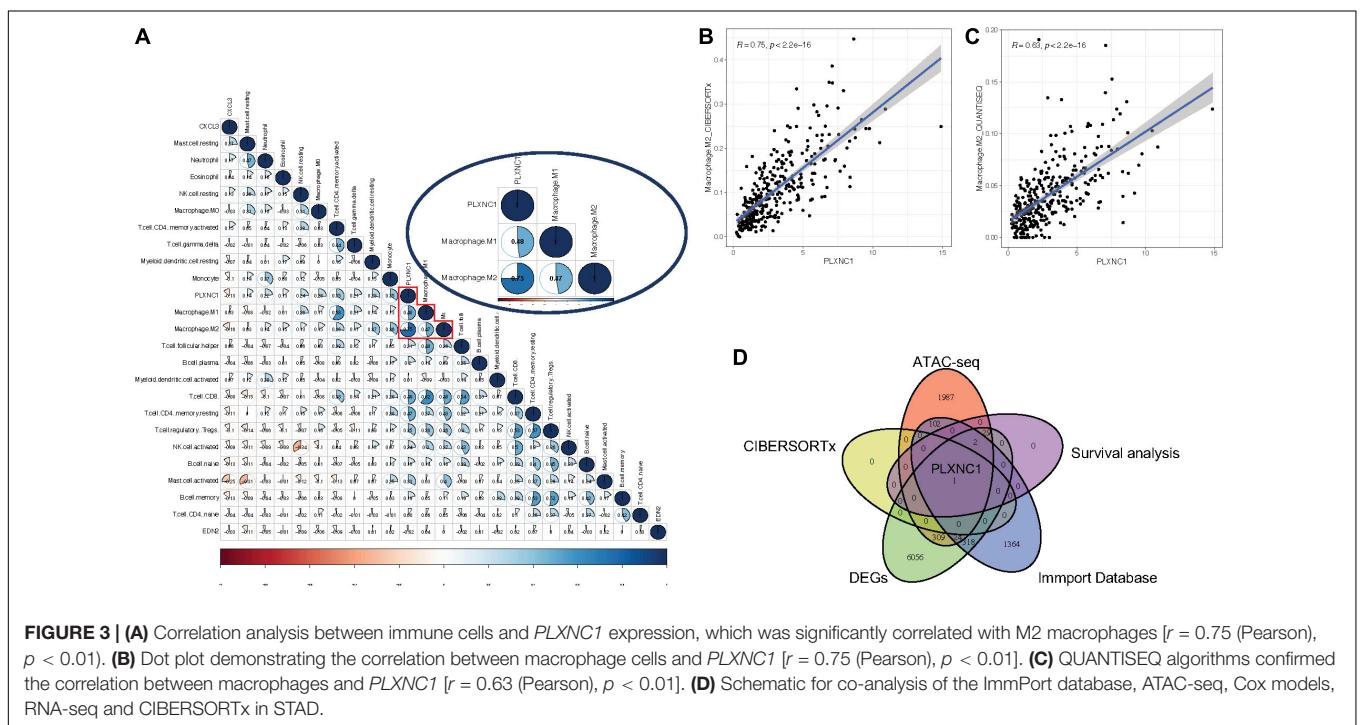
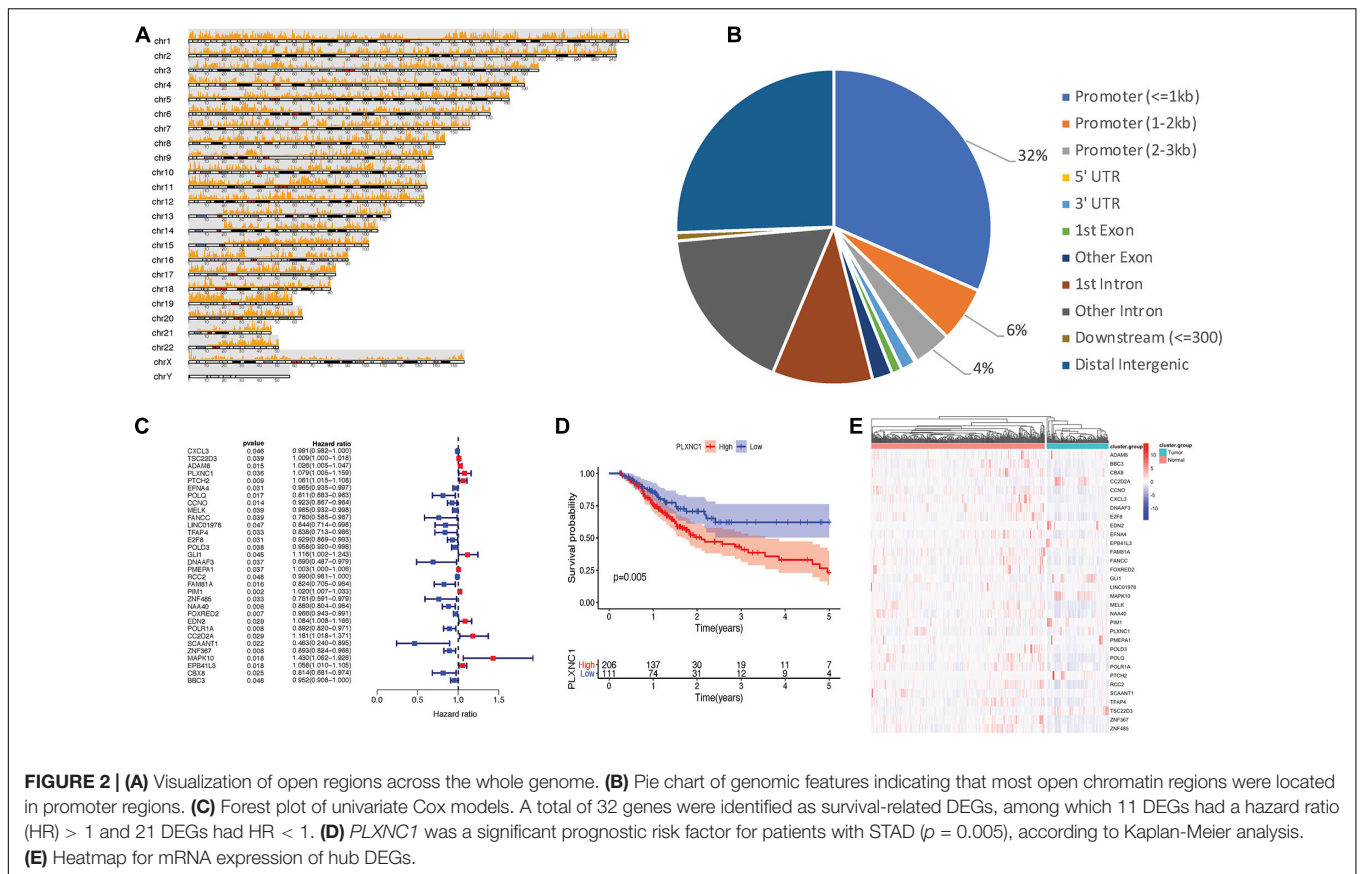


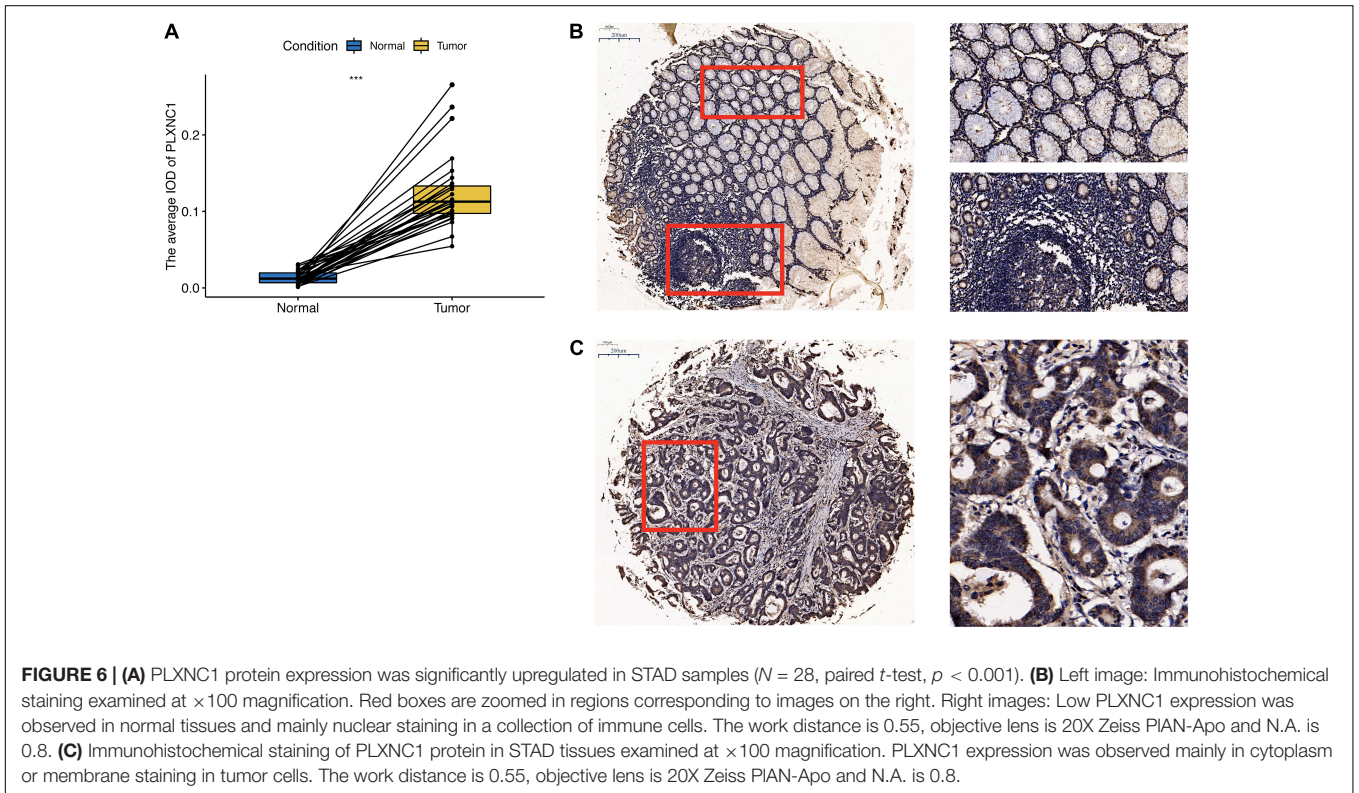
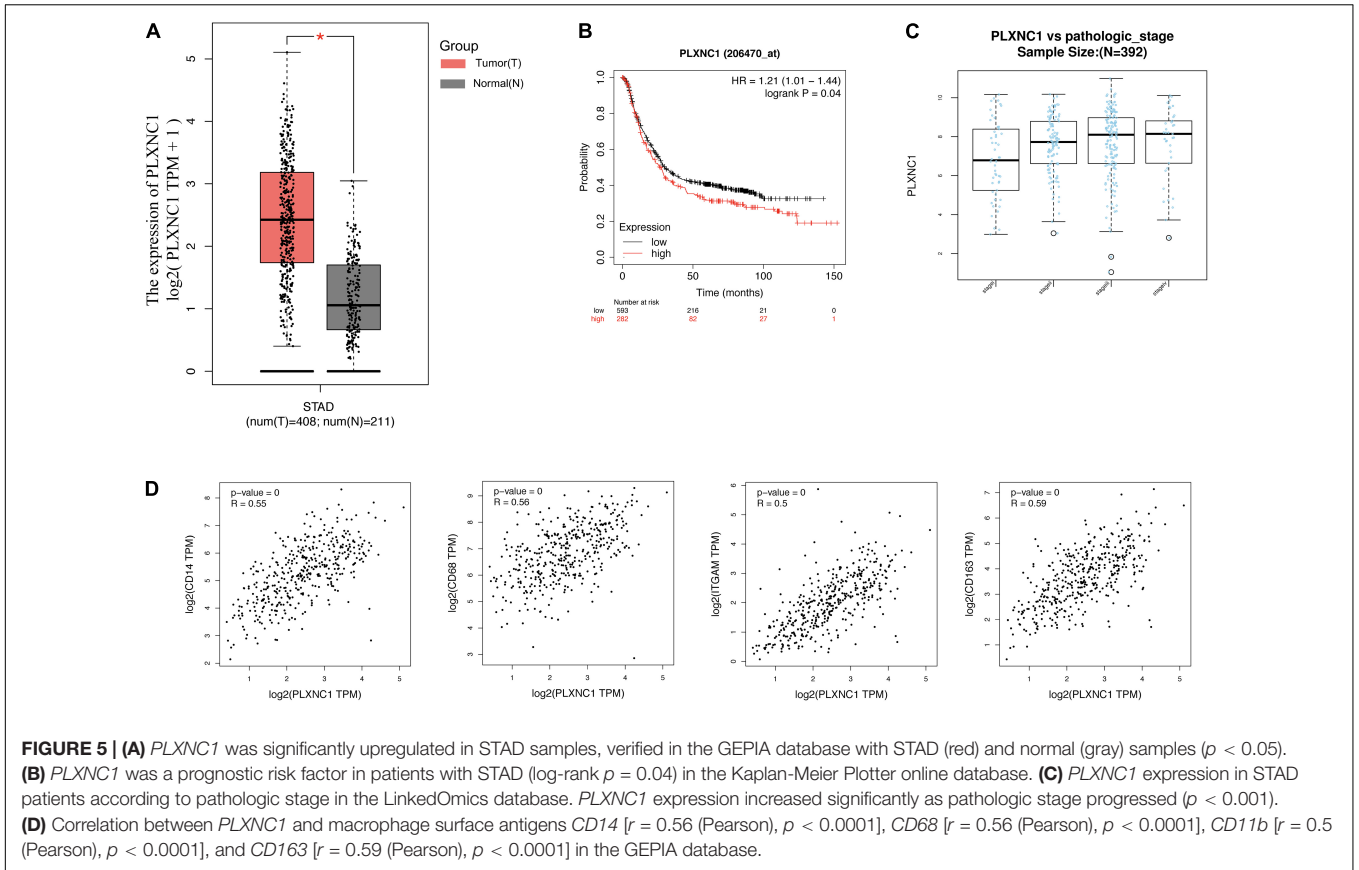
(Figure 2D). Figure 2E illustrates the mRNA expression of hub DEGs.

Correlation Between PLXNC1 and Tumor-Infiltrating Immune Cells

A list of 2,498 immune-related genes was downloaded from the ImmPort database, from which the hub DEGs were screened (Supplementary Table 5). The results suggested that *CXCL3*, *PLXNC1*, and *EDN2* were immune-related genes in STAD.

By applying the CIBERSORTx algorithm, the composition of 22 tumor-infiltrating immune cells was acquired for STAD (Supplementary Table 6). To further investigate the regulatory relationship between hub DEGs and immune cells, Pearson correlation analysis was performed (Figure 3A). Among the three genes, *PLXNC1* was the only gene correlated with various immune cells. Furthermore, *PLXNC1* showed the highest correlation with M2 macrophages ($r = 0.75$, $p < 0.01$) (Figure 3B). In order to authenticate the results and minimize bias, other methods (TIMER, quanTIseq, and EPIC) were





DISCUSSION

ATAC-seq, widely used for genome-wide identification of open chromatin, is based on the use of hyperactive Tn5 transposase to recognize and cleave DNA in open chromatin regions (Nordstrom et al., 2019). This method requires fewer cells to generate comprehensive maps. The relative simplicity of the protocol makes it a good choice for studies with a large number of samples, especially clinical cohorts. Using ATAC-seq profiles from TCGA and other bioinformatics approaches, we identified 2,454 genes with chromatin accessibility in promoter regions, of which 32 were survival-related DEGs. According to the ImmPort database and deconvolution algorithm validation, *PLXNC1* was significantly associated with macrophages.

PLXNC1 encodes a member of the plexin family located on chromosome 12, which serves as a receptor for Semaphoring 7A (Sema7A) (Liu et al., 2010). According to The Human Protein Atlas (Uhlen et al., 2015), *PLXNC1* expression is enhanced in macrophages, melanocytes, and Kupffer cells. Semaphorins are a large family of molecular signals regulating cell motility and migration, axon guidance, and the immune response (Zhou et al., 2008). These findings support that *PLXNC1* is associated with the process of inflammation and immune reactions. Furthermore, accumulating evidence has confirmed that *PLXNC1* is aberrantly expressed in a variety of human cancers and serves as either a cancer promoter or suppressor (Wang et al., 2018; Chen et al., 2020). According to the TIMER database (Li et al., 2017), differential expression of *PLXNC1* has been detected in 13 cancer types (CHOL, COAD, ESCA, HNSC, KICH, KIRC, LIHC, LUSC, PRAD, READ, STAD, THCA and UCEC). Among them, *PLXNC1* is highly expressed in CHOL, ESCA, HNSC, KIRC, LIHC, STAD and THCA compared with normal tissues (**Supplementary Figure 1**). König et al. (2014) demonstrated that *PLXNC1* directly influenced critical steps of leukocyte transmigration to promote acute inflammation *in vivo*. In another study, the Sema7A/PlexinC1 interaction was originally shown to induce activation of monocytes (Holmes et al., 2002). It was also found that *PLXNC1* deficiency enabled synaptotagmin 7-mediated macrophage migration and enhanced mammalian lung fibrosis (Peng et al., 2016). When it comes to its role in the multistep cancer process, *PLXNC1* was found to be involved in cancer progression (Balakrishnan et al., 2009). In these studies, *PLXNC1* was suggested to be a protective factor in melanoma, suppressing tumor development. These studies demonstrated that *PLXNC1* participates in the occurrence and development of tumors.

In addition, Chen et al. (2020) were the first researchers to identify that *PLXNC1* was up-regulated in STAD and associated with poor prognosis. They have confirmed that overexpression of *PLXNC1* significantly accelerated carcinogenesis in GC *in vitro* and *in vivo*. Furthermore, *PLXNC1* promoted proliferation and migration of GC cells through transcriptional activation of the interleukin 6 signal transducer (IL6ST).

In our study, *PLXNC1* was significantly correlated with M2 macrophages and most significantly correlated with the “hallmark_interferon_gamma_response” pathway, an immune-related pathway. Therefore, *PLXNC1* may be a key player in the inflammatory and immune responses. Our results also suggested

that *PLXNC1* has a prognostic value in STAD, although this was not the emphasis of our study. We focused on the interactions between *PLXNC1* and M2 macrophages during inflammation to enhance STAD proliferation and the regulation of *PLXNC1* by *IRF5*.

Our study has some limitations. First, we did not validate the prognostic value of *PLXNC1* in a clinical cohort, instead we used the Kaplan-Meier Plotter online database for verification. Studies with larger populations and longer follow-up duration will be conducted in the future to confirm our results. Second, our research did not investigate the mechanisms between *PLXNC1* and tumor infiltrating immune cells, especially tumor-infiltrating macrophages. Future studies will focus on these aspects. However, despite the above-mentioned limitations, this is the first study to infer that *PLXNC1* expression might be regulated by *IRF5* and correlated with macrophages in STAD using ATAC-seq and RNA-seq. Molecular biological experiments will be performed to validate these findings in the future.

CONCLUSION

Our study demonstrated that *PLXNC1* is regulated by *IRF5*. *PLXNC1* expression was upregulated in patients with STAD and associated with poor outcome. Furthermore, *PLXNC1* was strongly associated with the macrophage fraction. These results reveal that *PLXNC1* is associated with immunity and interacts with macrophages in some manner, suggesting the potential value of anti-*PLXNC1* therapy for STAD treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Shanghai Tongji Hospital (2018-LCYJ-005). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZN, CH, HZ, QH, and BG conceived the idea and wrote the manuscript. ZN, CH, HZ, JZ, MH, QC, QH, and BG designed the experiments and analyzed the data. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.662707/full#supplementary-material>

Supplementary Figure 1 | The expression pattern of PLXNC1 in other solid tumors.

Supplementary Figure 2 | The relative expression of PLXNC1 mRNA in six patients.

Supplementary Table 1 | The result of 1,639 transcription factors genes.

Supplementary Table 2 | The result of 4,287 genes with chromatin accessibility after removing duplicate genes.

Supplementary Table 3 | The result of 2,454 genes with chromatin accessibility in promoter regions.

Supplementary Table 4 | The result of DEGs with chromatin accessibility.

Supplementary Table 5 | A list of 2,498 immune-related genes.

Supplementary Table 6 | The results of CIBERSORTx in STAD.

Supplementary Table 7 | The Pearson correlation analysis between PLXNC1 and 1,639 TF genes at chromatin accessibility.

Supplementary Table 8 | The Pearson correlation analysis between PLXNC1 and the top 10 TF genes with chromatin accessibility at mRNA expression level.

Supplementary Table 9 | The acronyms are defined in Supplementary Table 9.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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