IgA- Tertiary Lymphoid Structures Correlates With a Better Prognosis in GADC Patients

Lizhou Jia

Bayannur Hospital

Tengqi Wang

Bayannur Hospital

Hao Chen

Wannan Medical College

Teer Ba

Inner Mongolia University

Bin Wang

Nanjing First Hospital

Xiaolei Wang

Nanjing First Hospital

Weiwei He

Nanjing City Hospital of Traditional Chinese Medicine

Youcai Zhao

Nanjing First Hospital

Wei Zhao (✉ zhaowei_njmu@163.com)

Nanjing First Hospital <https://orcid.org/0000-0001-8557-297X>

Research

Keywords: GADC, IgA, SSR4, TIBs, TLSs

Posted Date: October 5th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-944650/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: The roles of tumor infiltrating B lymphocytes (TIBs) and tertiary lymphoid structures (TLSs) in solid tumor genesis and tumor therapy have been recognized by researchers, but the specific formation and effect of the TLSs have not been fully understood. In this study, we used single-cell RNA sequencing, multiple immunofluorescence assays, and quantitative digital image analysis to study the formation and structure of TLSs in gastric adenocarcinoma (GADC). Furthermore, the study collected 165 cases of GADC with TLSs and analysis the relationships between TLS formation and clinicopathological characteristics and prognosis of GADC patients were analyzed.

Results: The result identified the type of IgA-TLSs which contained higher level of IgA⁺-B cells in GADC, and the major structures of the IgA-TLSs were determined. We found that immune cells in IgA-TLSs had higher levels of cellular interactions and migration ability. The expression of signal sequence receptor subunit 4 (SSR4) was characterized and found to higher expressed in the IgA-TLSs. Furthermore, IgA-TLSs correlated with age, differentiation, distant metastasis, TNM stage, chemotherapy effect, expression of programmed death-ligand 1, J-chain, and SSR4, and better overall survival.

Conclusions: Our research provided the information about CD79A/J-chain B cells in GADC and indicated that IgA-TLSs was associated with better prognosis for GADC patients.

Introduction

Gastric cancer (GC) is one of the five most common tumors worldwide and one of the most frequent malignant tumors of the digestive system in China.^[1] More than 80% of patients with GC in China present with middle and advanced stages at the time of diagnosis because of the absence of obvious symptoms. Gastric adenocarcinoma (GADC) is a kind of gastric cancer, which is caused by malignant changes of gastric gland cells. Its incidence rate accounts for 90% of gastric malignant tumors.^[1] The growth, invasion and metastasis of GADC is a complex and dynamic process, which involves the genetic abnormalities inherent in the tumor tissue and tumor-immune cell interactions in the local microenvironment.^[3, 4] Moreover, B cell mediated mucosal immunity and humoral immunity also plays an important role in gastrointestinal tumors.^[3, 5, 6] IgA is the most abundant immunoglobulin in the body and the main force of mucosal immunity against infection. IgA induces B and T cell responses and recruits them to various effector sites, differentiating into plasma cells that can continue to secrete IgA and play a mucosal protective role.^[7]

The tumor microenvironment (TME) of GADC is a dynamic network and is a key factor affecting the metastasis and promotion or inhibition of immune response against the tumor cells.^[8] Infiltrating immune cells in the TME, also known as tumor-infiltrating lymphocytes (TILs), mediate the anti-tumor response.^[9] In the gastric TME, there is bidirectional regulation between tumor cells and immune cells. The interaction between the two types of cells can disrupt homeostasis in the body, mobilize internal and external resources of cells, produce TMEs suitable for tumor cell growth, and affect the response of GC

cells to GC immunotherapy.^[3] Immunotherapy is a major treatment method following surgery, radiotherapy, chemotherapy, and targeted therapy.^[10] Currently, effective immunotherapy for GC mainly comprises immune checkpoint inhibitor (ICI) treatment with monoclonal antibodies against programmed cell death-1 or programmed cell death ligand-1.^[11, 12] Additionally, adoptive immunotherapy with chimeric antigen receptor T cells has shown good efficacy in the treatment of hematological tumors and some solid tumors.^[13] To date, strategies for predicting biomarkers and enhancing clinical immunotherapeutic responses have focused on T cells, with less attention has been paid to the role of other immune cell subsets that may contribute to anti-tumor immune responses.

The TME contains not only TILs but also tertiary lymphoid structures (TLSs).^[14, 15] TLSs, also known as ectopic lymphoid tissue, is a new type of lymphoid tissue found in recent years in areas that are stimulated by certain autoimmune diseases, graft rejection, and chronic inflammatory responses. Its structure and function are very similar to secondary lymphatic organs such as lymph nodes and spleen. TLSs can be seen in both primary and metastatic tumors,^[16] and mainly composed of T and B lymphocytes, follicular dendritic cells (FDC), and mature dendritic cells,^[17] which can penetrate into the “hinterland of the enemy” much like a “Trojan horse” and effectively inhibit tumor growth and inflammation.^[18] Furthermore, the density of TLSs and the ratio of TLSs to tumor area, as well as the number of tumor-infiltrating B lymphocytes (TIBs) were reported to be higher in patients who were sensitive to ICI treatment than in ICI-insensitive patients.^[19]

In our previous study, we collected 4 cases of fresh GADC tissue samples and found the potential importance of IgA-mediated humoral immunity in GADC patients with TLSs through single-cell RNA sequencing.^[19] To further explore the formation of IgA-TLSs in GADC, in present study we explored the information of CD79A/J-chain B cells in GADC tissue samples, and conducted Gene Ontology (GO) and Cellular Spatial Organization mapper (CSOmap) analysis on the CD79A/J-chain cell cluster. Next, we collected 24 cases of GADC with TLSs to compare structures and densities of tumor-associated TLSs using multiple immunofluorescence assays. We also analyzed the relationships between IgA-TLSs formation and clinicopathological characteristics and prognosis in patients with GADC through 165 cases of TMA analysis. Our research provided the information about CD79A/J-chain B cells in GADC and indicated that IgA-TLSs was associated with better prognosis for GADC patients.

Materials And Methods

Sample Collection

All sample collection was approved by the Human Research Ethic Committee of Bayannur Hospital and Nanjing First Hospital. 4 cases of GADC tissue samples (named as T1, T2, T3 and T4) with TLSs were immediately collected after resection and dissociated into a single-cell suspension. Single-cell sequencing data came from the previous research data of our team. Please refer to the information of the 4 patients^[19]. All paraffin tissue blocks were received from the department of Pathology at the Bayannur

Hospital and Nanjing First Hospital between Jan. 2008 to Dec. 2010, and preparation onto tissue microarray (all samples from 120 GADC patients). The formation of germinal center structures is determined by two experienced pathologist. An effective TLS is defined as an area of ectopic lymphocyte aggregation greater than a 200 × visual field (1mm in diameter). Two distinct regions of TLSs are observed under the microscope: the bright region and dark region. Clinical pathology information includes gender, age, differentiation, Tumor Node Metastasis (TNM) stage, chemotherapy effect and TLSs location. TNM stage standard according to American Joint Committen on Cancer (AJCC) Cancer Staging Manual Eighth Edition.^[20] We divided the locations of TLSs into tumor center, para-cancerous tissues and the junction between tumor (T) and normal (N) tissues (T/N junction). Overall survival (OS) period was defined from initial diagnosis to death. Matched normal tissue was obtained from sites displaced at least 5 centimeters from the tumor and was confirmed to lack tumor cells on histopathology review. All patients did not receive any other therapy before surgery, more details about samples please see Table 2.

Table 2
Associations between IgA-TLSs formation with clinicopathologic characteristics in
GADC patients

Characteristics	n	IgA-TLSs n (%)	nTLSs n (%)	p	χ^2
Total	165				
Gender				0.108	0.853
male	122	42(34.43)	80 (65.57)		
Female	43	16(37.21)	27(62.79)		
Age				17.442	<0.001
<60	54	31(57.41)	23(42.59)		
≥60	111	27(24.55)	84(76.36)		
Differentiation				6.253	0.014*
well-middle	75	34(45.33)	41(54.67)		
middle-poor	90	24(26.67)	66(73.33)		
T stage				2.241	0.524
T ₁	23	8(34.78)	15(65.22)		
T ₂	4	0(00.00)	4(100.00)		
T ₃	113	41(36.28)	72(63.72)		
T ₄	25	9(36.00)	16(64.00)		
N stage				4.022	0.17
N ₀	33	10(30.30)	23(69.70)		
N ₁	43	11(25.58)	32(74.42)		
N ₂	35	12(34.29)	23(65.71)		
N ₃₋₄	54	25(46.30)	29(53.70)		
M stage				4.425	0.049*
M ₀	137	53(38.69)	84(61.31)		
M ₁	28	5(17.80)	23(82.14)		
TNM stage				9.359	0.025*
I	17	7(41.18)	10(58.82)		

Characteristics	n	IgA-TLSs n (%)	nTLSs n (%)	<i>p</i>	χ^2
□	50	13(26.0)	37(74.0)		
□	68	32(47.06)	36(52.94)		
□	30	6(20.00)	24(80.00)		
chemotherapy effect				13.538	<0.001*
(CR+PR)	109	49(44.95)	60(55.05)		
(SD+PD)	56	9(16.07)	47(83.93)		
TLSs location					
tumor center	98	34(34.69)	64(65.31)	0.031	0.985
para-carcinoma tissue	34	12(35.29)	22(64.71)		
T/N junction	33	12(36.36)	21(63.64)		
PD-L1 expression				4.953	0.034*
≥5%	83	36 (43.37)	47 (56.63)		
<5%	82	22 (26.83)	60 (73.17)		
SSR4 expression				65.653	<0.001*
positive	90	56 (62.22)	34 (37.78)		
negative	75	2 (2.67)	73 (97.33)		
* means $P < 0.05$.					

Analysis of CD79A/J-chain B Cell Cluster Subgrouping in 10 × Single Cell Transcriptome

For clustering and dimension reduction analysis, t-distributed stochastic neighbor embedding (tSNE) was used in the algorithm, resolution was set at 0.5, and harmony was used to remove the batch effect. A total of 14,660 cells were retained after screening. Bimod algorithm is adopted in the difference analysis between different clusters, and p value is set as < 0.01 , the filter condition $\log_2FC \geq 0.26$.^[20]

Gene Ontology(GO) Analysis

The GO is an internationally standardized functional classification system for genes. It provides a dynamically updated standard vocabulary to comprehensively describe the properties of genes and gene products in an organism. There are three ontologies (ontologies) in GO. The molecular function, cellular component and biological process of the gene were described respectively. In this study, only the biological process was analyzed. The basic unit of GO is “term”, and each term corresponds to an attribute. Firstly, all the significantly differentially expressed genes were mapped to the terms of the GO

database, and the number of genes in each term was calculated. Then, hypergeometric test was applied to find out the GO items that were significantly enriched in the significantly differentially expressed genes compared with the whole genome background.^[22] GGPLOT2 was used to carry out enrichment analysis on the GO results and scatter plot display: Rich factor represents the number of differential genes located in the GO/the total number of genes located in the GO. The greater the Rich factor, the higher the GO enrichment degree.^[23]

Cellular Spatial Organization Mapper (CSOmap)

To reconstruct the cell spatial organization, we analyzed 2,557 ligand-receptor paired in single cell affinity matrix. The ligand-receptor interaction database in CSOmap is based on FANTOM5 (https://fantom.gsc.riken.jp/5/suppl/Ramilowski_et_al_2015/) for estimating the cell-cell affinity matrix.^[24,25] Function Annotation Of The Mammalian Genome (FANTOM) 5 compiled putative ligands from known interacting ligands, orphan Human Plasma Membrane Receptome (HPMR) ligands and from a set of secreted proteins that were not found in the set of known receptors. FANTOM5 compiled putative receptors from known interacting receptors, orphan HPMR receptors and from a set of PM proteins that were not found in the set of known ligands. And then FANTOM5 obtained predicted ligand–receptor pairs by searching for validated protein-protein interactions (Human Protein Reference Database and STRING32 databases) between putative ligands and putative receptors.^[25]

Immunohistochemistry, Multiple Immunofluorescence Assay and HALO Spatial Analysis

Tissue paraffin sections were incubated in the oven at 65°C for 1h, dewaxed by xylene for 3 times, gradient alcohol hydration was performed, and antigens were retrieved by microwave. Sections were pretreated and stained with monoclonal antibodies directed against CD79A (SP18, Abcam), J-chain (OTI2B1, Invitrogen), CD4 (EPR6855, Abcam), SSR4 (11655-2-AP, Proreintech), CD8 (SP16, Abcam), CD14 (SP192, Abcam) and PD-L1 (22C3, Dako), DAPI (D3571, Thermo Fisher Scientific) on a Leica-Bond III/max autostainer platform (Leica Biosystems). Incubated overnight at room temperature (RT), added biotin-labeled secondary antibody at RT for 1h, and adding horseradish peroxidase labeled streptomycin opaletin working solution. Incubated at 37°C for 30min, and using anti - immunoglobulin - coupled horseradish peroxidase with 3,3'- diaminobenzidine (DAB, OptiView Kit, Roche Diagnostics) as substrate. Nuclear counterstaining was performed with Mayer hematoxylin. The degree of J-chain expression in tissues were scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The frequency of positive cells was defined as follows: 0 < 5%, 1, 5% - 25%; 2, 26% - 50%; 3, 51% - 75%; and 4, greater than 75%. Multiply the two scores, and for statistical analysis, score of 0 - 7 were considered low or no expression and score of 8 to 12 considered high expression. PD-L1 positive defined as $\geq 5\%$.

Lymphocyte regions on tissue images were manually circled, based on Halo Highplex FL V4.0.4 (ICA Labs, Albuquerque) algorithm, the algorithm was adjusted according to debug the algorithm according to the localization and staining characteristics of different positive cells. The number and proportion of positive cells labeled by antibodies in the whole image and lymphocyte cells region were quantitatively

analyzed, and the morphology of each cell was quantitatively analyzed. The area to be analyzed on the tissue image was observed manually, and the infiltration degree of immune cells 300µm inside and outside the tissue boundary was calculated and analyzed. Each interval of 50µm was divided into 1 interval. The number of immune cells in each interval, the tissue area of each interval, and the cell density in each interval were calculated, and the histogram was automatically generated.

Statistical Analysis

All data were analyzed by SPSS 23.0 software. Survival curves were generated by the Kaplan-Meier method. The results of Kaplan-Meier method is displayed with HR and *P* or Cox *P*-values from a log-rank test. Pearson χ^2 test was used to compare the relationship between the expression levels of gene and clinicopathological parameters. The independent paired t test was used to compare the mean values of the two groups. *P* < 0.05 is considered a significant difference.

Results

The information of CD79A/J-chain B cells in GADC Tissue Samples

In our previous study, we performed single-cell RNA sequencing to analyze of 49,765 single cells from four GADC patients, which contained a larger number of B cells. In the part of B cell, we found CD79A and J-chain higher expressed. After clustering and dimension reduction analysis, 14,660 CD79A/J-chain B cells were re-clustered into 27 separate subsets through t-SNE algorithm (Figure 1A). The distribution of CD79A/J-chain B cells in tumor tissue samples is shown in the Figure 1B, and we found that they mainly existed in T1 and T2 (Figure 1C). Therefore, we choosed CD79A/J-chain B cells cluster from tissue samples to analysis and found that the cluster exhibited related biological process, including classical complement activation, leukocyte migration, and immune responsiveness through gene ontology (GO) analysis (Figure 1D). After performing enrichment analysis, we determined that the main functions of these cells were related to production of secretory and monomeric IgA immunoglobulin complexes, and the monomeric IgA immunoglobulin complex, and antibacterial humaral response (Figure 1E).

A mathematical optimization model (CSOmap) was used to analyze the dominant ligand-receptor pairs that contributed to the distribution of immune cells in the tumor tissue. The results showed that T1 and T2 samples, which contained higher number of CD79A/J-chain B cells, had a greater number of cells and different types of cellular interactions and migration. The dominant ligand-receptor pairs (*P* < 0.01) included CXCL13-CXCR3 for FDC and B cell interactions, CCL4-CCR8 for T regulatory cells and mononuclear macrophages, and HLA-E-KLRC1 for B cells and natural killer T cells (Figure 1F).

Correlation between SSR4 and Immune Cells in GADC

Through cluster analysis, the study discovered that the 27 types of sub-clusters had higher gene expression of immunoglobulin heavy chain alpha (IGH) A1, IGHA2 and signal receptor subunit 4 (SSR4)

genes (Figure 2A). Therefore, we speculated that the expression of SSR4 gene is related to the function of CD79A/J-chain B cells.

The expression levels of SSR4 were evaluated in immune cells through Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia.cancer-pku.cn/>), and the results indicated that SSR4 gene was higher expressed in immune cells, especially in B cells and T cells. Furthermore, the expression levels of SSR4 were significantly different between distinct types of B cells in gastric tissue (Figure 2B).

The study also found significant correlations between SSR4 and marker genes of B cell, T cells, tumor-associated macrophage (TAM), such as CD79A, CD3D, IL10, HLA-DPB1, and IFN- γ in Tumor IMmune Estimation Resource (TIMER) database (<https://cistrome.shinyapps.io/timer/>) (Table 1).

Table 1
Correlation analysis between SSR4 and relate genes and markers of immune cells in GADC

Description	Gene markers	NONE		Purity	
		Cor	P	Cor	P
CD8+ T Cell	CD8A	0.105	0.032	0.111	0.030
	CD8B	0.116	0.018	0.125	0.015
T Cell	CD3D	0.147	**	0.151	**
	CD3E	0.154	0.002	0.162	**
	CD2	0.100	0.044	0.102	0.046
B Cell	CD19	0.075	0.128	0.090	0.090
	CD79A	0.189	***	0.195	***
Monocyte	CD86	0.091	0.065	0.0775	0.132
	CD115(CSF1R)	0.027	0.586	0.016	0.757
TAM	CCL2	-0.014	0.774	-0.041	0.421
	CD68	0.175	**	0.154	**
	IL10	**	0.730	**	0.987
M1 Macrophage	INOS (NOS2)	-0.061	0.217	-0.078	0.130
	IRF5	0.109	0.027	0.117	0.023
	COX2(PTGS2)	-0.137	**	-0.160	**
M2 Macrophage	CD163	-0.031	0.535	-0.061	0.238
	VSIG4	-0.012	0.811	-0.037	0.472
	MS4A4A	0.017	0.737	**	0.985
Neutrophils	CD66b (CEACAM8)	-0.123	0.012	-0.118	0.021
	CD11b (ITGAM)	-0.005	0.926	-0.018	0.723
	CCR7	0.022	0.662	0.023	0.674
Natural killer cell	KIR2DL1	-0.043	0.385	-0.028	0.591
	KIR2DL3	-0.126	0.010	-0.114	0.026
	KIR2DL4	0.101	0.040	0.113	0.028
	KIR3DL1	-0.017	0.727	-0.009	0.865
	KIR3DL2	**	0.842	0.021	0.677

Description	Gene markers	NONE		Purity	
		Cor	P	Cor	P
	KIR3DL3	0.032	0.521	0.063	0.221
	KIR2DS4	0.020	0.688	0.032	0.539
Dendritic cell	HLA-DPB1	0.158	**	0.158	**
	HLA-DQB1	0.137	**	0.130	0.011
	HLA-DRA	0.133	**	0.136	**
	HLA-DPA1	0.164	**	0.167	**
	BDCA-1(CD1C)	-0.030	0.542	-0.036	0.482
	BDCA-4(NRP1)	-0.071	0.146	-0.099	0.054
	CD11c (ITGAX)	-0.028	0.562	-0.042	0.406
Th1	T-bet (TBX21)	0.072	0.143	0.074	0.149
	STAT4	-0.022	0.659	-0.016	0.761
	STAT1	0.053	0.282	0.068	0.183
	IFN- γ (IFNG)	0.153	**	0.164	**
	TNF- α (TNF)	0.039	0.429	0.021	0.683
Th2	GATA3	0.025	0.607	0.028	0.580
	STAT6	-0.101	0.039	-0.107	0.037
	STAT5A	-0.091	0.064	-0.093	0.071
	IL13	-0.051	0.300	-0.045	0.380
Tfh	BCL6	-0.170	**	-0.190	***
	IL21	0.119	0.015	0.130	0.011
Th17	STAT3	-0.123	**	-0.140	**
	IL17A	0.188	***	0.185	***
Treg	FOXP3	0.162	**	0.161	**
	CCR8	**	0.936	**	0.903
	STAT5B	-0.217	***	-0.235	***
	TGF β (TGFB1)	-0.026	0.595	-0.040	0.438
T cell exhaustion	PD-1 (PDCD1)	0.146	**	0.162	**

Description	Gene markers	NONE		Purity	
		Cor	P	Cor	P
	CTLA4	0.120	0.014	0.123	0.017
	LAG3	0.189	***	0.200	***
	TIM-3 (HAVCR2)	0.047	0.339	0.038	0.461
	GZMB	0.185	***	0.186	***
TAM, tumor-associated macrophage; Th, T helper cell; Tfh, Follicular helper T cell; Treg, regulatory T cell; Cor, R value of Spearman's correlation; None, correlation without adjustment. Purity, correlation adjusted by purity; ** $P < 0.01$; *** $P < 0.001$.					

These results further confirm that SSR4 is significantly related with immune infiltrating cells in GADC, which suggests that SSR4 plays a important role in the immune microenvironment.

Different Structures and Densities of Tumor Associated TLSs

The study collected 24 cases of paraffin tissue blocks, and each samples with TLSs. Twelve of these tissues contained TLSs that formed germinal centers (bright region and dark region). Multiple immunofluorescence assays detected the morphology and structure of the TLSs in these tissues. We selected anti-CD79A antibody as a marker for B cells, anti-CD4 antibody for helper T (Th) cells, anti-CD8 antibody for CD8⁺ T cells, anti-CD14 antibody for mononuclear macrophages, and anti-SSR4 antibody as a marker for immune cells (Figure 3A-B). The results indicated that there were different types of TLSs in GC tissues. CD79A⁺-B cells existed mainly in the core of germinal center in the TLSs. Th cells mainly existed at the outer margins of the TLSs with germinal center. The interaction between Th cells/mononuclear macrophages and CD79A⁺-B cells were involved in the formation and development of germinal centers. We also found that the central region of TLSs forming germinal centers is highly expressed SSR4 and CD79A. By quantitative analysis, we determined that the densities of total cells in the TLSs, which CD79A⁺-B cells existed mainly in the core, were higher than those with CD79A⁺-B cells in the edge of TLSs. We suggested that the CD79A⁺-B cells higher expression in the core of the TLSs could recruit more types of immune cells and promot the formation of germinal centers (Figure 3C-D).

The information of IgA-TLSs (CD79A+/J-chain+) and the Correlation with Clinicopathological Characteristics in Patients with GADC

To analyze the function of IgA-TLSs, we determined the relationships between IgA-TLS in GADC and clinicopathological parameters. We collected 165 samples from 120 GADC patients that contained TLSs and analyzed protein expression using tissue microarray. Each sample point detected the formation of TLSs by determining the location of CD79A protein and the expression of J-chain. We defined IgA-TLSs as CD79A expressed in the core of the TLSs and simultaneously detected the expression of J-chain. We

also detected the expression levels of PD-L1, and SSR4 proteins and combined analyses with clinicopathological information was performed (Figure 4).

By using IHC, we found that the formation of IgA-TLSs, as determined by CD79A expression in the core of TLSs, was present in 58/165 (35.15%) of GADC tissue. Furthermore, we found the expression of IgA-TLSs was correlated with age ($\chi^2 = 17.442$, $P < 0.001$), differentiation ($\chi^2 = 6.253$, $P = 0.014$), M stage ($\chi^2 = 4.425$, $P = 0.049$), TNM stage ($\chi^2 = 9.359$, $P = 0.025$), chemotherapy effect ($\chi^2 = 13.538$, $P < 0.001$), PD-L1 expression ($\chi^2 = 4.953$, $P = 0.034$), and SSR4 expression ($\chi^2 = 65.653$, $P < 0.001$); however, no significant relationship was found for gender, T stage, N stage, or TLS location (Table 2).

IgA-TLS was Associated with Better Prognosis for GADC Patients

To determine the relationship between IgA-TLSs and overall survival (OS) of GADC patients, Kaplan-Meier survival curve analyses were performed and it was suggested that IgA-TLSs formation was significantly associated with a better prognosis for GADC patients (log rank = 13.604, $P < 0.001$) (Figure 5). In univariate analysis, IgA-TLS formation was associated with a better OS period (HR, 2.065; 95% CI, 1.380–3.091; $P < 0.001$), and other prognostic markers, including M stage (HR, 2.702; 95% CI, 1.181–6.182; $P = 0.019$), TNM stage (HR, 1.972; 95% CI, 1.316–2.955; $P = 0.001$), chemotherapy effect (HR, 4.166; 95% CI, 2.088–8.312; $P < 0.001$), TLS location (HR, 1.57; 95% CI, 1.014–2.434; $P = 0.044$). In multivariate analysis, the formation of IgA-TLSs (HR, 1.900; 95% CI, 1.051–3.437; $P = 0.034$), TNM stage (HR, 2.651; 95% CI, 1.614–4.356; $P < 0.001$), chemotherapy effect (HR, 3.782; 95% CI, 1.649–8.669; $P = 0.002$), and TLSs location (HR, 1.845; 95% CI, 1.085–3.138; $P = 0.024$) were associated with better OS period (Table 3). These results indicated that IgA-TLSs may predict a better treatment outcome and a longer survival prognosis for GADC patients.

Table 3
Univariate and multivariate analysis of prognostic markers for overall survival in GADC

	Univariate analysis			Multivariate analysis [#]		
	HR	p-value	95%CI	HR	p-value	95%CI
IgA-TLSs and nTLSs						
IgA ⁺ -TLSs <i>versus</i> nTLSs	2.065	<0.001*	1.38-3.091	2.004	0.003*	1.260-3.186
Age						
≤60 <i>versus</i> ≥60	0.747	0.416	0.371-1.506	—	—	—
Gender						
Male <i>versus</i> Female	1.457	0.333	0.68-3.122	—	—	—
Differentiation						
well-middle <i>versus</i> middle-poor	1.02	0.952	0.532-1.957	—	—	—
T stage						
T ₁ <i>versus</i> T ₂ <i>versus</i> T ₃ <i>versus</i> T ₄	0.706	0.102	0.465-1.072	—	—	—
M stage						
M0 <i>versus</i> M1	2.702	0.019*	1.181-6.182	0.338	0.146	0.078-1.458
AJCC TNM stage						
Ⅱ+Ⅲ <i>versus</i> Ⅳ	1.972	0.001*	1.316-2.955	2.651	<0.001*	1.614-4.356
chemotherapy effect						
CR+PR <i>versus</i> SD+PD	4.166	<0.001*	2.088-8.312	4.325	0.001*	1.867-10.021
TLSs location						
tumor center <i>versus</i> para-carcinoma <i>versus</i> T/P junction	1.57	0.044*	1.014-2.434	1.775	0.029*	1.060-2.973
PD-L1 expression						
≥1% <i>versus</i> <1%	1.693	0.114	0.882-3.25	—	—	—
SSR4 expression						

	Univariate analysis			Multivariate analysis [#]		
Negative <i>versus</i> Positive	1.832	0.068	0.955-3.513	—	—	—
[#] Significant factors in univariate analysis were used to construct model for multivariate analysis.* means $P < 0.05$.						

Discussion

In recent years, it has been suggested that the TLSs and the TIBs may regulate the efficacy of immunotherapy in several types of tumors, and may closely related to the prognosis of immunotherapy. Therefore, these possibilities open a new immunotherapy research direction. The roles of TIBs and TLSs in tumorigenesis and immunotherapy have been recognized by researchers, but the specific mechanisms of action are not fully understood.^[26, 27]

In our study, the results showed that a large number of B cells in GADC tumor tissues expressed CD79A/J-chain gene, and most of these cells were IgA+ plasma cells (high expression of IGHA1 and IGA2 genes). Through GO enrichment analysis, the study also found CD79A/J-chain B cells have complement activation, B cell actication and antibacterial humoral response. These results confirmed that CD79A/J-chain B cells are involved in mucosal and humoral immunity and were mainly IgA-B cells cluster.

Next, we determined the spatial structure of single cells through CSOmap, and found that T1 and T2 samples contained more cells and different types of cellular interactions and migration ability compared with cells in T3 and T4 tissues. Moreover, we observed increased expression of receptors and ligands associated with chemokines, and these data suggests that the immune cells in T1 and T2 samples may have a stronger ability to migrate. Denton et al., confirmed that CXCL13 can recruit immune cells to generate an environment permissive for germinal center formation in the lung.^[28]It supports our results that the TLSs contained in T1-2 tissues form germinal centers.

The germinal center is located in the follicular region of secondary lymphatic organs. It is a special structure formed by the aggregation of B cells in the humoral immune response, and it is also a transient and dynamic micro-anatomical structure.^[29]Antigen-specific B cells gather in this particular structure, and then they expand to produce somatic high frequency mutations, antibody type changes and eventually become high affinity B cells for specific antigens.^[30]

Through cluster analysis, CD79A/J-chain B cells expressed high levels of the IgA1, IgA2, and SSR4 gene. We continue to find that SSR4 plays a important role in the immune microenvironment through GEPIA and TIMER database analysis. IgA is the most abundant antibody produced in mammals. IgA produced in the gastrointestinal mucosa is secretory IgA (SIgA), which is mainly composed of dimers.^[31, 32]The two monomers are linked by a J-chain protein, forming an important immune protective layer on the mucosal surface. The J-chain is a glycoprotein with a molecular weight of approximately 15 kD and is

mainly synthesized by IgA or IgM plasma cells and connects two monomeric units of IgA or IgM.^[33] Although J-chain can polymerize IgA and IgM, IgM mainly exists in blood, and SIgA plays an important role in mucosal immunity of the body. Based on this, it is reasonable to speculate that CD79A/J-chain B cells play a role in mucosal immunity.

The SSR4 gene encodes the translocon-associated protein δ (TRAP δ) subunit. The TRAP complex comprises four transmembrane subunits (α , β , γ and δ), which exist in the ER and are involved in protein transport across the ER membrane. Studies suggest that the TRAP complex is involved in the regulation of humoral immunity by guiding the secretion and transport of immunoglobulins.^[34] It is reasonable to speculate that the immune cells with high expression of SSR4 may be related to the performance of humoral immunity in human body.

In order to obtain more information about TLSs, we continued to collect 24 cases of GADCs tumor samples with or without germinal centers. We compared the structural differences by multiple immunofluorescence and digital quantitative analysis techniques. And found that CD79A+B cells and SSR4-immune cells existed mainly in the core of germinal center in the TLSs. Moreover, The interaction between immune cells were involved in the TLSs with germinal centers. These results also support the analysis results of CSOmap prediction model.

B cells are a type of lymphocytes, which enters the peripheral lymphoid tissue after maturation of the fetal liver and bone marrow mature.^[35] Subsequently, B cells proliferate and mutate in germinal centers, differentiate into memory B cells and plasma cells, and secrete antibodies to induce humoral immunity and mediate cellular immunity. The germinal center is the site of clonal expansion and affinity maturation of B cells.^[30, 36] TIBs exists in some tumor tissues and are also an important part of TLSs. At present, there is still some controversy about the role of TIBs in anti-tumor immunity. Some studies showed that some immunosuppressive TIBs subtypes promoted tumor progression;^[37] Another study suggested that TIBs promotes tumor immunity and inhibits the growth of tumor cells by producing tumor-specific antibodies and presenting tumor antigens.^[38] The function of TIBs and the formation of TLSs will likely affect the response to immunotherapy in GADC patients.

To understand more about the role of TIBs and TLSs in GADC, we collected 165 tissue samples from 120 patients with GADC who developed TLSs, and each case included complete clinicopathological information, treatment and prognosis information. IHC analyzed results indicated that the formation of IgA-TLSs was correlated with age, differentiation, M stage, TNM stage, chemotherapy effect, PD-L1 expression, and SSR4 expression. Moreover, the presence of IgA-TLS in GADC patients was associated with better OS period.

In this study, we provided the newly insight about TLSs in GADCs and found SSR4 higher expressed in the IgA-TLSs, which may promote a better prognosis for GADC patients. Future studies will be required to confirm that the presence of SSR4 in the core of TLS is predictive of an IgA-immune response and promotes an improved prognosis in patients with GADC.

Declarations

Availability of data and material

All data included in this study are available upon request by contacting with the corresponding author.

Ethics approval and consent to participate

All sample collection was approved by the Human Research Ethic Committee of Bayannur Hospital and Nanjing First Hospital.

Authors' contributions

WZ supervised the research, designed experiments, and revise the manuscript. LJ and TW performed experiments and wrote the paper; HC modified the article and performed molecular experiments; XY, BW and XW helped write the manuscript and analysis data. WH and YZ contributed to clinical sample and data preparation. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by grants from National Natural Science Foundation of China (82060438), Natural Science Foundation of Inner Mongolia Autonomous Region (2020MS08002, 2020MS08060, 2020MS08149), Inner Mongolia Autonomous Region Science and Technology Plan Project (2020GG0273, 201802138), Doctoral Research Station of Bayannur (BYZ2019-1-2), Academician workstation(YSZ2018-2).

Acknowledgments

We wish to thank LC-Bio for its single-cell sequencing technology services. We thank Yong Wang (Institute of Applied Mathematics, Academy) of Mathematics and Systems Science, Chinese Academy of Sciences) for their technical discussions.

Competing interests

The author(s) declare no competing financial interests

Consent for publication

Not applicable

References

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries[J]. CA Cancer J Clin, 2021.

2. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries[J]. *CA Cancer J Clin*. 2018;68(6):394–424.
3. Sathe A, Grimes SM, Lau BT, et al. Single-Cell Genomic Characterization Reveals the Cellular Reprogramming of the Gastric Tumor Microenvironment[J]. *Clin Cancer Res*. 2020;26(11):2640–53.
4. Dangaj D, Bruand M, Grimm AJ, et al. Cooperation between Constitutive and Inducible Chemokines Enables T Cell Engraftment and Immune Attack in Solid Tumors[J]. *Cancer Cell*. 2019;35(6):885–900.
5. Nie S, Yuan Y. The Role of Gastric Mucosal Immunity in Gastric Diseases[J]. *J Immunol Res*, 2020,2020:7927054.
6. Geri JB, Oakley JV, Reyes-Robles T, et al. Microenvironment mapping via Dexter energy transfer on immune cells[J]. *Science*. 2020;367(6482):1091–7.
7. Yamamoto M, Rennert P, McGhee JR, et al. Alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the gastrointestinal tract[J]. *J Immunol*. 2000;164(10):5184–91.
8. Li X, Wenes M, Romero P, et al. Navigating metabolic pathways to enhance antitumour immunity and immunotherapy[J]. *Nat Rev Clin Oncol*. 2019;16(7):425–41.
9. Wang SS, Liu W, Ly D, et al. Tumor-infiltrating B cells: their role and application in anti-tumor immunity in lung cancer[J]. *Cell Mol Immunol*. 2019;16(1):6–18.
10. Kataoka K, Shiraishi Y, Takeda Y, et al. Aberrant PD-L1 expression through 3'-UTR disruption in multiple cancers[J]. *Nature*. 2016;534(7607):402–6.
11. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer[J]. *N Engl J Med*. 2012;366(26):2455–65.
12. Zhao W, Jia L, Zhang M, et al. The killing effect of novel bi-specific Trop2/PD-L1 CAR-T cell targeted gastric cancer[J]. *Am J Cancer Res*. 2019;9(8):1846–56.
13. June CH, O'Connor RS, Kawalekar OU, et al. CAR T cell immunotherapy for human cancer[J]. *Science*. 2018;359(6382):1361–5.
14. Kim SR, Shin K, Park JM, et al. Clinical Significance of CLDN18.2 Expression in Metastatic Diffuse-Type Gastric Cancer[J]. *J Gastric Cancer*. 2020;20(4):408–20.
15. Cabrita R, Lauss M, Sanna A, et al. Tertiary lymphoid structures improve immunotherapy and survival in melanoma[J]. *Nature*. 2020;577(7791):561–5.
16. Tang H, Zhu M, Qiao J, et al. Lymphotoxin signalling in tertiary lymphoid structures and immunotherapy[J]. *Cell Mol Immunol*. 2017;14(10):809–18.
17. Marin ND, Dunlap MD, Kaushal D, et al. Friend or Foe: The Protective and Pathological Roles of Inducible Bronchus-Associated Lymphoid Tissue in Pulmonary Diseases[J]. *J Immunol*. 2019;202(9):2519–26.

18. Helmink BA, Reddy SM, Gao J, et al. B cells and tertiary lymphoid structures promote immunotherapy response[J]. *Nature*. 2020;577(7791):549–55.
19. Jia L, Wang T, Zhao Y, et al. Single-cell profiling of infiltrating B cells and tertiary lymphoid structures in the TME of gastric adenocarcinomas[J]. *Oncoimmunology*. 2021;10(1):1969767.
20. Seeruttun SR, Yuan S, Qiu H, et al. A comprehensive analysis comparing the eighth AJCC gastric cancer pathological classification to the seventh, sixth, and fifth editions[J]. *Cancer Med*. 2017;6(12):2804–13.
21. Luecken MD, Theis FJ. Current best practices in single-cell RNA-seq analysis: a tutorial[J]. *Mol Syst Biol*. 2019;15(6):e8746.
22. Lambrechts D, Wauters E, Boeckx B, et al. Phenotype molding of stromal cells in the lung tumor microenvironment[J]. *Nat Med*. 2018;24(8):1277–89.
23. Combes F, Loux V, Vandenbrouck Y. GO Enrichment Analysis for Differential Proteomics Using ProteoRE[J]. *Methods Mol Biol*. 2021;2361:179–96.
24. Ren X, Zhong G, Zhang Q, et al. Author Corrections: Reconstruction of cell spatial organization from single-cell RNA sequencing data based on ligand-receptor mediated self-assembly[J]. *Cell Res*, 2021.
25. Ramilowski JA, Goldberg T, Harshbarger J, et al. A draft network of ligand-receptor-mediated multicellular signalling in human[J]. *Nat Commun*. 2015;6:7866.
26. Xiao X, Lao XM, Chen MM, et al. PD-1hi Identifies a Novel Regulatory B-cell Population in Human Hepatoma That Promotes Disease Progression[J]. *Cancer Discov*. 2016;6(5):546–59.
27. Lu Y, Zhao Q, Liao JY, et al. Complement Signals Determine Opposite Effects of B Cells in Chemotherapy-Induced Immunity[J]. *Cell*. 2020;180(6):1081–97.
28. Denton AE, Innocentin S, Carr EJ, et al. Type I interferon induces CXCL13 to support ectopic germinal center formation[J]. *J Exp Med*. 2019;216(3):621–37.
29. Wang Y, Shi J, Yan J, et al. Germinal-center development of memory B cells driven by IL-9 from follicular helper T cells[J]. *Nat Immunol*. 2017;18(8):921–30.
30. Mayer CT, Gazumyan A, Kara EE, et al. The microanatomic segregation of selection by apoptosis in the germinal center[J]. *Science*, 2017,358(6360).
31. Tortajada A, Gutierrez E, Pickering MC, et al. The role of complement in IgA nephropathy[J]. *Mol Immunol*. 2019;114:123–32.
32. Tezuka H, Abe Y, Iwata M, et al. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells[J]. *Nature*. 2007;448(7156):929–33.
33. Xiong E, Li Y, Min Q, et al. MZB1 promotes the secretion of J-chain-containing dimeric IgA and is critical for the suppression of gut inflammation[J]. *Proc Natl Acad Sci U S A*. 2019;116(27):13480–9.
34. Losfeld ME, Ng BG, Kircher M, et al. A new congenital disorder of glycosylation caused by a mutation in SSR4, the signal sequence receptor 4 protein of the TRAP complex[J]. *Hum Mol Genet*. 2014;23(6):1602–5.

35. Sabatino JJ, Probstel AK, Zamvil SS. Publisher Correction: B cells in autoimmune and neurodegenerative central nervous system diseases[J]. *Nat Rev Neurosci*. 2020;21(1):56.
36. Petitprez F, de Reynies A, Keung EZ, et al. B cells are associated with survival and immunotherapy response in sarcoma[J]. *Nature*. 2020;577(7791):556–60.
37. Xiao X, Lao XM, Chen MM, et al. PD-1^{hi} Identifies a Novel Regulatory B-cell Population in Human Hepatoma That Promotes Disease Progression[J]. *Cancer Discov*. 2016;6(5):546–59.
38. Lu Y, Zhao Q, Liao JY, et al. Complement Signals Determine Opposite Effects of B Cells in Chemotherapy-Induced Immunity[J]. *Cell*. 2020;180(6):1081–97.

Figures

expression in CD79A/J chain-B cell cluster through GO enrichment analysis. The Rich Factor represents the number of differential genes/the total number of genes. (F)Parts of whole showing the dominant ligand-receptor pairs contributing to distribution of single cells in tumor tissue through CSOmap analysis ($P < 0.01$).

Figure 2

Correlation between SSR4 and immune cells in GADC (A) Ridge plot of the expression level of IgA1, IgA2 and SSR4 genes in CD79A/J chain-B cell cluster. (B) Histogram of SSR4 expression level grouped by tissue (up) and cell type (down) though GEPIA database.

Figure 3

Different structures and densities of tumor associated TLSSs (A-B) Representative multiple immunofluorescence assay images of CD4, CD8, CD14, CD79A and SSR4 in tertiary lymphatic structures

with germinal center (A) or not (B). 200×magnification. The blue circles represent the manually delineated HALO spatial analysis areas. (C-D) Digital image analysis of the density of TLSs with germinal center (C) or not (D) by HALO Spatial Analysis; Ordinate: number of positive cells; Abscissa: 0 indicates the analysis boundary, negative value indicates within the delineated region, and positive value indicates outside the delineated region.

Figure 4

Representative images of CD79A, SSR4, J-chain and PD-L1 in the IgA-TLSs and the normal TLSs of GADC IgA-TLSs and nTLSs with positive CD79A expression. IgA-TLSs with positive SSR4 expression and nTLSs with negative SSR4 expression. IgA-TLSs with high J-chain expression and nTLSs with low or no J-chain expression. IgA-TLSs with negative PD-L1 expression and nTLSs with positive PD-L1 expression. Line a is staining with $\times 20$ (bar = 500 μm), line b is staining with $\times 200$ (bar = 50 μm).

Figure 5

Survival curves for GADC patients with the IgA-TLS or the nTLSs using the Kaplan-Meier method and the log-rank test Overall survival curves for patients with the IgA-TLS (green line) or the nTLSs (blue line); Log Rank = 13.604, $P < 0.001$.