Single-cell sequencing reveal Renin-Angiotensin-System regulator patterns guide intercellular communication of tumor microenvironment that contribute to gastric cancer progression and immunotherapy

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Research Article

Keywords: gastric cancer, renin-angiotensin system, Tumor microenvironment, prognosis

Posted Date: June 26th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3067874/v1

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Abstract

Background

The renin-angiotensin system (RAS), which is involved in this process, is well-known for its function in blood pressure regulation. In addition to a systemic RAS, the preponderance of target organs have a local RAS. Consequently, RAS hormones and receptors are expressed variably in various types of cancer, the heart, blood vessels, and kidneys. RASi therapy has recently demonstrated promise as a cancer treatment, despite a number of obvious adverse effects, such as hypotension. Consequently, it is essential to perceive how RAS functioned within the tumor microenvironment.

Methods

Single-cell RNA-seq data were acquired from gastric cancer (GC) tumor tissues, and nonnegative matrix factorization (NMF) was used to identify 16 RAS regulators. We evaluated the prognosis and immunological response of TME clusters using GC and Immunotherapy cohorts retrieved from a public repository.

Results

For each cell type (fibroblasts, myeloid cells, T cells, endothelial cells, and mast cells), two or three subclusters were identified based on similar biological processes and marker genes. A connection was discovered between RAS regulatory elements and the clinical and biological aspects of GC, and the pseudotime trajectory of the main TME cell types was also identified. The results of bulk sequencing indicate that these RAS-related TME cell subgroups have a significant immunological response in patients undergoing ICB therapy, especially in CAFs and Tregs, and have a high prognostic value for GC patients. Among the associations uncovered by CellChat's research was the fact that certain TME cell subgroups were associated with RAS. Further investigation revealed that MIF-(CD74 + CXCR4) and MIF-(CD74 + CD44) ligand receptors play a role in RAS-related subgroups' communication with TME cells.

Conclusion

Our research uncovered a previously unknown RAS pathway in the microenvironment of gastric cancer. This route has implications for both the progression of the disease and immunotherapy.

Background

The Renin-Angiotensin System (RAS) is commonly targeted in the treatment of cardiovascular problems, but RASi also have tremendous potential in the treatment of cancer. Directly and indirectly, the RAS affects the microenvironment of cancer cells, making it an essential player in the pathophysiology of the
RAS activation primarily aids in the maintenance of blood pressure and cardiac output through vasoconstriction and ventricular hypertrophy. Activation of the receptor activator of kinases (RAS) has been shown to benefit cardiovascular health\(^7\,^8\). When it comes to this complex system of bioactive peptides that communicate via multiple receptors, angiotensin II (AngII) is the key effector responsible for maintaining tissue homeostasis by exerting regulatory and counterregulatory functions\(^5\). The many receptors that Angiotensin II (AngII) has allow it to regulate and counter-regulate several physiological processes. Angiotensinogen (AGT) is hydrolyzed by renin (REN), which is made by juxtaglomerular cells in the kidney\(^3\). In this process, angiotensin I (AngI) is created. The majority of AngI is made by endothelial cells in the pulmonary vasculature, and is then processed by ACE to create the physiologically active AngII. Angiotensin III, IV, 1–7, and 1–9, as well as angiotensin A and alamandine, have all been shortened to reveal novel bioactive peptides\(^9\,^10\). Seven-transmembrane receptors AT1R and AT2R modulate the effects of AngA. In contrast to Ang(1–7), which primarily works via the MAS receptor (MASR), alamandine interacts with and transmits signals via the MAS-related G protein-coupled receptor D (MRGD)\(^11\). AngIV has a binding action that causes it to bind to insulin-regulated aminopeptidase (IRAP; also known as AT4R and LNPEP). There are many different RAS proteins. Some examples include THOP1, MAS1, CMA1, Chymase 1, CPA3, Carboxypeptidase A3, MAS2 (Mas-Related G Protein-Coupled Receptor 2), CTSG (Cathepsin G), and MAS2\(^7\,^12\,^13\).

Most target organs, in contrast to the systemic RAS, have a local RAS that primarily regulates cellular-level processes like proliferation, growth, and metabolism\(^3\). This is observed in several of the intended tissues. The local RAS cooperates with the global RAS even though it functions autonomously. The differential expression of RAS-related genes (RRGs), which have been implicated in the development and progression of cancer, renal disease, and cardiovascular disease. Through its interactions with a variety of receptors, the primary RAS effector angiotensin II (AngII) controls the progression of tissue fibrosis and inflammation. In contrast to angiotensin-(II), angiotensin-(1–7) (Ang(1–7)) is hypothesized to prevent tissue fibrosis and inflammation. It has been found that the ratio of AngII to Ang-(1–7) is a crucial factor in the onset and course of the disease\(^14\). RAS has also been linked to the development and spread of cancer. Increased expression of the angiotensin II receptor type 1 (AT1R) is linked to advanced cancer stages and a poorer prognosis in studies of cancer biology\(^15\). In contrast to the AngII/AT2R and Ang-(1–7)/MAS signaling pathways, which are both expected to inhibit tumor growth, the AngII/AT1R axis is thought to promote tumor growth. Angiogenesis in solid tumors may be stimulated by AngII/AT1R signaling. Microvessel density (MVD) and VEGF/VEGFR expression have both been associated to AT1R expression in various human malignancies\(^3\,^16\).

Heart disease treatment frequently involves renin-angiotensin system inhibitor (RASi) medication. RASi medications include, but are not limited to, angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARB), throughout the mid-1990s\(^17\), captopril was found to be the first orally active angiotensin-converting enzyme inhibitor (ACEi), and throughout the twenty-first century, losartan was found to be the first orally active selective angiotensin receptor blocker\(^18\). Since then,
numerous ACEis and ARBs have been developed and are now commonly used to treat a wide range of conditions, including the most common ones such arterial hypertension, heart failure, myocardial infarction, and chronic kidney disease.

The tremendous effectiveness of ACEi and ARB in cancer, especially in tumor immunity, is the outcome of more than two decades of use outside of cancer. Earlier research suggested that extensive fibrosis in tumor tissue could serve as a physical barrier. This barrier prevents T cells from entering tumor tissue, which results in constricted blood arteries and reduced blood flow. This creates an acidic and oxygen-depleted environment, both of which are detrimental to the proper function of immune cells. Immune cells' ability to specifically target tumor cells is diminished, and the production of chemicals that inhibit immunological checkpoints, including programmed death-ligand 1 (PD-L1), is increased, all thanks to the tumor microenvironment. In patients with solid tumors, RASi has been proven to significantly reduce tissue fibrosis. Studies have demonstrated that the therapeutic benefits of anticancer drugs and nanotherapeutic agents can be enhanced by the addition of losartan by increasing blood flow, decreasing hypoxia in tumors, and enhancing distribution. Thomas.S. claims that ACEi or ARB can lessen the severity of liver metastatic sclerosis by preventing the contraction of fibroblasts and the deposition of extracellular matrix. However, anti-angiogenic drugs may be less effective when used with RASi therapy. It has been shown that the use of bevacizumab in conjunction with RASi increases patient survival. The results suggest that RAS has a role in tumor development and therapy, and that decreasing RAS activity can increase patient survival and the success of immunotherapy. However, RASi therapy is not widely used for the treatment of tumors due to its considerable side effects, such as chronic hypotension.

Gastric cancer (GC) is one of the most common malignancies worldwide, with a new case being identified every minute of every day. Late detection contributes significantly to the high fatality rate associated with gastric cancer, which is the third most frequent cancer worldwide. It's unfathomable that GC caused 784,000 deaths in a single year. The effects of GC have been felt most keenly in East Asia, Eastern Europe, and South America. More males than women have been diagnosed with GC in the previous 100 years. Recent data show a downward trend in both the overall rate of GC and the total number of deaths owing to the disease. Population decline in industrialized countries has led to a decline in the overall number of persons with GC, but the percentage of those with GC who seek medical attention has remained stubbornly high. More cases of GC are expected to be identified and treated by medical professionals in the near future. More research into the disease's molecular mechanisms is needed, as evidenced by the fact that GC is being diagnosed at younger and younger ages, according to epidemiological statistics from a variety of high-income nations. Immune checkpoint blockade (ICB), and more specifically PD-1/L1 and CTLA-4 inhibition, has shown extraordinary therapeutic success in a subset of patients with solid malignancies. Those who have GC and whose tumors have progressed despite first- or second-line treatment are given immune checkpoint inhibitors. Nivolumab, an anti-programmed death-1 (PD-1) monoclonal antibody, helped Asian patients in the randomized phase III ATTRACTION-2 trial more than a placebo plus best supportive treatment. Patients with GC who had not responded to chemotherapy were given pembrolizumab in the nonrandomized phase II KEYNOTE-059 study, and overall survival rates were
similar between the two treatment groups. Immunotherapy has shown negligible to no benefit in clinical trials for the vast majority of people with GC. It is not advised to use immune checkpoint inhibitors as a primary treatment for this condition. Understanding the mechanisms that lead to a decline in tumor immune responses and modifications to the tumor microenvironment is crucial for enhancing immunotherapy's effectiveness. It would be interesting and useful to have a better understanding of how different subsets of cells connected to RAS affect and predict the success of immunotherapy. More research is needed into this matter. If we can answer these issues, we can alter the standard approach to treating GC and create RASi with less side effects.

This work examines the expression patterns of RAS-related genes in critical tumor microenvironment (TME) cells like stromal cells, myeloid cells, T cells, mast cells, and endothelial cells using single-cell RNA sequencing data from patients with gastric cancer. Data was collected from patients who have undergone therapy for stomach cancer. Twenty-three RAS-related genes were also analyzed using non-negative matrix factorization (NMF). The RNA sequencing data from TCGA stomach cancer cases were analyzed using the NMF approach. Different cell subpopulations in the tumor microenvironment (TME) of gastric cancer patients have variable RAS-related gene expression. This results in a dynamic range of immunological, metabolic, transcriptional regulatory, interpersonal, and prognostic responses. Our innovative strategy incorporates both single-cell and large-scale analyses. The study's major goal is to ascertain whether or not local RAS has a role in the development of GC.

**Materials and Methods**

**Dataset Acquisition and Tissue Specimens**

The gastric cancer RNA sequencing and clinical data were entered into the Cancer Genome Atlas (TCGA) database (https://portal.gdc.com). The Gene Expression Omnibus (GEO) database was used to acquire single-cell mRNA sequencing (scRNA-seq) data from 12 gastric cancer patient samples, including 8 tumor tissues and 4 surrounding normal tissues. We constructed phenotypic and gene expression matrices for 16,087 scRNA-seq datasets by analyzing 16 RRGs expression patterns and integrating initial samples. Both the Cancer Genome Atlas (TCGA) and the GEO database contain datasets of gastric cancer samples containing a substantial amount of mRNA sequencing or microarray data. The data used in this investigation can be obtained without cost from published works or the public domain.

**Gene Collection and RNA Sequencing Analysis**

The RAS gene set was obtained from GSEA (https://www.gseamsgdb.org/gsea/msigdb/human/geneset/KEGG RENIN ANGIOTENSIN SYSTEM). The 'limma' utility in the R program was used to examine the expression pattern and fold change of these genes in gastric tumor and normal tissues using the TCGA dataset. Adjustments were made to P values to accommodate for false positive results. The criteria for screening were "adjusted P0.05 and Log (fold change) > 1 for up-regulation and Log (fold change) -1 for down-regulation." These gene signature scores
were calculated with the GSVA instrument. GSVA RAS_score (GSVA_score_UP - GSVA_score DN) was calculated using the RAS gene set analysis results (GSVA_score_UP - GSVA_score DN).

Non-negative Matrix Factorization of RRGs in TCGA Database and TME Cells

To investigate the potential function of RAGs in GC, the TCGA patients were divided into three groups using the non-negative matrix factorization algorithm (NMF, R package version 0.20.6). The "PCA" application was utilized to conclude the major component analysis. Using Kaplan-Meier analysis, the overall survival (OS) durations of the three subgroups were compared. To best assess the impact of RRGs expression on TME cell types, the non-negative matrix factorization algorithm (NMF R package, version 0.20.6) was used to conduct a dimension reduction analysis for 16 RRGs across all TME cell types using scRNA-seq. Multiple cell subtypes have been identified for each of these cell types based on the expression matrix of scRNA. The order of these steps was the same as in previous investigations. All these steps were performed in a manner similar to the previous studies\textsuperscript{29,30}.

Identification of the Marker Genes of RAS-related Cell Subtypes in TME Cells

FindAllMarkers was utilized to identify the markers for each NMF cluster and cell type. The corrected p value of 0.05 was used to screen genes for future analysis, while min.pct and logfc.threshold values were set to 0.15. The Dotplot program was utilized to illustrate the highest levels of gene expression in each NMF cluster. Using the AddModuleScore function and differentially expressed genes (DEGs), signature scores were computed for these NMF cell clusters. Utilizing the FeaturePlot function of the GC TME, the distribution of specific NMF cluster score signatures was illustrated. Additional File 1 comprises a list of the unique gene sets used to compare clusters associated with RAS.

Functional Enrichment Analysis SCENIC Analysis for NMF RAS-related Subcelltypes

Using the clusterProfiler R package, NMF clusters in distinct TME cell types were sought among Kyoto Encyclopedia of Genes and Genomes (KEGG) marker genes. The transcription factor (TF) gene regulation network in GC was investigated using the SCENIC program. The transcription start site (TSS) and gene regulatory networks were identified using two gene-motif rankings from the RcisTarget database (hg19-tss-centered-10 kb and hg19-500 bp-upstream) in the GC scRNA-seq data. TFs with Benjamini-Hochberg false discovery rates (BH-FDR) of 0.05 were considered for the investigation that follows.

Cell–Cell Communication and Metabolism Analysis

Cellchat, a previously described R package containing human and mouse ligand-receptor interaction datasets, can be used to investigate intercellular communication networks derived from scRNA-seq data categorized by distinct cell clusters\textsuperscript{31}. Using SCmetabolism assessed the activity of cellular metabolic
pathways. Using the VISION method, the metabolic activity of various subtypes of epithelial cells was evaluated.

**Analyses with RAS-related Subcelltype Signatures in Public Bulk RNA-sequence Datasets**

The FindAllmarker function of the Seurat R package was used to construct RAS-related gene signatures for each NMF cell cluster. Using scRNA data, the main cell type of the GC TME was also determined. The gene signature scores in the GC public datasets were then calculated utilizing the GSVA method. Using the R software package 'limma' and the TCGA and ACRG datasets, it was also investigated how RAS-related NMF signatures were expressed in stomach tumor and normal tissues. The log-rank test and Cox proportional hazard regression were utilized to investigate the association between RAS-related NMF signatures and patient prognosis, including overall survival (OS) rate. Using the survminer R utility, which was used to generate Kaplan-Meier curves, the cutoff values for various NMF cell signatures in available datasets were determined.

**Protein-peptide docking**

Chimear was utilized to generate 3D models of PLL and ICG, while Autodock 4.2 was utilized for protein-peptide docking. Before protein and peptide coupling, polar hydrogen atoms were added and the Gasteiger method was used to correct charge. All other docking parameters were left at their default settings. The parameters of the docking box were modified to encompass the protein wholly, with the protein as the focal point.

**Immunohistochemical Staining**

Anti-AGT antibody (1:150, Protentech, 68020-1-Ig AGT monoclonal antibody) and secondary antibody (ZSGB-BIO) were incubated overnight at 4°C on the samples. Utilizing the landlord and landlord analysis modules of the visiopharm software, the HDAB-DAB filter was applied, the area of interest (ROI) was segmented based on staining intensity, and the corresponding labels (0–75 strong positive; 76–120; 121–160 weakly positive; 161–212 negative) were assigned. In order to achieve semi-quantitative tissue staining, histochemical scoring, a histological scoring technique for immunohistochemistry, converts the number of positive cells and intensity of staining in each slice into corresponding numerical values. H-Score (H-SCORE=(pi)= (percentage of feeble intensity1)+(percentage of moderate intensity2)+ (percentage of strong intensity3), where Pi represents the proportion of pixels with a positive signal and I represents the color intensity.

**Collection of immunotherapy transcriptomic**

4 Immune immunotherapeutic cohorts with TPM or FPKM transcriptomic were collected from the public datasets, included 2 melanoma datasets GSE91061\textsuperscript{32} and GSE78820\textsuperscript{33}, and 2 non-melanoma cohorts (IMvigor210 (2018, anti-PDL1, Urothelial Cance\textsuperscript{34}); Braun et al.( 2020, anti-PD1,CCRCC\textsuperscript{35})). All the patients had the immune response in these cohorts.
Statistical Analysis

Data analysis and statistics were performed using R (version 4.1.3; R Foundation for Statistical Computing). The Student’s t-test, Wilcoxon rank-sum test, Kruskal-Wallis test, and Chi-square test were used to examine continuous target or category variables in these cell subgroups. The correlation between TME cell signatures or gene expressions was demonstrated by Pearson correlation analysis. The ComplexHeatmap and pheatmap programs displayed variable-scale target data expressions in NMF clusters derived from TME GC cell types. In standard statistical analyses using R 4.0 software, a two-sided p-value of less than 0.05 was regarded as statistically significant.

Results

The landscape of RAS regulators in TME cells in GC

As stated previously, the GC scRNA-seq dataset was utilized to investigate the landscape of RAS regulatory factors in TME cells of gastric cancer (Figs. 1A,B). This action was taken to better comprehend the situation. Using graph-based uniform manifold approximation and projection (UMAP), we separated 16,087 high-quality TME cells from 12 samples obtained from four colon cancer patients into 11 significant clusters. Using the expression of identified marker genes, we then annotated these clusters for the main cell types. Following preprocessing, integration, and single-cell sequencing of the GSE167297 data set, principal component analysis (PCA) was applied. This outcome was accomplished. Figures 1F and G depict histograms with layers. This histogram illustrates the distribution of various cell types throughout the total population. Normal stomach tissues contained significantly fewer myeloid cells and T cells, particularly CD4 + T cells, than tumor tissues (Fig. 1G). The discovery that the number of cell subtypes in various samples varies significantly lends support to the notion that stomach cancer is a highly diverse form of the disease. We utilized Seurat’s AddModuleScore instrument to determine the typical RNA expression of RRGs in TME cells from patients with gastric cancer. (Fig. 1H,I) Eleven distinct types of GC cells expressed RAS regulatory factors at substantially different levels. Consequently, we decided to concentrate our research on fibroblasts, endothelial cells, regulatory T cells, macrophages, and myeloid cells by analyzing the expression profiles and biological characteristics of RAS regulatory factors in various cell types.

Novel RAS-related fibroblasts contributed to the TME of GC

A significant proportion of the disease-induced microenvironment of gastric cancer is composed of fibroblasts, which are a primary contributor to AGT. Using the limma package, we determined that the RNA expression levels of AGT, CTSA, NLN, and THOP1 were substantially higher in tumor tissues than in adjacent normal tissues (Fig. 2A). In contrast, CMA1, CTSG, AGTR1, CPA3, and ACE2 had lower RNA expression levels. As shown in Fig. 2B, the expression of RAS regulatory factors such as AGT, AGTR1, CMA1, CPA3, and ENPEP affected the prognosis of gastric cancer patients. The evidence indicated that
this was the case. In cases of gastric cancer, abnormally high AGT expression levels indicate
dysregulation of RAS in the tumor microenvironment. Prior research has demonstrated that certain
medications, including ACE, CMA1, and CTSG, are responsible for the conversion of AGT to AngII. AGTR1,
the most important AngII receptor, promotes inflammation and fibrosis by means of a pathway leading to
its downstream effect. In conclusion, our research suggests that gastric cancer may produce a significant
quantity of AngII and that RAS regulation may be abnormal in this condition. Figure 2C depicts how data
from the TCGA Tumor Match, TCGA Normal, and GTEx indicate that AGT expression levels were
substantially elevated in patients with gastric cancer. The immunohistochemistry labeling of gastric
cancer patient tissue samples and the sequencing results derived by the TCGA were consistent (Fig. 2D,
E). Using NMF to evaluate single-cell data, we identified two clusters of RAS-associated fibroblasts. The
remaining fibroblasts were designated Non-RAS-CAF-C1. fibroblasts are the primary source of AGT in
gastric cancer, and the aberrant activation of AGT-fibroblasts contributes to the dysregulation of RAS in
the tumor microenvironment, according to our research. Glutamyl aminopeptidase is an extracellular zinc-
binding domain-containing type II integral membrane protein. The glutamyl aminopeptidase gene
encodes for this specific protein. By cleaving the N-terminal aspartic acid of AngII to form AngIII, this
protein regulates angiogenesis and stimulates tumor growth. It is probable that the ENPEP + CAF
subpopulation is the population that secretes AngIII to promote tumor growth and modulate angiogenesis
in the GC TME. Moreover, pseudotime analysis revealed that AGT expression occurred during the middle
of fibroblast development, whereas ENPEP expression occurred later (Fig. 2I). scMetabolism was used to
analyze the metabolic changes that occurred in RAS-associated subpopulations, and 30 of the most
significant metabolic changes were chosen for visualization (Fig. 2J). Several metabolic pathways were
significantly enriched in the ENPEP + CAF subgroup, while the AGT + CAF subgroup substantially enriched
sucrose and lipid metabolism pathways. On the basis of prior research, we also evaluated Pan-CAF
characteristics, such as collagen synthesis, extracellular matrix (ECM), matrix metalloproteinases
(MMPs), transforming growth factor beta (TGFb), Neo-angio, contractile, and RAS pathways (Fig. 2K). In
the AGT + CAF subgroup, the proliferative CAF (pan-dCAF) is associated with increased expression of
MMP family and collagen genes (DCN, LUM, and TAGLN). It was discovered that the ENPEP + CAF
subgroup expressed a large number of inflammatory factor genes associated with pan-inflammatory CAF.
As illustrated by the pathway heatmaps (Figs. 2L and M), genes associated with the Kars pathway are
strongly associated with AGT + CAF, whereas genes associated with the NOTCH pathway are strongly
associated with ENPEP + CAF.

ANPEP promotes tumor immune evasion in GC through
myeloid cell

Monocytes, macrophages, and dendritic cells were further separated into myeloid cell subpopulations by
assessing the expression of known marker genes in each myeloid cell subpopulation (Figs. 3A,C). We
found six different RAS-myeloid subgroups expressing RAS-regulatory factors using the NMF approach
(Fig. 3D,G): ANPEP + MAC-C2, CTSA + MAC-C3, ANPEP + DC-C2, CTSA + DC-C1, and THOP1 + DC-3.
Furthermore, scMetabolism was used to assess the metabolic profiles of the various RAS-related
subgroups, and the 30 pathways with the highest expression level differences were displayed (Figs. 2E and 2H). The metabolic pathway activation levels in the ANPEP + DC-C2 and ANPEP + MAC-C2 groups were considerably greater than in the other groups. We discovered a collection of genes implicated in cytotoxicity and exhaustion in CD8 + T lymphocytes and used Seurat’s AddModuleScore tool to examine the average RNA expression of these genes in RAS-related myeloid cells. The expression of genes associated with fatigue rose significantly in both the ANPEP + MAC and ANPEP + DC groups. Angiogenesis, tumor growth, and metastasis have all been linked to mutations in the ANPEP gene, demonstrating the gene's importance in these processes. Furthermore, these alterations have been linked to certain types of leukemia and lymphoma. Our data suggest that it may have an influence on tumor formation via pathways related with CD8 + T cell depletion. The expression levels of 26 immunological checkpoint genes varied statistically substantially between myeloid subgroups differentiated by RAS status, according to the Kruskal-Wallis test (Figs. 2J and 2L). Using the Python pySCENIC module, we compared the average AUC of three different macrophage groups. The resulting heat map demonstrated that the TF's activity varied significantly. The level of activity of the TFs was assessed using AUCcell. The gene regulatory network analysis revealed that the expression of 16 transcription factors (TFs) differed considerably between the three groups. Figure 2K shows that the transcription factors ETS2, PRDM1, REL, and NFKB1 were significantly greater in the ANPEP + MAC-C2 cluster. Using pseudotime analysis, we discovered that ANPEP was expressed earlier in the development of myeloid cells than CSTA (Fig. 4M).

**NMF clustering of RAS-associated Mast/Treg/EC in GC**

Previous has linked the presence of regulatory T cells (Tregs), mast cells, and endothelial cells (ECs) to the local RAS of gastric cancer. The subsequent sections analyze these three cell types in depth. Successful tumor angiogenesis requires ECs. Figure 4A identifies four RAS-related EC subgroups in the microenvironment of gastric cancer (ACE + ENDO-C1, LNPEP + ENDO-C3, CTSA + ENDO-C4, and one non-RAS-ENDO-C2). Angiotensin-converting enzyme (ACE) is a protein that catalyzes the conversion of inactive angiotensin I to the physiologically active form, angiotensin II. By constricting blood vessels and stimulating aldosterone, Angiotensin II regulates blood pressure and fluid-electrolyte balance. Additionally, it inhibits the production of vasodilator kinins. ACE inhibitors (ACEi) can alleviate hypertension resulting from the body’s natural production of active peptides. The LNPEP gene encodes a protein that can degrade numerous peptide hormones, including proenkephalin A, oxytocin, vasodilator-stimulated phosphoprotein, enkephalin, and antidiuretic hormone. This AT4 receptor protein catalyzes the angiogenic tensinogen to angiogenic tensin IV (AT4) conversion. Analysis of the gene regulatory network (Fig. 4B) revealed that 15 transcription factors (TFs) were substantially more highly expressed in the ACE + ENDO-C1 subgroup. Figure 4F depicts the expression profiles of ACE, CTSA, and LNPEP and demonstrates that all three genes are expressed in mature ECs. Specifically, the LNPEP-positive EC subgroup may have a higher level of activity in the FGFR1 growth factor-related pathway (Fig. 4C), indicating that ACE and LNPEP play a role in the synthesis of angiotensin II and IV, respectively.

Figure 4E demonstrates that CTSG + MAST-C1 mast cells, CTSG + CMA1 + MAST-C1 mast cells, but not CPA3 + MAST-C4 mast cells, expressed RAS regulatory factors. The enzyme that converts angiotensin I to
the vasoactive peptide angiotensin II is a member of the S1 family of peptidases and is produced by the CMA1 gene. Graph F demonstrates that there were significant disparities in TF activity between the four subtypes of mast cells. The gene regulatory network analysis revealed that the expression of 15 TFs varied significantly between the four groups. Next, we utilized pseudo-time analysis to examine RRG expression levels in RAS-associated mast cells over time. Figure 4G demonstrates that CTSG and CMA1 growth followed CPA3. Mast cells in the tumor microenvironment appear to be implicated in AngII production, based on our observations.

Identical methods were utilized to determine whether or not distinct Treg subtypes played a function in the local RAS regulation of the gastric cancer tumor microenvironment (TME). Non-RAS-Treg-C1 is one of the four fundamental RAS Treg subtypes that does not express RAS regulatory elements (Fig. 4I), whereas the other three do (CSTA-Treg-C2, LNPEP-Treg-C3, and THOP1-Treg-C4). 13 transcription factors are statistically significantly overexpressed in the THOP1-Treg-C4 subgroup, according to pySCENIC data (Fig. 4J). Figure 4M depicts the expression of 26 immunological checkpoint genes in Treg subgroups associated with RAS. THOP1 is the gene that codes for zinc-dependent kinase. This gene encodes a protein with the function of inhibiting the adhesion of cytoplasmic peptides to antigen-presenting cells. This protein degrades angiotensin I to create shorter neuropeptides (20 AA) and angiotensins (1–7). The binding mechanism between THOP1 and angiotensin I was investigated using molecular docking studies (Fig. 4K). The docking investigation revealed an energetic interaction between THOP1 and the polypeptide AngI. Important for binding are the interactions between the histidine and arginine residues of AngI and the proline and lysine residues of THOP1. The optimal role of THOP1 in RAS regulation and the molecular binding conformation of the AngI-THOP1 complex were confirmed by our results.

The Communication Patterns and Prognostic Typing Mediated by RAS

We have constructed a map of the regulatory landscape of RAS in the primary cell types that constitute the tumor microenvironment (TME) of patients with gastric cancer using the previously discussed research findings. Included in this category are mast cells, myeloid cells, stromal cells, and T cells. This section analyzes the intercellular communication between RAS-associated cell subpopulations and the rest of the TME in greater depth. Cell-to-cell communication has demonstrated that the intercellular connections between cells are extremely diverse (Fig. 5A). Figure 5B indicates that MIF-(CD74 + CXCR4), MIF-(CD44 + CXCR4), and CXCL12-CXCR4 were the most effective intercellular communication channels. We analyzed the intercellular connection between CD8 + T cells, CD4 + T cells, epithelial cells, Treg cells, mast cells, endothelial cells, and fibroblasts and found that all of these cell types are implicated in RAS signaling (Fig. 5C-I). Two myeloid cell subpopulations known to be associated with RAS, ANPEP + DCs and ANPEP + MACs, have been found to engage in extensive communication with a wide variety of TME cell types. In the past, it has been demonstrated that ANPEP promotes the development of tumor blood vessels and metastasis. These two subpopulations have more extensive high-intensity communication and express more genes associated with T cell exhaustion, which may be a mechanism by which ANPEP
promotes the malignant progression of gastric cancer. This is only one of numerous potential mechanisms.

We desired a deeper understanding of the potential benefits of RAS-related TME cells, so we decided to combine the results of an RNA-seq aggregate data analysis with a thorough single-cell data analysis. To conduct a meta-analysis on TCGA-STAD cohort patients, we derived RAS-related feature scores and differentially expressed genes (DEGs) of RAS-related TME cells using GSVA. The tumors and adjacent tissues of individuals with gastric cancer were analyzed to determine RAS-related feature scores, which were then displayed (Fig. 5J). Then, we evaluated the efficacy of sixteen RRGs using GSVA, and we referred to the resulting score as the RAS_score. As shown in the box diagram of Fig. 5J, the levels of RRG expression in the tumors of patients with gastric cancer vary in comparison to the contiguous tissues. On the basis of these results, RAS regulation abnormalities may exist in the microenvironment of gastric cancer.

To better inform clinical decision-making based on the likelihood of local RAS regulatory abnormalities in gastric cancer, we constructed an NMF classification of patients based on 16 RRGs and analyzed the intergroup prognosis levels of subtypes. This was done in an effort to enhance the clinical decision-making process regarding the possibility of gastric cancer. The three categories into which NMF clustering classified the TCGA-STAD data are displayed in Fig. 5L-N. The PCA principal component analysis revealed the distribution of samples within these categories, whereas the KM curve revealed the prognostic differences between patients within these groups. Patients in group 3 with high expression of both CTSG and CPA3 genes had the worst prognosis (P < 0.05), while those in group 2 with high expression of both NLN and THOP1 genes had the best prognosis. Ang(1–7) and its regulatory genes may play a crucial role in determining the prognosis of gastric cancer patients, as evidenced by the elevated expression of Ang(1–7) producing genes THOP1 and NLN in group 2 with the best prognosis. Because it inhibits the Ang II-related route, traditional RASi has the potential to induce hypotension refractoriness in addition to other undesirable side effects. By using a novel RASi that stimulates Ang(1–7) and its associated pathways, it may be possible to achieve the same biological effects with fewer deleterious effects.

**RAS-Mediated TME Patterns Impact Prognosis and Immunotherapy in GC**

We recalculated their DEGs in the GC scRNA dataset and identified the top 30 as cell markers in order to characterize the numerous significant GC TME cell types and their unique properties. This research aimed to identify the pertinent GC TME cell types. Then, we performed a meta-analysis of data on overall survival (OS) from seven distinct GC cohorts. Using GSVA and all DEGs of RAS-related TME cells, we created RAS scores and evaluated their prognostic value in GC patients (Fig. 6A). Diverse subclusters of m6A-mediated cells exhibited differential survival in relation to variations in the primary m6A loci. There were fibroblasts, macrophages, Tregs, and endothelial cells among the subclusters. To further evaluate the predictive utility of RAS-related TME cell characteristics in immunotherapy patients, we performed a
meta-analysis of overall survival (OS) in four common cancer cohorts (melanoma, RCC, and bladder cancer; Fig. 6F). Figure 6D depicts the application of logistic regression to examine the impact of RAS-related TME cell prediction on the immunological response of immunotherapy patients in two distinct cohorts. The data we used originated from TCGA and ACRG research on gastric cancer. Certain subgroups of GC patients with RAS-related mutations significantly contributed to prognosis and immunotherapy response, whereas others with RAS-related mutations significantly contributed to neither. Patients in the RAS-related categories ACE + ENDO, AGT + CAF, ENPEP + CAF, LNPEP + ENDO, and THOP1 + Treg had the greatest impact on immunotherapy response rate and prognosis. The results of the immunotherapy cohort study utilizing IMvigor210 concurred with those of the TCGA and ACRG. In the immunotherapy response analysis, box plots were utilized to compare RAS-related TME cell characteristics between the CR/PR and SD/PD groups. Within the immunotherapy response group, the characteristics of the ACE + ENDO, AGT + CAF, ENPEP + CAF, and ANPEP + MAC subgroups were reduced, whereas the characteristics of the THOP1 + Treg and LNPEP + Treg subgroups were elevated, with P < 0.05 being the significant level. THOP1 + Treg was identified as a beneficial cell subset for patient prognosis, with its characteristics yielding consistent results across numerous gastric cancer datasets. This is in stark contrast to previous Treg cell research. Figures 6B, C, and E illustrate how the patient’s characteristics can be used to predict the patient’s prognosis and treatment response. Multiple occurrences of the THOP1 + Treg subpopulation were discovered while analyzing gastric cancer tissue samples (Fig. 6H).

**Discussion**

The relationship between RAS and GC pathology has only been briefly examined. This is the first study to analyze the patterns of RRG expression in the immune microenvironment of gastric cancer, as well as the variety of single-cell interactions between RAS-related TME subtypes and other cells in the microenvironment. We can now comprehend how these diverse cell subtypes impact the prognosis of GC patients as a result of this ground-breaking and novel research. Numerous RAS hormones and receptors are expressed differently by the kidney, blood vessels, heart, and other malignancies, and the majority of target organs have both local and systemic RAS. It is one of the most essential peptide synthesis and metabolism systems in the body and promotes tumor growth. TME cells including stromal cells, macrophages, T cells, endothelial cells, and mast cells were found to have diverse RRGs expression patterns in the microenvironment of stomach cancer, leading to the establishment of a local RAS system distinct from the body as a whole. According to studies of solitary cells, each RAS subgroup has an active relationship with TME cells. In addition, pathway analysis revealed that interactions between MIF (CD74 + CXCR4), MIF (CD74 + CD44), MDK-NCL, LGALS9-CD45, and CXCL12-CXCR4 ligand-receptor couplings enhance communication between RAS-associated subgroups and TME cells.

CAFs (cancer-associated fibroblasts) serve a crucial role in the malignancy microenvironment. They are divided into four categories based on their molecular characteristics: pan-myCAFs, pan-dCAFs, pan-iCAFs, and pan-nCAFs. The potential functions of CAFs in the RAS system have not been thoroughly
investigated. Angiotensinogen, or AGT, is the essential and fundamental component of the RAS system, which other RAS proteins convert into angiotensin II, angiotensin (1–7) or other orientations, thereby influencing the fibrosis and inflammatory levels of gastric cancer. AGT is more likely to be converted to angiotensin II in the conventional direction when CMA1, ACE, or CTSG are present, which promotes tumor spread, increases fibrosis, and worsens patient prognosis. When TOP1, ACE2, or MME are present, AGT is more likely to be converted to angiotensin (1–7), which suppresses inflammation and fibrosis and improves patient prognosis. We concluded that fibroblasts are the primary source of AGT in the cancer microenvironment because we were able to identify a percentage of AGT-positive fibroblasts in patients with gastric cancer. Simultaneously, the TCGA's gastric cancer data center observed an increase in AGT expression levels, indicating that RAS may play a role in the development of gastric cancer and that this AGT + fibroblast subpopulation is likely to play a key role in RAS aberrations in gastric cancer. In addition, AGT + CAFs were more frequently associated with tumor epithelial cells than non-RAS-related fibroblasts. This may be because tumor epithelial tissue in the microenvironment increased and controlled AGT expression, causing RAS system dysfunction and altering the prognosis of the patient. AGT + CAFs were identified as a type of dCAF that facilitated collagen secretion, presumably by secreting DCN, ILUM, and MMPs, thereby promoting tumor fibrosis and inhibiting immune cell infiltration. They were also associated with increased expression of extracellular matrix (ECM) and matrix metalloproteinase (MMP). Several metabolic pathways, including the Kras pathway, were associated with this AGT + CAF subgroup. By cleaving Ang II into Ang III, the ENPEP gene encodes the glutamyl aminopeptidase enzyme, which regulates vascular expansion and tumor development in tissues as well as increases blood pressure. ENPEP expression negatively predicts gastric cancer patients. According to our findings, the ENEPEP + CAF subpopulation signature has a negative effect on the prognosis and immune treatment responsiveness of patients with gastric cancer. According to an analysis of data from the Mvigor210 immune treatment cohort, elevated levels of ENEPEP + CAF subpopulation signature expression are associated with CR/PR patient outcomes. These data suggest that ENPEP could be a suitable target for RASi and warrant additional research.

A growing body of evidence implies that the disease microenvironment is dependent on a delicate balance between angiotensin II's pro-inflammatory and pro-fibrotic actions and angiotensin's anti-inflammatory and anti-fibrotic qualities (1–7). We divided the TCGA-STAD data into three independent groups using the NMF clustering approach in order to investigate the importance of this equilibrium in stomach cancer in further depth. Individuals with a better prognosis were more likely to exhibit genes that speed up the conversion of AGT to angiotensin (1–7). Individuals with a worse prognosis, on the other hand, were more likely to have CTSG, a gene that aids in the conversion of AGT to angiotensin II. Researchers concluded that RAS is implicated in the evolution of gastric cancer because patients who expressed more genes that increase angiotensin production (1–7) had greater 5-year survival rates.

Mast cells, according to earlier research, may contribute to tissue fibrosis. We propose that RAS-associated mast cells mediate local angiotensin II signaling based on the results of our NMF clustering analysis of single-cell data. Mast cells express RRGs at the highest level of any cell type in the
microenvironment of stomach cancer. Furthermore, a subset of the positive genes expressed by mast cells is responsible for the generation of angiotensin II by mast cells. According to the findings, mast cells are engaged in the synthesis of angiotensin II in the surroundings of stomach cancer and may enhance angiotensin II production as one of their potential tactics for enhancing tumor fibrosis. This is supported by the presence of mast cells in the microenvironment of stomach cancer.

According to our findings, THOP1 + Treg, a subset of RAS-related regulatory T cells, may play an important role in regulating the amount of angiotensin II produced by mast cells. Multiple studies have revealed that regulatory T cells, or Tregs, aid in the immune evasion of malignancies and metastases by suppressing cytotoxic T cells and producing inhibitory cytokines like transforming growth factor. The encoded protein of THOP1 (Thimet Oligopeptidase 1) is a neuropeptidase capable of cleaving neuropeptides with fewer than 20 amino acids. This increase in angiotensin production has been attributed to THOP1 (Thimet Oligopeptidase 1) (1–7). The THOP1 + Tregs signature was discovered to have both predictive and positive associations with the survival time of gastric cancer patients. Furthermore, the THOP1 + Tregs signature is substantially expressed in the IMvigor210 immunotherapy cohort's medication response group. According to TIDE statistics on immunotherapy for gastric cancer, this is connected with increased patient survival and has a favorable correlation with patient response rate.

Due to the diversity of Treg cells, THOP1 + Tregs have been proven to be beneficial to patients, contrary to the findings of previous studies. We hypothesize that different inflammatory activation factors, such as angiotensin II, which promotes inflammation and increases fibrosis, may have different effects on the microenvironment, and that regulatory T cells (Tregs) can suppress inflammation and immunological processes through a variety of mechanisms. Treg subsets are responsible for improving patient prognosis and clinical performance by lowering inflammatory factor production. We conducted immunofluorescence assays on gastric cancer tissue samples to confirm the presence of THOP1 + Treg subgroups in gastric cancer patients' malignant tissues.

Using NMF clustering of single-cell data, we were also able to identify endothelial cells, macrophages, and dendritic cells as being related with RAS. The conventional component that stimulates angiotensin II production, angiotensin-converting enzyme (ACE), has a deleterious impact on the prognosis and immunotherapy outcomes of patients with gastric cancer. Aminopeptidase N, commonly known as ANPEP, has the ability to induce angiogenesis, tumor development, and metastasis. It has also been associated to certain types of leukemia and lymphoma. ANPEP can also stimulate the synthesis of angiotensin IV. ANPEP + DC and ANPEP + MAC cells were discovered to express more genes related with CD8 + T cell fatigue and to interact more extensively with other cells in the microenvironment. These findings support the idea that ANPEP increases tumor growth.

In conclusion, our findings shed light on the RAS-related subpopulations in the microenvironment of gastric cancer that regulate the synthesis of various angiotensins as components of the local RAS system, thereby influencing the course and prognosis of gastric cancer patients. The signatures of
multiple subpopulations can predict patient prognosis and immune therapy response rates. Our exploratory investigation is limited by the limited profundity of scRNA-seq, the small sample size, and the need for additional validation in more patients. The presence of more zero observations in scRNA-seq data for specific RAS regulatory factors in GC than in total RNA-seq data may have affected our clustering technique. Despite this, the scRNA-seq study reveals the uniqueness of RAS regulatory factors in various TME single cells, as well as their impact on patient prognosis and immunological treatment, which represents a significant advance in clinical practice.

**Declarations**

**Acknowledgments**

We appreciate all team members at Bioinfo_composer, the leading bioinformatics platform in China, for their selfless help.

**Author Contributions**

Conceptualization, QX, LF; Formal analysis, QX, SX; Funding acquisition, LF; Investigation, QW; Methodology, QX, SX, QW and FL; Writing—original draft, QX, YZ; Writing—review & editing, QX, SX, QW, JL, YZ and LF All authors have read and agreed to the published version of the manuscript.

**Funding**

This work was supported by National Science Foundation of China (No. 8217100675) subaward.

**Conflicts of Interest**

The authors declare no conflict of interest.

**Ethics approval and consent to participate**

Not applicable.

**Availability of data and materials**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://cancergenome.nih.gov/, TCGA and Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/).

**References**


Figures
Figure 1

The landscape of RAS regulators in TME cells in GC

(A) A description of the scRNA-Seq methodology. (B) The primary environmental regulatory mechanisms of RAS-related peptides. (C) The UMAP diagram depicts the clustering outcomes of eleven main cell types derived from 16,087 high-quality single cells extracted from normal and malignant stomach tissues.
Different colors represent distinct types of cells. A heatmap displaying the most prevalent marker genes in each main cell type. The most abundant marker genes in each main cell type are depicted on a dot map. The magnitude of the dot represents the proportion of cells in each primary cell type that express the marker gene, while the color represents the average expression level of the marker gene in each primary cell type. Red represents intense emotion, whereas blue represents a milder expression. A stacked histogram depicting the proportion of eleven main cell subtypes in total cells derived from normal and cancerous stomach tissues. Histogram depicting the proportion of three main cell types in normal and cancerous stomach tissues. (H-I) RAS regulatory factor RNA expression levels in distinct gastric cancer TME cell subtypes.
Figure 2

Novel RAS-related fibroblasts contributed to the TME of GC

(A) The volcano plot demonstrates RRGs expression levels. (B) The column graph illustrates the prognostic significance of RRGs. (C) The box plot depicts the elevated AGT expression levels in gastric cancer. (D-E) Immunohistochemical staining of AGT in specimens of gastric cancer. (H) Subtyping of
NMF according to RAS regulatory factors. The expression of RRGs in fibroblasts is revealed by pseudotime analysis. (J) scMetabolism investigates intergroup metabolic differences in RAS-associated subgroups. The heatmap illustrates differential expression levels of common signaling pathway genes, including collagen, ECM, MMPs, TGFβ, Neo-Angio, Contractile, RAS, and Proinflammatory. (L-M) Clustering analysis of enrichment. The heatmap reveals ($p<0.05$) distinctions in proliferative pathways between the main subgroups of fibroblasts associated with RAS. The heatmap depicts activated KEGG pathways in the principal RAS-related fibroblast subgroups ($p<0.05$). Different clusters of RAS-related fibroblasts are linked to previously identified characteristics ($p<0.05$).
Figure 3

ANPEP promotes tumor immune evasion in GC through myeloid cell

(A) UMAP diagram depicts the distribution of myeloid cells in the dataset between three distinct cell types: monocytes, macrophages, and dendritic cells. (B) CellChat analysis reveals that the three primary myeloid cell subtypes exhibit differing levels of pathway activity. Violin plots illustrate which genes are
highly expressed in each main cell type. Using NMF, macrophages are classified based on RAS regulatory factors into distinct subtypes. Using scMetabolism analysis, metabolic differences between RAS-associated macrophage subsets are identified. The heatmap displays the average levels of cytotoxic and fatigued gene expression in CD8+ T cells. Dendritic cells are classified into distinct subtypes based on RAS regulatory factors using NMF. Using scMetabolism analysis, metabolic differences between RAS-associated dendritic cell subgroups are identified. This heatmap depicts the expression levels of cytotoxic and fatigued genes in CD8+ T lymphocytes. Levels of immune checkpoint gene expression in macrophages associated with RAS. Using AUCell to evaluate TF activity, a heatmap reveals significant differences in TF activity between the three RAS-associated macrophage subgroups (Kruskal-Wallis test, \( p<0.001 \)). (L) Immune checkpoint gene expression levels in RAS-associated dendritic cells. (M) Through pseudotime analysis, the temporal expression pattern of RRGs in myeloid cells is revealed.
Figure 4

NMF clustering of RAS-associated Mast/Treg/EC in GC

(A) Endothelial cell classification according to the NMF. (B) A heatmap depicts the disparities in TF activity among the four RAS-related mast cell subgroups (Kruskal-Wallis test, \( p<0.001 \)). (C) GSEA analysis of the distinct pathways of four subgroups of mast cells associated with RAS. (D) Trajectory Analysis.
reveals that RRGs expression levels in mast cells vary with time. (E) Classification of endothelial cells with NMF. (F) The heatmap demonstrates that TF activity differs significantly between the four RAS-related subgroups of endothelial cells (Kruskal-Wallis test, $p<0.001$). (G) Trajectory Analysis displays endothelial cell RRGs expression levels. (H) Variations in immune checkpoint gene expression among subgroups of mast cells associated with RAS. (I) Regulatory T cells are classified utilizing the NMF method. (J) The Kruskal-Wallis test revealed statistically significant differences in TF activity between subgroups of Treg cells associated with RAS ($p<0.001$). (K) Molecular docking analysis illustrates THOP1's molecular association with AngI. (L) Trajectory Analysis explains the temporal levels of RRG expression in regulatory T cells. (M) Various subtypes of regulatory T cells express immune checkpoint genes.
Figure 5

The Communication Patterns and Prognostic Typing Mediated by RAS

A cell-cell communication analysis revealed relationships between the most prevalent RAS-related cell types and TME cells. (B) The heatmap depicts the primary RAS-related subgroups and TME cells that interact with ligands. Research on cell-to-cell communication indicates connections between RAS-related
cell types and TME cells. (J) The box plot illustrates the expression levels of RAS-related subgroup cell characteristics. (K) TCGA data differential GSVA scores of 16 RRGs. TCGA-based NMF classification. N classification NMF PCA analysis. The (N) KM curve demonstrates that prognostic differences exist between NMF subtypes.

![Figure 6](image)
Overall prognosis and immunotherapy response of RAS-related cell types were analyzed in bulk sequence data from public cohorts.

Using the remaining R programs, cutoffs were calculated. (A) OS analysis was conducted using information from seven GC cohorts. (B,C) KM curve-based survival analysis of THOP+Treg subgroups. The OS analysis was conducted using information from four immunotherapy cohorts. KM curve-based survival analysis of THOP+Treg subgroups. Using data from the TIDE database, an analysis of immunotherapy response was conducted, and response rates were provided. Box diagrams are presented. Immunofluorescence assays reveal the presence of THOP1+Treg in gastric cancer tissue. FOXP3 (green), DAPI (blue), and THOP1 (red) are shown here.

Figure 7

The landscape of renin-angiotensin system of GC TME

Supplementary Files
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