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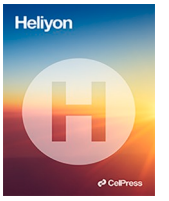

Metformin promotes cGAS/STING signaling pathway activation by blocking AKT phosphorylation in gastric cancer

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ABSTRACT

The cGAS/STING signaling pathway plays a pivotal role in regulating innate immunity. Emerging novel drugs aim to regulate the anti-tumor immune response by activating innate immunity. The anti-diabetic drug metformin has been reported to exhibit anti-cancer effect against various types of cancer. However, the role of metformin in regulating the cGAS/STING signaling pathway in gastric cancer remains unknown. In our study, we first used bioinformatic analysis to detect that metformin is closely related to tumor immunity in multiple tumors. Next, we validated the function of metformin in activating the cGAS/STING signaling pathway in gastric cancer cell lines. In addition, KEGG pathway enrichment analysis showed that metformin is negatively correlated with the PI3K/AKT signaling pathway in gastric cancer. We further verified that metformin activates the cGAS/STING signaling pathway by blocking AKT phosphorylation. Moreover, we found that metformin regulates the AKT signaling pathway by mediating the transcription factor SOX2. Thus, our study indicates that metformin activates the cGAS/STING signaling pathway by suppressing SOX2/AKT and has promising potential in gastric cancer immunotherapy.

1. Introduction

Gastric cancer (GC) is the fifth most common malignant cancer and the fourth most common cause of tumor-related mortality worldwide [1]. Owing to the occult onset and rapid progression of gastric carcinoma, most patients are at an advanced stage at their first diagnosis. Radical surgery combined with chemoradiotherapy is the standard cure for patients with advanced gastric cancer [2]. Although chemoradiotherapy has made great progress, the overall outcome of patients with gastric cancer remains unsatisfactory [3]. With the advancement of immunotherapy, a series of immunotherapeutic drugs are available for patients with advanced gastric cancer, and more options are under clinical investigation. Based on the NCCN Guidelines for gastric cancer, nivolumab plus chemotherapy is regarded as the first-line therapy strategy for patients with HER2-negative advanced gastric cancer [4]. However, immune checkpoint inhibitor (ICI) therapy seems to provide limited benefits for patients with advanced gastric cancer [5]. Therefore, seeking novel hypotoxic reagents or old drugs with new applications is a potential strategy to enhance the antitumor effect of

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immunotherapy.

Metformin is a first line treatment for type-2 diabetes mellitus (T2DM). It decreases glucose production by blocking gluconeogenesis in the liver and exerts an anti-diabetic effect [6]. Metformin can activate AMPK by inhibiting mitochondrial respiratory chain complex I, which increases the AMP/ATP ratio and suppresses the expression of genes encoding specific gluconeogenic enzymes [7]. In addition to its hypoglycemic function, metformin can reduce the risk of cancer among diabetic patients [8]. Furthermore, recent clinical research has suggested that combining metformin with chemoradiotherapy can improve the 2-year overall survival (OS) and progression-free survival (PFS) rates compared with historical controls in patients with locally advanced head and neck squamous cell cancer [9]. The conversion of metformin from an old antidiabetic drug to an encouraging anti-tumor reagent probably contributes to raising the therapeutic effect of immunotherapeutic reagents in gastric cancer. However, whether metformin can activate immunity and the specific molecular mechanism underlying the anti-tumor effect of metformin in gastric cancer remain unclear.

In our research, we found that metformin could activate the cGAS/STING signaling pathway and promote the release of downstream inflammatory factors via SOX2/AKT in gastric cancer cells, which provides the basis for the clinical application of metformin in combination with immunotherapies in gastric cancer.

2. Material and methods

2.1. Transcriptomic data and cohorts

Transcriptome profiling and clinical information of tumor samples treated with metformin were downloaded from the Genomic Data Commons Data Portal of TCGA (https://cancergenome.nih.gov/) and Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) datasets. In addition, we collected publicly available raw microarray expression data from GEO for the eligible datasets. GSE190076, mRNA profiles of Huh-7 cells treated with and without metformin for 48 h; GSE207122, expression profiling of CAL27 cells co-treated for 48 h with either PBS vehicle or 30 mM metformin; GSE208773, gene expression profiling analysis of glioblastoma cell lines obtained from RNA sequencing (RNA-seq) data of six metformin-treated groups versus six control groups. GSE180962 [10], fresh-frozen pretreatment breast tumors (n = 233:106 ganitumab/metformin arm, 127 control arm) from the neoadjuvant ISPY2 trial for high-risk and early-stage breast cancer were analyzed using GPL16233 (n = 70) or GPL20078 (n = 163) expression arrays, and patients treated with ganitumab/metformin were explored in this study. The data from GEO and TCGA were public and open access, so approval from the ethics committees was not required.

2.2. Differentially expressed gene analysis

Differential tests were performed on samples with metformin treatment and placebo control. Differentially expressed genes (DEGs) in GSE196343 and GSE180962 were selected with the Q-value (adjusted *P*-value) <0.05 and $|\text{Log2 fold change (FC)}| > 1.50$ using the "limma" package in R [11]. The DEGs with $|\text{Log2 FC}| > 1.50$ were identified as specific genes with metformin treatment.

2.3. Genes enrichment and pathway analysis

KEGG API (https://www.kegg.jp/kegg/rest/keggapi.html) was used to obtain the latest gene annotations of the KEGG pathway and GO enrichment in the R package org.Hs.eg.db (version 4.0.1) [12]. The R software package "cluster-Profiler" (version 4.0.1) was used to conduct enrichment analysis and obtain the results. As the background of enrichment, the minimum gene was set to 5 and the maximum gene was set to 5000 with a statistically significant *P*-value <0.05 (modified) and FDR <0.25 (modified).

2.4. Analysis of tumor infiltrated immune cells

We used the CIBERSORT algorithm to calculate tumor-infiltrating immune cells. CIBERSORT is an analytical tool used to quantify infiltrated immune cell components, which includes a gene expression signature matrix containing 547 marker genes [13]. LM22 is a gene expression signature matrix defining 22 immune cell subtypes, which can be downloaded from the CIBERSORT web portal (http://cibersort. stanford. edu/) (Table S1). LM22 distinguishes the 22 human immune cell phenotypes, including T cells, naïve and memory B cells, plasma cells, NK cells, monocytes, M0-M2 macrophages, dendritic cells, mast cells, eosinophils, and neutrophils. CIBERSORT *P* and RMSE (root mean squared error) were calculated in each sample file to improve the accuracy of the deconvolution algorithm. A default signature matrix of 100 permutations was used in the algorithm. We only selected data with CIBERSORT $P < 0.05$ for further analysis. We used the CIBERSORT algorithm to analyze the immune cell components of all samples from TCGA and GEO cohorts.

2.5. RNA-seq

We used RNAiso Plus (9109, Takara) to extract total RNA according to the manufacturer's instructions. After quality inspection, the RNA samples were subjected to RNA-sequencing (BGI, Shenzhen, China). Each group consisted of three replicates. Genes with a fold change >1.5 and a $P < 0.05$ were considered to be differentially expressed.

2.6. Cell lines, antibodies, and reagents

Human gastric cancer cell lines BGC823, AGS, and SGC7901 were purchased from the National Collection of Authenticated Cell Cultures of China and were authenticated using Short Tandem Repeat (STR) analysis by the users' lab. Cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin in a humid incubator with 5% CO_2 at 37 °C. The primary antibodies used were anti-p-TBK1 (1:1000; #5483, Cell Signaling Technology), anti-TBK1 (1:1000; #67211-1-Ig, Proteintech), anti-p-IRF3 (1:1000; #29047, Cell Signaling Technology), anti-IRF3 (1:1000; #11312-1-AP, Proteintech), anti-p-AKT (1:1000; #4060, Cell Signaling Technology), anti-AKT (1:1000; #9272, Cell Signaling Technology), anti-SOX2 (1:1000; #11064-1-AP, Proteintech), anti-STING (1:1000; #13647, Cell Signaling Technology), anti-cGAS (1:1000; #26416-1-AP, Proteintech), anti-FLAG (1:5000; #66008-4-Ig, Proteintech), and anti-GAPDH (1:3000; #60004-1-Ig, Proteintech). The secondary antibodies used were HRP-conjugated goat anti-rabbit (1:3000; #SA00001–15, Proteintech) and anti-mouse (1:3000; #SA00001–1, Proteintech). Metformin HCl (#S1950) and SC79 (#S7863) were purchased from SELLECK (TX, USA).

2.7. Plasmid construction and transfection

Wild-type SOX2 was cloned into pLVX-3FLAG-IRES-puro for expression in a human gastric cancer cell line. Plasmid transfection was performed using the Neofect transfection reagent (Neofect Biotechnology) according to the manufacturer's instructions.

2.8. Western blot analysis

RIPA buffer (V900854, Sigma, MO, USA) was used to extract the total protein with 1% protease inhibitor (B14001, Bimake, TX, USA) and phosphatase inhibitor (G2007, Servicebio, Wuhan, China). The proteins were separated using 7.5%–12.5% SDS-PAGE and transferred to PVDF membranes. Next, membranes were blocked in 5% skim milk for 1 h and incubated with primary antibodies overnight at 4 °C. After washing with TBST three times, we incubated the membranes with secondary antibodies for 1 h. Finally, we used the Invitrogen iBright CL1000 imaging system (Thermo Fisher Scientific, USA) to screen the membranes after incubation with ECL reagents (Thermo Fisher Scientific, USA). The results were analyzed using ImageJ.

2.9. RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

We used RNAiso Plus (9109, Takara) to extract total RNA, which was then reverse-transcribed into cDNA using PrimeScript RT Master Mix (RR036A, Takara). Subsequently, mRNA expression levels were examined using SYBR® Premix Ex TaqTM (RR820A, Takara) on a StepOnePlus™ Real-Time PCR System. The results were standardized by GAPDH and then calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$). The primers were as follows: GAPDH, former primer: 5′-ACAACTTTGGTATCGTGGAAGG-3′, reverse primer: 5′-GCCATCACGCCACAGTTTC-3'; CCL5, former primer: 5′-CCAGCAGTCGTCTTTGTCAC-3′, reverse primer: 5′-CTCTGGGTTGGCACACACTT-3'; CXCL10, former primer: 5′-GTGGCATTCAAGGAGTACCTC-3′, reverse primer: 5′-TGATGGCCTTC-GATTCTGGATT-3'; CCL20, former primer: 5′-TGCTGTACCAAGAGTTTGCTC-3′, reverse primer: 5′-CGCACACAGACAACTTTTTCTTT-3'.

2.10. Immunofluorescence staining

The cells were incubated with metformin (5 mmol/L) for 2 d after seeding on the coverslips of 6-well plates. Next, the cells were fixed with 4% formaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 20 min, blocked with 5% BSA for 1 h, and incubated with anti-p-TBK1 (1:100) overnight at 4 °C. The coverslips were incubated with FITC-conjugated secondary antibodies (1:200) for 1 h and DAPI for 5 min. Finally, the coverslips were imaged using a fluorescence microscope.

2.11. RNA interference

Specific SOX2 siRNA was synthesized by Tsingke Biotechnology. AGS cells were seeded in 6-well plates at a confluence of 30–40% overnight and transfected with SOX2 or non-target siRNA using Lipofectamine 6000 (Thermo Fisher Scientific, USA). The following are the siRNA sequences of SOX2 and non-target control: SOX2: 5′-CGCTCATGAAGAAGGATAAGT-3'; non-target control: 5′-TAAGGC-TATGAAGAGATAC-3'.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software. All results are expressed as the mean ± standard deviation (SD), and a two-tailed *t*-test was performed to analyze statistical differences between the two groups. Bioinformatics was performed using R v4.0.1. Significance levels were as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns: not significant.

3. Results

3.1. Metformin affects tumor immunity in multiple tumors

It has been recently reported that metformin has anti-tumor activity and can maintain high cytotoxic T lymphocyte (CTL) immune surveillance [14]. To analyze the relationship between metformin and immune function, we found that the expression of immune regulator genes was enhanced by metformin in liver cancer, glioblastoma, and head and neck squamous cell carcinoma (Fig. 1A, Table S2). Furthermore, some immune regulatory pathways, including the TNF, IL-17, and chemokine signaling pathways, appeared in the upregulated KEGG pathways (Fig. 1B). Moreover, we performed immune infiltration analysis to explore the influence of metformin on tumor-infiltrating immune cells. After filtering with CIBERSORT $P < 0.05$, we analyzed 22 immune cell types in 106 tumor tissues treated with ganitumab/metformin. The most common immune cells in tumor tissues with pCR were M0 macrophages, M1 macrophages, M2 macrophages, CD4 T cell memory activated, CD8 T cells, NK cell activated, follicular helper T cells, and gamma delta T cells. The immune cells with significantly higher fractions in tumor tissues with pCR compared with tissues without pCR were CD8+T cells, memory-activated CD4+T cells, and follicular helper T cells (all $P < 0.05$). Plasma cells, M2 macrophages, and neutrophils were higher in tumor tissues with non-pCR compared with tumor tissues with pCR (all $P < 0.05$) (Fig. 1C and D). These results indicate that metformin is closely related to immunity.

3.2. Metformin promotes cGAS/STING signaling pathway activation in gastric cancer

Based on the above-mentioned bioinformatic analysis results, metformin is closely related to tumor immunity. We further explored whether metformin activated the cGAS/STING signaling pathway in gastric cancer. We found that cGAS, STING, phosphorylated-TBK1 (p-TBK1), and phosphorylated-IRF3 (p-IRF3) obviously increased in a time-dependent manner in BGC823, AGS, and SGC7901 cells in the Western blot assay (Fig. 2A). In addition, the immunofluorescence staining assay showed that the p-TBK1 positive cell ratio was significantly increased after metformin treatment in the three gastric cancer lines (Fig. 2B). Next, we found that the expression levels of CCL5, CXCL10, and CCL20, which are the downstream inflammatory cytokines of the cGAS/STING signaling pathway, were upregulated in the metformin-treated group than those in the control group (Fig. 2C). These data indicate that metformin enhances cGAS/STING signaling pathway activation in gastric cancer cells.

3.3. Metformin down-regulates PI3K/AKT signaling pathway in gastric cancer

Having explored the obvious activation of the cGAS/STING signaling pathway in metformin-treated cells, cGAS/STING regulation

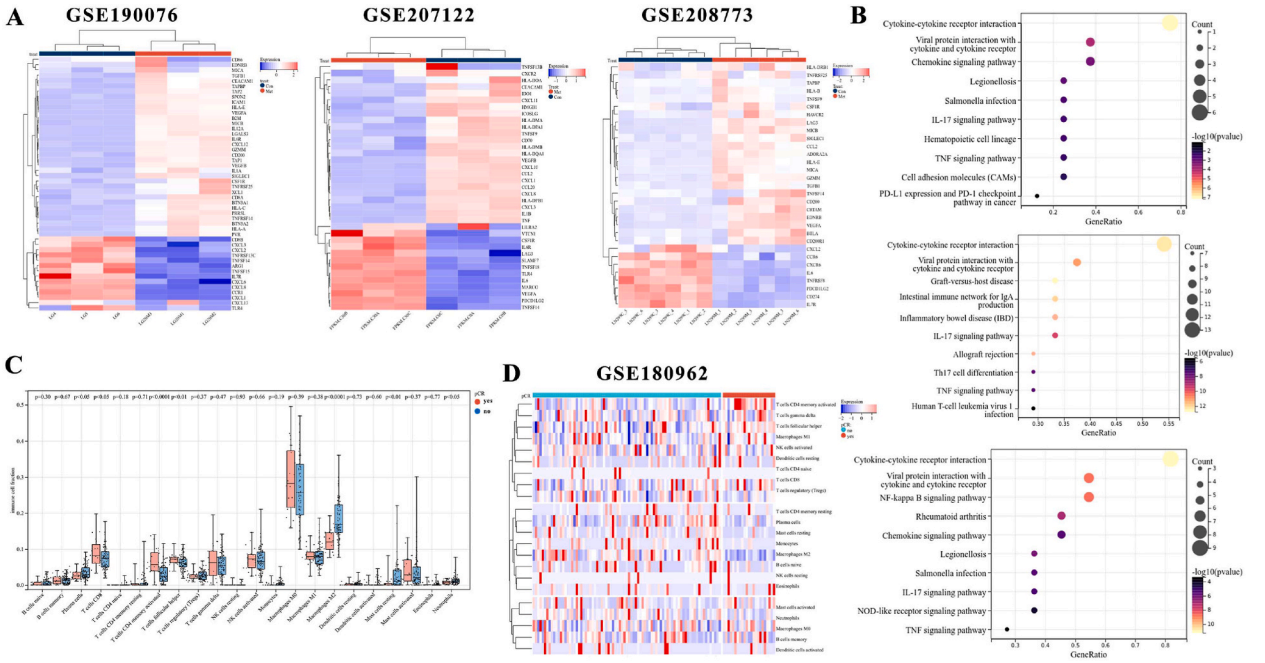

Fig. 1. Metformin effects on tumor immunity in multiple tumors. **(A)** The heatmap of DEGs in GSE190076, GSE207122 and GSE208773. **(B)** The bubble chart of top 10 up-regulated KEGG pathways in GSE190076, GSE207122 and GSE208773. (**C**) Comparisons of immune cells between tumor tissues with pCR and with non-pCR in GSE180962. (**D**) Correlation of CD4 memory activated T cells, M0 macrophages, M1 macrophages with other immune cells in GSE180962. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$.

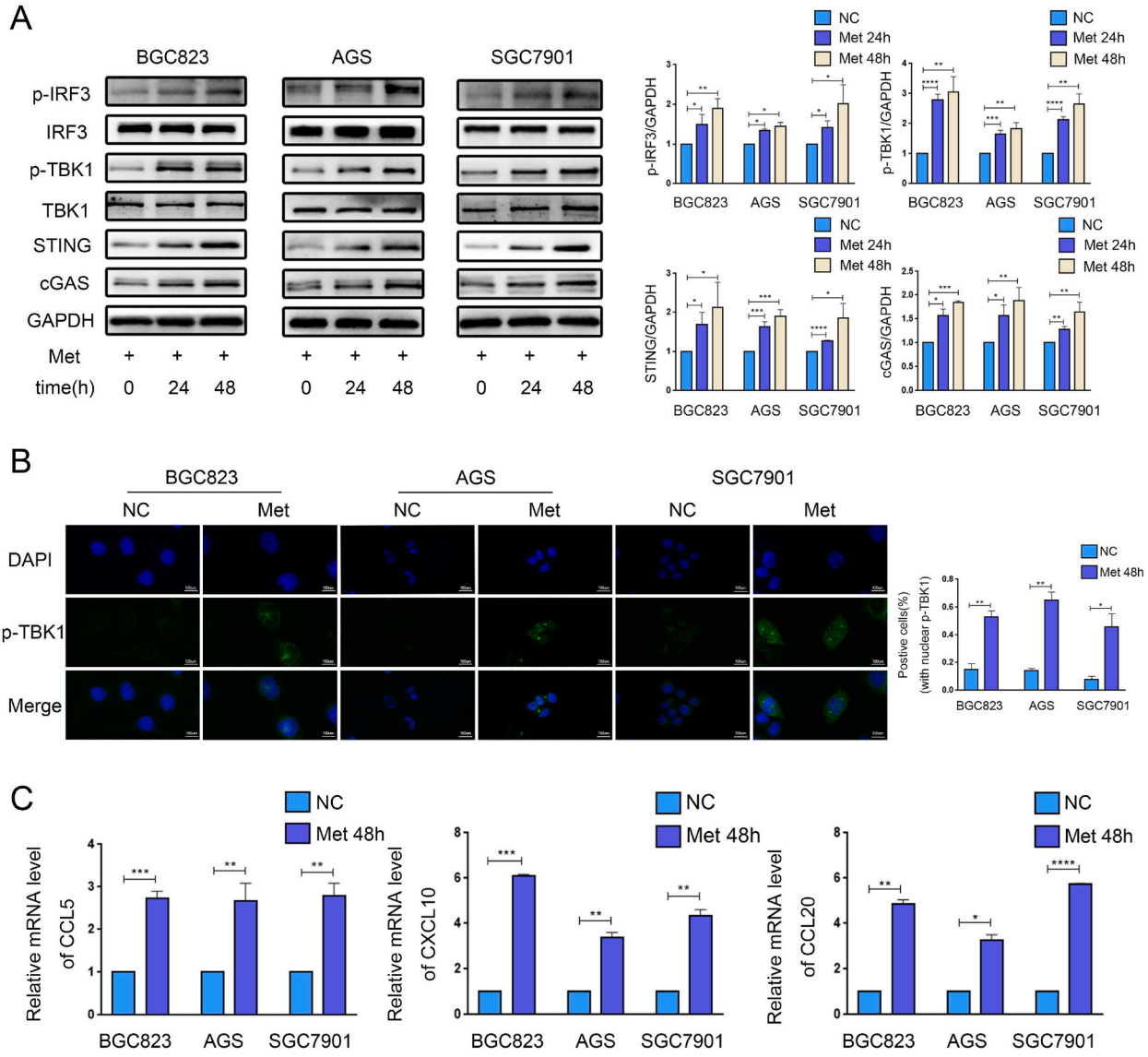

Fig. 2. Metformin promoted cGAS/STING signaling pathway activation in gastric cancer cells. **(A)** Western blot analysis of phosphorylated IRF3 (p-IRF3), total IRF3, phosphorylated TBK1 (p-TBK1), total TBK1, STING and cGAS in BGC823, AGS and SGC7901 cell lines with treatment of metformin (5 mM) for 0 h, 24 h or 48 h respectively. Data are presented as mean ± SD of three independent experiments. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$. **(B)** Immunofluorescence to detect the expression of p-TBK1 (Ser172) in BGC823, AGS and SGC7901 cell lines with treatment of metformin (5 mM) for 48 h. The data are reported as mean ± SD (n = 3).*$P < 0.05$. **$P < 0.01$. **(C)** qRT-PCR analysis to show the mRNA expression level of CCL5, CXCL10 and CCL20 in BGC823, AGS and SGC7901 cell lines with treatment of metformin (5 mM) for 48 h. The data are reported as mean ± SD of three independent experiments. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$.

by metformin is still unclear. We conducted RNA-seq with gastric cancer cell line AGS and the volcano plot and heatmap are shown in Fig. 3A and B. A total of 324 DEGs were obtained from analysis with Q value < 0.05 and $|\text{Log2 FC}| > 2.00$ (Table S3). Next, DEGs enrichment analysis was performed on the upregulated and downregulated gene sets. As shown in Fig. 3C, D, and E, complement and coagulation cascades and chemical carcinogenesis were included in up-regulated KEGG pathways, and the significantly enriched GO terms for biological process were mainly transcription of DNA templates. Meanwhile, the PI3K/AKT signaling pathway showed a significant change in the downregulated KEGG pathways (Fig. 3F and G) and GO enrichment analysis (Fig. 3H), which showed that metformin was negatively correlated with the PI3K/AKT signaling pathway in gastric cancer.

3.4. Metformin activates cGAS/STING signaling pathway via blocking AKT phosphorylation in gastric cancer cell lines

Further, we explored whether metformin regulated the cGAS/STING signaling pathway by inhibiting the PI3K/AKT pathway in

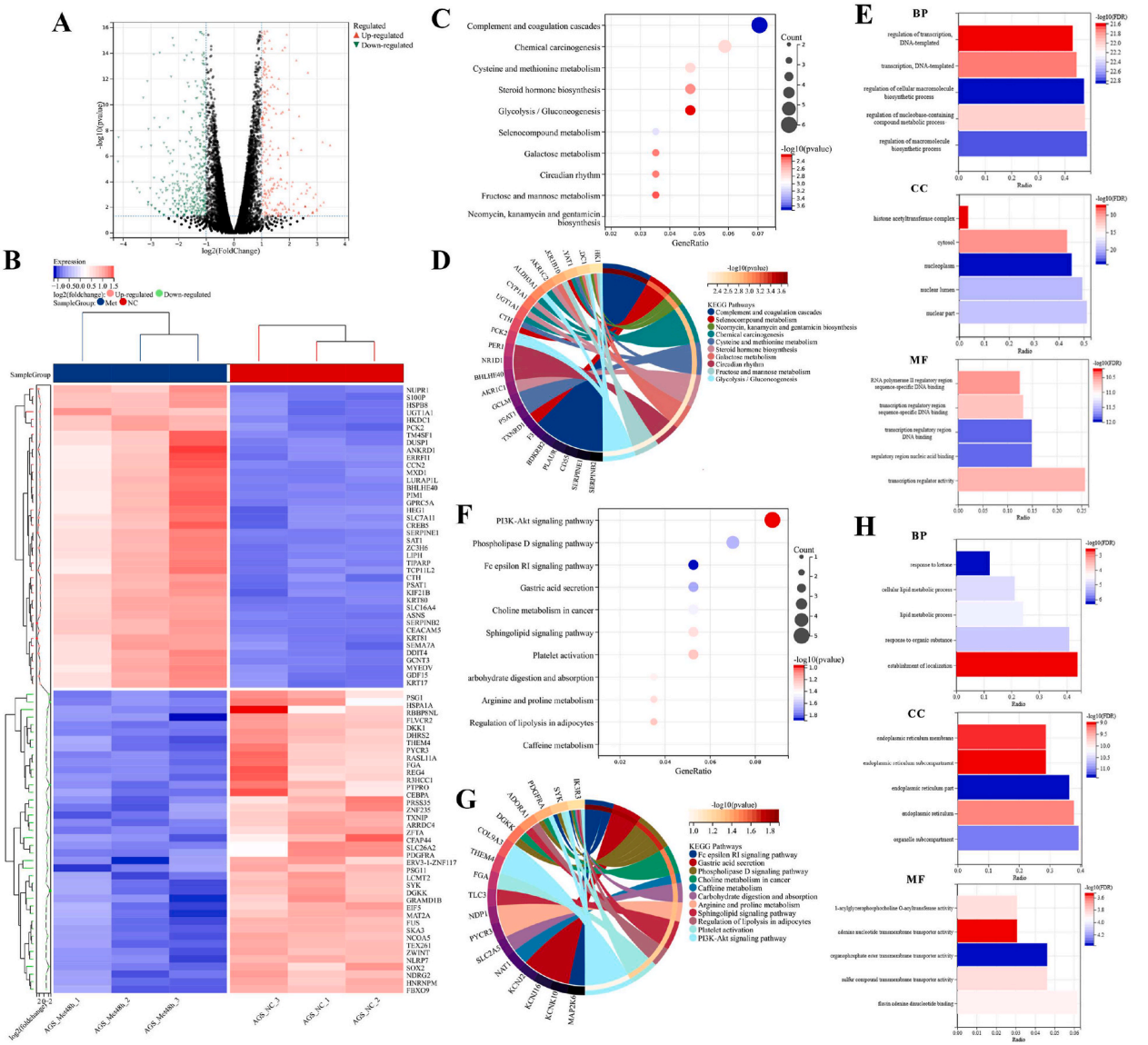

Fig. 3. Differentially expressed gene analysis and genes enrichment and pathway analysis of RNA-seq. **(A)** The volcano plot with Q value < 0.05 and $|\text{Log2 FC}| > 2.00$. **(B)** The heatmap of top 40 DEGs. **(C)** The bubble chart of top 10 up-regulated KEGG pathways. **(D)** The circle plot of top 10 up-regulated KEGG pathways. **(E)** Histograms of GO terms of up-regulated gene set showed the top 5 terms with Q value < 0.05. **(F)** The bubble chart of top 11 down-regulated KEGG pathways. **(G)** The circle plot of top 11 down-regulated KEGG pathways. **(H)** Histograms of GO terms of down-regulated gene set showed the top 5 terms with Q value < 0.05. DEGs differentially expressed genes, BP biological process, CC cellular component, MF molecular function.

gastric cancer. We observed that the phosphorylation of AKT was suppressed after metformin treatment in BGC823, AGS, and SGC7901 cells (Fig. 4A). Next, we conducted a rescue assay with metformin and the AKT agonist SC79 to explore the role of AKT in the process by which metformin activates the cGAS/STING signaling pathway. However, the up-regulation effect of cGAS, STING, p-TBK1, and p-IRF3 in the metformin-treated cells was reversed after co-treatment with SC79 in both BGC823 and AGS cells (Fig. 4B). Likewise, CCL5, CXCL10, and CCL20 increased in the metformin-treated group but decreased in the SC79-treated group and combination group (Fig. 4C). These results demonstrate that metformin regulates the cGAS/STING signaling pathway by inhibiting AKT phosphorylation.

3.5. *Metformin mediates AKT signaling pathway through regulating SOX2 in gastric cancer cell lines*

Among the total DEGs, we screened out the fifty most increased and most decreased genes and performed *in vitro* experiments to verify the role of these genes. A significant decrease in SOX2 expression was detected in the metformin-treated gastric cancer cell lines AGS and SGC7901 (Fig. 5A), which is consistent with the results in GSE190076, GSE207122, and GSE208773 (Fig. S1). Meanwhile, we

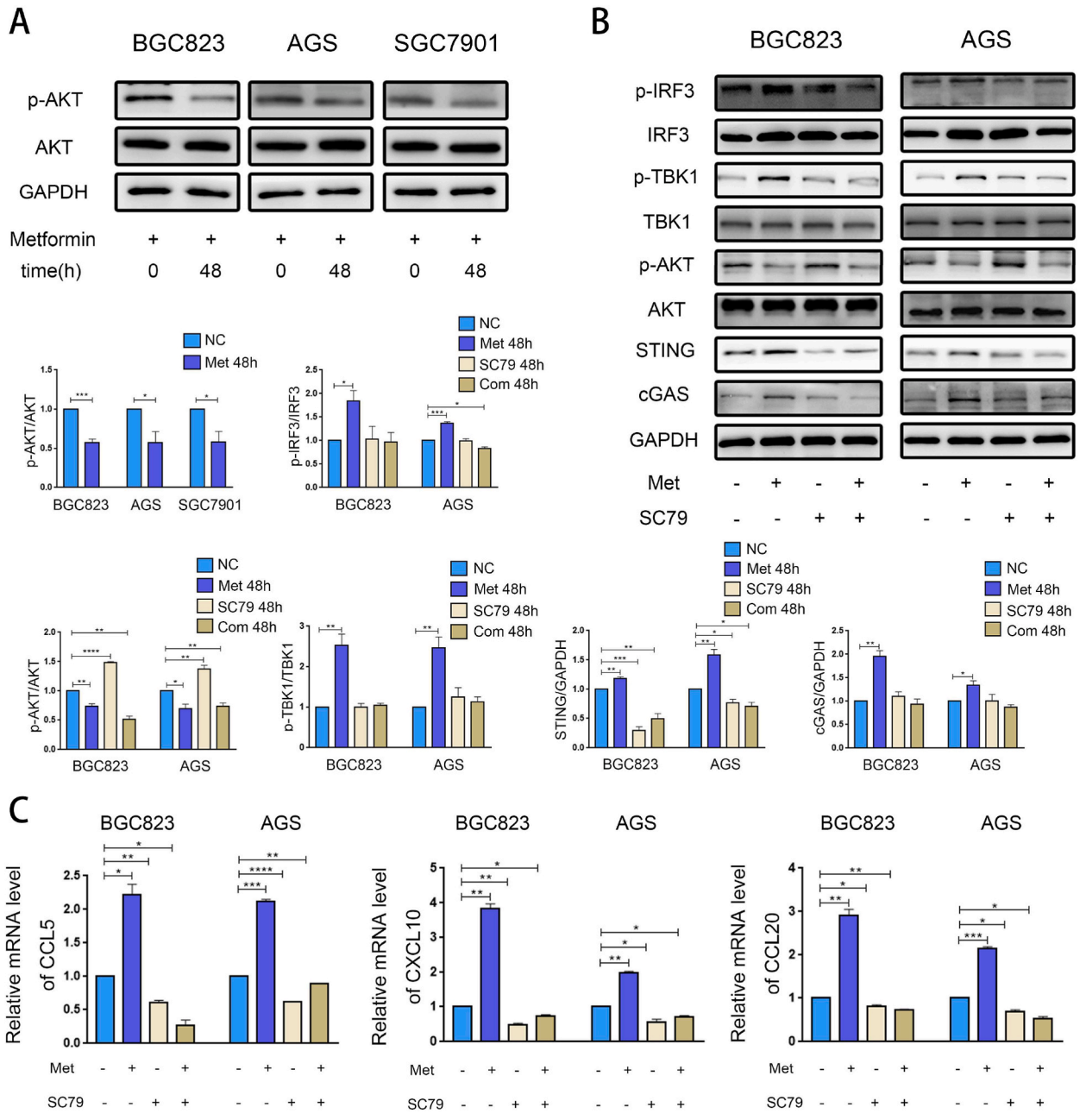

Fig. 4. Metformin activated cGAS/STING signaling pathway via blocking AKT phosphorylation in gastric cancer cells. **(A)** Western blot analysis of phosphorylated AKT (p-AKT) and total AKT in BGC823, AGS and SGC7901 cell lines with treatment of metformin (5 mM) for 48 h. Data are presented as mean ± SD (n = 3). $^{*}P < 0.05$. $^{***}P < 0.001$. **(B)** Western blot analysis of phosphorylated IRF3 (p-IRF3), total IRF3, phosphorylated TBK1 (p-TBK1), total TBK1, phosphorylated AKT (p-AKT), total AKT, STING and cGAS in BGC823 and AGS cell lines with treatment of metformin (5 mM), SC79 (20 μM), or combination therapy for 48 h. Data are presented as mean ± SD of three independent experiments. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$. **(C)** qRT-PCR analysis to show the mRNA expression level of CCL5, CXCL10 and CCL20 in BGC823 and AGS cell lines with treatment of metformin (5 mM), SC79 (20 μM), or their combination for 48 h. The data are reported as mean ± SD (n = 3). $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$.

found that the increased p-TBK1 and p-IRF3 levels in metformin-treated cells were reversed after overexpressing SOX2 (Fig. 5B). Likewise, CCL5, CXCL10, and CCL20 levels increased in the metformin-treated group but decreased in the SOX2-overexpressing and combination groups (Fig. 5C). These data suggest that metformin exerts its regulatory function by activating the cGAS/STING signaling pathway by regulating SOX2. Furthermore, we explored the relationship between AKT and SOX2. As shown in Fig. 5B and D, the expression level of phosphorylated-AKT (p-AKT) was increased in the SOX2-overexpressing group and reduced in the siSOX2-treated

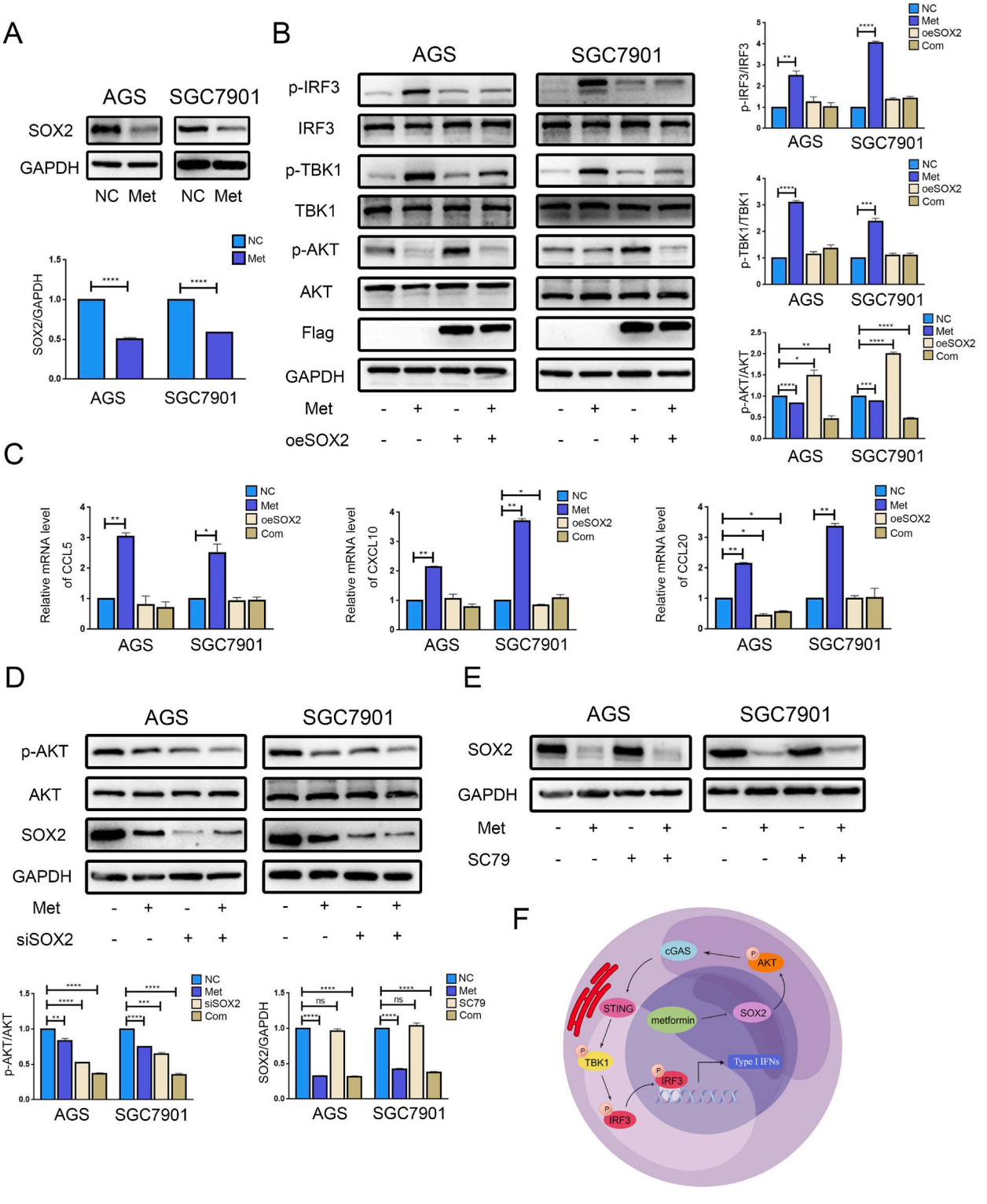

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Fig. 5. Metformin mediated AKT signaling pathway through regulating SOX2 in gastric cancer cell lines. **(A)** Western blot analysis of SOX2 in AGS and SGC7901 with metformin (5 mM) for 48h. **(B)** Western blot analysis of phosphorylated IRF3 (p-IRF3), total IRF3, phosphorylated TBK1 (p-TBK1), total TBK1, phosphorylated AKT (p-AKT), total AKT and SOX2 in AGS and SGC7901 with metformin (5 mM) for 48h, oeSOX2 or combination. **(C)** The mRNA expression level of CCL5, CXCL10 and CCL20 in AGS and SGC7901 with metformin (5 mM) for 48h, oeSOX2 or combination. **(D)** Western blot analysis of p-AKT and total AKT in AGS and SGC7901 with metformin (5 mM) for 48h, siSOX2 or combination. **(E)** Western blot analysis of SOX2 in AGS and SGC7901 with metformin (5 mM) for 48h, SC79 for 48h or combination. **(F)** Schematic of metformin regulating cGAS/STING signaling pathway through SOX2/AKT in gastric cancer. The data are reported as mean ± SD (n = 3). $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$. ns, not significant.

group compared to the control group. However, as shown in Fig. 5E, no difference was detected in the expression level of SOX2 between the control group and AKT agonist SC79 group. Thus, metformin activated cGAS/STING signaling pathway through regulating SOX2/AKT in gastric cancer according to the results above.

4. Discussion

Numerous studies have shown that metformin could exert its anti-tumor effect in several cancer types, including lung cancer, pancreatic cancer, breast cancer, prostate cancer, and colon cancer [15–19]. Previous studies have shown that metformin exerts anti-tumor effects mainly via inhibiting the mTOR1 signaling pathway in an AMPK-dependent or -independent manner in breast cancer [20,21]. Additionally, metformin could inhibit the IL-6/STAT3 signaling pathway by suppressing mesothelin expression in ovarian cancer, which led to the inhibition of tumor cell growth and migration [22]. In this study, we confirmed that metformin could activate the cGAS/STING signaling pathway by suppressing the SOX2/AKT axis in gastric cancer, which showed that metformin might be a promising strategy to amplify the anti-tumor efficacy of immunotherapy in gastric cancer.

The PI3K/AKT signaling pathway plays an essential role in regulating cell proliferation, metabolism, survival, and others [23]. Previous research has shown that metformin inhibits colorectal cancer cell growth by inhibiting the TGF-β/PI3K/AKT pathway [24]. Additionally, metformin can suppress the migration of human cervical cancer cells by inhibiting the PI3K/AKT axis [25]. Porcine circovirus 2 (PCV2), considered the primary pathogen of porcine circovirus-associated diseases (PCVAD), targets the lymphatic system and induces immunosuppression in pigs [26]. Recent research found that PCV2 promoted the phosphorylation of cGAS at S278 through activation of the PI3K/AKT signaling pathway [27]. In our study, we validated that metformin activated the cGAS/STING signaling pathway by inhibiting AKT phosphorylation. Further, we verified that metformin exerted its regulatory function in the AKT signaling pathway by suppressing the expression level of SOX2 in gastric cancer (Fig. 5F).

The transcriptional factor SOX2 is a member of the sex-determining region Y-box (SOX) gene family, which plays a fatal role in embryonic development and maintains the self-renewal characteristics of embryonic stem cells [28]. In our study, we found that SOX2 expression was downregulated after treatment with metformin using RNA-seq. Previous research found that metformin suppressed nicotine-induced esophageal squamous cell carcinoma by inhibiting the CHRNA7/JAK2/STAT3/SOX2 axis [29]. Another study found that activated STAT3 could move into the cell nucleus to directly bind to the SOX2 promoter, which upregulates SOX2 expression [30]. According to these two studies, metformin might inhibit SOX2 expression by regulating STAT3. A recent study showed that TGF-β induced the expression of SOX2 by targeting SOX4 in glioma stem cells [31]. Metformin may regulate SOX2 by inhibiting the TGF-β/SOX4/SOX2 axis. Further studies are needed to explore the regulatory mechanisms of metformin and SOX2.

In recent years, immunotherapy for advanced gastric cancer has made great breakthroughs, and ICIs have played a certain anti-tumor role in first-line, second-line, or post-line treatment, especially in first-line treatment [32–34]. However, most patients with advanced gastric cancer are insensitive to monotherapies [2]. Exogenous drugs enhancing the effect of ICI monotherapies is a feasible strategy, such as WEE1 inhibitors and ATM inhibitors [35,36], but in reality, most reagents have not been approved for clinical application due to drug toxicity. Metformin has been widely applied in clinical care, and our study validated the ability of metformin to activate immune-associated signaling pathways in gastric cancer, which showed that metformin had a great potential in improving the overall prognosis of patients with advanced gastric cancer in combination with immunotherapeutic reagents.

Undoubtedly, our study had limitations. *In vivo* experiments should be performed to confirm the ability of metformin to activate the cGAS/STING signaling pathway. Additionally, the mechanism underlying the regulation of SOX2 by metformin remains to be verified in further studies. Taken together, metformin combined immunotherapy is likely to sensitize the anti-tumor effect of immunotherapeutic drugs owing to its low toxicity and ability to activate innate immunity.

5. Conclusion

In this study, we confirmed that metformin activated the cGAS/STING signaling pathway by blocking AKT phosphorylation. Moreover, metformin regulated the AKT signaling pathway by mediating the transcription factor SOX2. Thus, our study indicates that metformin activates the cGAS/STING signaling pathway by suppressing SOX2/AKT and has promising potential in gastric cancer immunotherapy.

Declarations

Author contribution statement

Yuping Yin; Kaixiong Tao: Conceived and designed the experiments.
Qian Shen; Lei Yang: Performed the experiments; Wrote the paper.
Chengguo Li; Tao Wang: Analyzed and interpreted the data.
Yao Lin; Jianbo Lv; Weizhen Liu: Contributed reagents, materials, analysis tools, or data.

Data availability statement

Data will be made available on request.

Funding statement

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Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18954.

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