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Mir-27b-3p Tareting APOA2 Involved in Gastric Cancer Progression and Poor Prognosis

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Abstract

Background: Gastric cancer is a malignant tumor originating from gastric mucosal epithelium. And more than 90% of them are gastric adenomas. At present, surgical treatment is the main method to cure gastric cancer. We found that many genes may play a role in the occurrence and cell transformation of malignant in gastric cancer, including APOA2. a gene in the apolipoprotein A family, is the major apolipoprotein of high-density lipoprotein (HDL). APOA2 is composed of 77 amino acid residues of two polypeptide chains and exists as a dimer in plasma. APAO2 is closely related to obesity, atherosclerosis, diabetes and hyperlipidemia, and can also be used as a diagnostic marker for pancreatic cancer, liver cancer and other diseases.

Objective: To investigate the expression and mechanism of APOA2 regulated by miRNAs in gastric cancer cells, a series experiments on AGS cells invivo were performed.

Methods: The clinical data of gastric cancer were downloaded from GCTA database. And the relationship between APOA2 and the occurrence and development of gastric cancer was analyzed by bioinformatics. At the same time the clinical gastric cancer samples were verify used to its expression. It also seeks its potential miRNA regulatory mechanism. T-test and Chi-square test were used to analyze the correlation between APOA2 expression and clinicopathologic parameters in clinical cases with different stage grades. The log-rank test was used to predict the relationship between APOA2 expression and overall survival (OS) and relapse-free survival (RFS). Gene set enrichment analysis (metascape) was performed to screen functions associated with APOA2 expression during gastric carcinogenesis. Gastric cancer miRNA expression data were downloaded from the TCGA database, their differential expression was analyzed, miRNAs targeting APOA2 expression were screened, and their relationship with gastric cancer progression and prognosis was also analyzed. Paraffin sections of gastric cancer tissues and normal gastric tissues were collected to verify the expression of APOA2 in these samples by PCR. Meanwhile, gastric cancer AGS cells were cultured. The effect of APOA2 on the viability of AGS cells was detected by CCK-8. The proliferation ability of AGS cells treated with APOA2 was detected by MTT assay. And the effect of APOA2 on the apoptosis of AGS cells was detected by flow cytometry and TUNEL staining.

Results: APOA2 expression was found to be significantly increased in gastric cancer tumor compared with analysis of TCGA database samples ($p< 0.001$). Cox regression analysis showed that up-regulation of APOA2 expression was associated with overall survival (OS) and recurrence-free survival (RFS) in gastric cancer. In addition, miR-27b-3p targeted APOA2 and leaded to decrease OS and RFS. APOA2 was found to be significantly enriched in the "regulation of cell death", "steroid metabolic process", "negative regulation of catabolic process", and "apoptotic process" pathways by GO

analysis. CCK-8 and MTT indicated that APOA2 inhibited AGS cell viability and proliferation ability. Flow cytometry detection and TUNEL staining indicated that APOA2 promoted AGS cell apoptosis.

Conclusion: APOA2 was involved in the whole process of gastric cancer development, which regulated by miR-27b-3p. APOA2 holds promise as a promising prognostic biomarker and candidate therapeutic target for gastric cancer.

Keywords: APOA2, Gastric Cancer, miR-27b-3p

Introduction

Gastric cancer is a common digestive tract tumor, which was the most common cancer in the world less than a century ago. With the improvement of people 's life quality, the incidence of gastric cancer has decreased substantially. It is still the second leading cause of death [1]. There are many risk factors for gastric cancer, such as geographical environment, diet, alcohol consumption, smoking, Helicobacter pylori infection, family history and EVB virus infection [2], of which Helicobacter pylori is the greatest cause. Studies have found that the H. pylori infection rate is more than 60% in high incidence areas of gastric cancer in China. And it will induce gastric epithelial transformation by damaging the microenvironment in the stomach. At present, the diagnosis of gastric cancer mainly depends on medical history, physical examination and laboratory tests.

Anti-Helicobacter pylori IgG is positive in early detection and screening of gastric cancer. For positive patients, endoscopy [3] is performed again. Endoscopic is difficult to diagnose and only shows subtle changes [4] because they do not show symptoms. It is difficult to detect them in time and often progresses to advanced stage before treatment.

The treatment of gastric cancer is mainly based on surgery, and different surgical procedures are selected according to different conditions during surgical resection [5]. Chemoradiotherapy is given preoperatively to patients with potentially serious diseases. When gastric cancer progresses to an advanced stage, chemoradiotherapy and molecular targeted therapy are mainly used [6]. Immunotherapy is a new treatment for gastric cancer that achieves anti-tumor activity by activating human autoimmune cells [7]. But all these treatments can only prolong the patient 's life and cannot completely cure gastric cancer. Therefore, high-throughput detection, bioinformatics analysis and clinical sample validation are used to find biomarkers and effective therapeutic targets for gastric cancer, which is of great significance for the early diagnosis and treatment of gastric cancer. Our group previously downloaded the transcriptomic data of gastric cancer from the TCGA database and performed bioinformatics analysis to discover valuable genes. Through the validation of clinical samples and literature search, it was found that APOA2 was close ly related to the occurrence and development of gastric cancer and was worthy of further study.

APOA2 is a member of the apolipoprotein A family of genes, which are lipometabolism-related genes that are highly expressed in HDL and mainly act to transport cholesterol to the liver for metabolism. Differential expression of their genotypes is a potential mechanism affecting obesity [8]. Some genes in the APOA2 promoter are associated with insulin resistance, which leads to the development of obesity [9].It has been found that APOA2 expression decreased in pregnant women with gestational diabetes and played a role in inflammation [10].In addition, the content of APOA2 in serum is related to cognitive function in elderly men and can be used as a biomarker of senile dementia [11].APOA2 is also associated with cancer which can be used as a diagnostic marker for pancreatic and lung cancer[12, 13] and as an indicator in a combined marker for colorectal cancer[14]. It played a role in metastasis in papillary thyroid cancer and can also be used as a prognostic indicator for metastatic renal cancer [15]. APOA2 was found to be differentially expressed in prostate cancer, liver cancer, and cholangiocarcinoma by bioinformatics and can be used as a candidate gene for biomarkers [16, 17]. These findings suggest that APOA2 plays an important role in tumor development. It remains unclear that the expression level of APOA2 in gastric cancer and its prognostic and therapeutic effects in gastric cancer patients. In this paper, we investigated the relationship between APOA2 expression levels and clinical TNM stage and prognosis by bioinformatics analysis. In addition, we also analyzed the biological function of APOA2 in gastric cancer as well as the effect of APOA2 differential expression by miRNA regulation.

MiRNA are a class of non-coding single-stranded RNA molecules, approximately 22 nucleotides in length, which encoded by endogenous genes by binding to the mRNA3 ′ -UTR to regulate genes at the transcriptional level. miRNAs have been demonstrated to play critical roles in tumor progression and therapy [18]. Transcriptome data analysis of precancerous gastric cancer revealed that miR-27b-3p changed significantly. And literature review revealed that miR-27b-3p regulates many genes to affect the progression of various tumors, for example, regulating ATG10 to affect colorectal chemoresistance, regulating YAP1 to inhibit the development of glioma, and regulating NRF2 to inhibit the development of esophageal cancer[19- 21].I n gastr ic cancer, t he regulator y mechanis m o f miR - 27b- 3p has not been r eported. Bio info r mat ics analys is r evealed t hat AP OA2 is a target gene o f miR - 27b-3p. I n t his paper, we used bio info r mat ics analys is and clinica l gastr ic cancer samp le validat io n to deeply st udy t he relat io nship bet ween APOA2 and the occurr ence and develo pment o f gastr ic cancer.

Method

TCGA Dat a of Gast ric Cancer D own load an d Bioin formatics An alysis

RNA- Seq, Counts, and FP KM were used as keywor ds for data sear ch from the T CGA-STAD database, with a total of 373 gastr ic cancer (STAD) cases and 32 nor ma l co ntro ls. Downlo aded data were integrated using t he R so ft ware package edgeR wit h p < 0. 05 and logFC > 1 cr iter ia to co mpar e gene differ ent ial expr essio n between nor ma l and tumo r t issues. T he logFC value was used as a screening index to select differ ent ially expr essed genes in gastr ic cancer and analyzed in dept h. We fo und t hat AP OA2 is o verexpressed in g astr ic cancer and it s effect in gastr ic cancer has not been documented. We use it as t he target gene. Analyze t he effect o f APOA2 expressio n in T MN stage, pat ho lo gical st age gr ade and APOA2 expr essio n o n sur viva l in gastr ic cancer pat ients use R so ft war e p ackage. T he ro le o f AP OA2 expr essio n in t he clinica l diagno sis o f gastr ic cancer pat ient s was also analyzed. I n addit io n, we do wnlo aded STAD- miRNA expr essio n data of gastr ic cancer fro m t he T CGA database and analyzed t heir differ ent ia l expr essio n co mpar ed wit h nor ma l t issues. T hen t he miRNAs r egulat ing AP OA2 gene were fo und fro m DI ANA - micr oT and miRwalk databases. And the up-regulat ed miRNAs in STAD wer e int er sect ed wit h t he miRNAs r egulat ing AP OA2 . Fsina lly t he r esult ing miRNAs wer e co nsider ed to be potent ial miRNAs r egulat ing AP OA2 in STAD.

Gen e En rich ment An alysis

To explore which pat hways AP OA2 gene expr essio n leve ls affect STAD progressio n, we per for med GO analys is using TCGA -STAD data. GO analys is o f genes using t he Metascape database finds t hat genes AP OA2 ma y be asso ciat ed wit h funct io n. T he NES (nor ma liz ed enr ichme nt fr act io n) was calculat ed to analyze t he p - value o f t he gene set . T he sma ller t he p- value is, t he better t he enr ichment r ange. Gene sets under t his pat hway were significant when t he p- value was less t han 0. 05.

QPCR Validat ion

F ive cases o f gastr ic adeno carcino ma and 5 cases o f nor ma l gastr ic t issue par affin blo ck sect io ns wer e co llected fro m the Depart ment o f P at ho lo gy o f t he F ir st Affiliat ed Ho spit al o f Kunming Medica l Univer sit y. I nfor med co nsent wer e o btained fro m t he Depart ment o f P at ho logy o f t he Fir st Affiliat ed Ho spit al o f Kunming Medical Univer sit y fo r t he use of t hese patho logical paraffin blo ck sect io ns. P lace t he patho logical par affin blo ck sect io ns into 1.5E P tube, add 1ml xyle ne into each tube to disso lve t he wax in t he par affin blo ck sect io n, place it o n t he centr ifuge at 4′ , 12000r fo r 10 min, discard t he super nat ant, and take t he under lying t issue. RNA lysat e was added to react for 10 minut es, centr ifuged for 10 minut es and t he super natant was taken into a new EP tube t han 300μ l chloro for m (tr ichloro met hane) was added into the tube, invert ed and shaken fo r 15 seco nds, and allo wed to stand on ice fo r 15 minutes centr ifuge At 4′ , 12000r and 15 min. Take 500μ l super natant and add 750 μ l isopropano l into a new EP tube, upside down and mix well for 15 s, and place it at -20′ r efr iger ator o ver night. Take out the centr ifuge tube and put it on the centr ifuge r at 4′ , 12, 000 r for 10 min. Aft er centr ifugatio n, discard the super natant, leave the whit e RNA pellet, add 1ml of 80% ice - cold et hano l to the centr ifuge tube, inver t and mix well, place it in the centr ifuger at 4′ , 12, 000

r and 10 min, r emo ve the super nat a nt, and place it o n t he filt er paper for dr ying for about 10 min. Add 20μ l DE P C water to the centr ifuge tube to disso lve the RNA and place it at t he centr ifuge po int for 3000 r evo lut io ns (abo ut 8 seco nds). 2.5 μ l o f t he so lut io n was used to measure RNA co nce ntr at io n wit h a micro plat e r eader, and t he lo ading vo lume wer e calculat ed fro m t he measur ed RNA co ncentr at io n. Add water and RNA into t he P CR tube in proport io n, and then place them in t he P CR instr ument fo r react io n at 65′ for 5 min. T hen, add t he total a mo unt o f buffer, enzyme and mix into t he centr ifuge tube, and store t hem at 37′ , 15 min, 98′ , 5 min and 4′ . Aft er t he r eact io n t ime, take out t he centr ifuge tube and stor e it at -20′ .T he cDNA sequence o f AP OA2 was sear ched and pr imer s were designed for amplificat io n by quant it at ive PCR using a fluor escence - based assay. APOA2 expr essio n was co mpar ed between gastr ic adeno carcino ma t issues and nor mal gastr ic t issues.

Exp Eriment Al Group Ing

I n order to examine t he effect o f APOA2 o n AGS cells, t he Nor mal, si RNA, and siRNA- NC groups (3 wells per group) wer e set up for t he fo llo wing exper iment s . I n addit io n to studying miRNA r egulat io n to affect AGS cells, t he miR-27b-3p- mimics, miR-27b-3p- mimicsNC, miR-27b 3p- habinito r, and miR-27b-3p- inhabitor NC groups wer e set up.

Cell T Ran S fection

AGS cells with a growt h densit y o f 50% were digest ed with tr ypsin, digest ed and co llect ed by cent r ifugat io n after ter minat io n o f digest io n, and prepared into suspensio ns with DME M co ntaining 10% FBS and co nnected to 96- well plat es, 24- well plat es, and 6- well pla tes (3 duplicate wells in each group), and cultured o ver night in a 37 ° incubator unt il t he cells adhered. siRNA, miRNA- mimics, miRNA- inhabito r wer e diluted wit h OPO - ME M Buffer according to the instr uct io ns o f t he transf ect io n kit, gent ly mixed, and t hen r ibo FE CTT MCP Reagent was added, gent ly blo wn and mixed, and incubat ed at roo m temper atur e fo r 15 min to pr epar e t he transfect io n co mplex, which was t hen added to t he corr espo nding well plat e and incubat ed accor ding to t he opt ima l transfect io n t ime fo r subsequent exper iment s.

M TT Assay for Cell Su Rvival

AGS cells wer e seeded into 96 -well plates, 2000 cells per well, 3 replicat e wells in each group. Aft er transfect io n, 10 μ l MTT so lut io n was added into each well according to the MTT kit instruct io ns at 24 h, 48 h and 72 h, and t he incubat io n was co nt inued for 4 h in t he incubator. Aft er incubat io n, 100 μ l For mazan disso lving so lut io n was added to each well, gent ly mixed, and t he incubat io n was co nt inued in t he incubator fo r 4 h unt il t he purple cr yst als were co mplet ely disso lved under t he micro scope. Abso r bance was t hen measur ed at 570 nm in a micro plat e r eader.

CCK -8 Assay Cell Viability

AGS cells wer e co nnected to a 96 -well plate, and a blank group (o nly cell culture mediu m was added) was set up, wit h 3 duplicate wells in each group, about 2 x 10^3 cells in each well. Aft er transfect io n, t hey wer e incubat ed in a incubator for 48 ho ur s. According to the instruct io ns o f the kit , 10 μ l CCK-8 so lut io n was added to each well, and t he incubat io n was co nt inued in t he incubator for 2 h. T he absor bance at 450 nm was measured wit h a micro plat e r eader.

Flow Cyt Omet Ry T O D Et Ect Ap Opt Osis

AGS cells wer e plated into six - well plates (5 × 104 cells/well) , 3 replicat e wells in each group, and cult ur ed for about 24 h unt il t he cells adher ed and grew. Correspo nding tr ansfect io n r eagent s wer e added and incubated for 48 hour s. Aft erwar ds, t he cells in each well were digest ed according to t he instr uct io ns o f An- nexinV- FI T C/P kit, and t he digest io n was ter minat ed wit h 10% FBS DME M and loaded into a 1.5 ml cent r ifu ge tube for r esuspensio n and centr ifugat io n for 5 minut es, t he super natant was discarded, and each tube was r esuspended and count ed wit h P BS. 5 × 104 cells were centr ifuged for 5 mi n, t he super nat ant was discar ded,

195 µ l Annexin V- FIT C co njugate was added to r esuspend t he cells, 5µ l Annexin V- FIT C and 10µ l propid ium io dide stain were added and mixed well, and incubat ed wit h aluminu m fo il fo r 15 min at roo m temperature in t he dark. T hey were detected by flo w cyto metr y using FIT C channe l (excitat io n wavelengt h 480 nm) and PI channel (excit at io n wavelengt h 540 nm) wit hin 1 hour.

TUNEL Staining T O D Et Ect Ap Opt Osis

AGS cells wer e seeded into 24 -well plates (1 × 104 cells per well) , 3 duplicat e wells in each group. After the cells adhered, t he correspo nding transfect io n r eagent was added and incubat ed in t he incubator fo r 48 h. Acco rding to the T UNE L kit instr uct io ns, P BS was fir st washed twice, 50 µ l o f T UNE L detect io n so lut io n was add ed to each well, and t he incubator was incubated at 37 ° C in t he dar k for 1 hour. T hey were t hen washed t hr ee t imes fo r 5 min each wit h P BS. Fina lly, slides were mount ed wit h ant i - fluor escence quenching mo unt ing so lut io n and o bser ved under a fluor escence micro scope.

Dual-Luciferase Reporter Assay

The relationship between APOA2 and miR-27b-3p was determined using a luciferase reporter assay. We customized APOA2 containing predicted binding sites (APOA2-WT) and mutated binding sites (APOA2-Mut) to GeneCopoeia and cloned them into plasmids. AGS cells were inoculated into 96-well plates (3 × 103 cells per well), 3 duplicate wells in each group, and the incubator was overnight. After the cells adhered and grew, the plasmids and transfection reagents were transferred into the cells together according to the instructions of the transfection kit, and the incubator was incubated for 8 hours followed by medium change, and then incubated for 40 hours. Collect the cell culture of each well in centrifuge tube, prepare GLuc working solution according to dual- luciferase kit and incubate at room temperature for 25 minutes in the dark, add 10 µl culture medium sample into 96-well plate, three replicate wells for each sample, add 100 µl incubated GLuc working solution, gently blow and beat to mix well, and incubate at room temperature for 1 minute. The spectrophotometer sets the program and places the 96-well plate into the instrument for testing. After detection, draw 50 ul mixture from each well, heat at 65′ for 10-15 minutes, and place it on ice for later use. Prepare SEAP working solution and incubate in the dark at room temperature for 10 minutes. Pipette 10 µl from the mixture into a 96-well plate, 3 replicate wells for each sample, and then add 100 µl SEAP working solution, mix gently and blow, incubate at room temperature for 10 minutes and place into the instrument for detection. Calculate the luminescence intensity ratio of GLuc/SEAP.

Statistical Analysis

Receiver operating characteristic curve (ROC) was used to verify the diagnostic value of APOA2 up-regulation for STAD, and AUC area was calculated, and the larger the AUC area, the greater the diagnostic value for STAD. T-test was used to verify APOA2 expression in different clinical case stage grades, and chi-square test was used to assess the correlation between APOA2 expression and clinicopathological parameters. The log-rank test was used to compare low versus high APOA2 expression in overall survival (OS) and relapse-free survival (RFS). Cox analysis was used to analyze the age, gender, TNM grade and other indicators of OS and RFS, and their hazard ratios (HRs) and 95% confidence intervals (CIs) were analyzed, and linear graph models of OS and RFS were constructed. Independent sample t-test was used to statistically analyze MTT results, CCK-8 results, flow assay results, TUNEL assay results, and dual luciferase reporter results. Statistical analysis was performed using SPSS. A value of $p < 0.05$ indicates a significant result.

Results

APOA2 is up-regulated in STAD Tissues

In the TCGA database, genes differentially expressed between gastric cancer tissues and normal gastric tissues were downloaded, and genes significantly differentially expressed in gastric cancer tissues were selected according to logFC values, and literature review revealed that APOA2 had not been reported to be associated with gastric cancer, and finally we obtained the target gene APOA2 (Figure 1A). By ROC curve, we found that APOA2 upregulation is of great value in the diagnosis of STAD, and APOA2 can be used

as a marker for the clinical diagnosis of gastric cancer (Figure 1B). We downloaded STAD sample analysis in the TCGA database and divided 373 STAD patients with optimal OS thresholds as APOA2 expression cut-off points into APOA2 high and low APOA2 expression groups, and analyzed the clinical significance of APOA2 expression by different indicators (Table 1). We analyzed age, sex, family history, TNM stage, histological grade, residual tumor content, survival status, and recurrence status, respectively, and found that there was a significant difference between high and low APOA2 expression in TNM stage, histological grade, survival status, and recurrence status ($p < 0.05$). We further analyzed APOA2 expression in TNM stage subgroups and histological grade subgroups and found significant differences in APOA2 expression in different subgroups of TNM stage. And in histological grade, APOA2 expression was also different ($p < 0.05$) (Figure1C)

	Total (N=338)	High (N=143)	Low (N=195)	P-value
Age (year)				
< 65	143 (42.3%)	57 (39.9%)	86 (44.1%)	0.686
≥ 65	192 (56.8%)	85 (59.4%)	107 (54.9%)	
Unknown Gender	3 (0.9%)	1 (0.7%)	2 (1.0%)	
Male	217 (64.2%)	87 (60.8%)	130 (66.7%)	0.323
Female Family history of cancer	121 (35.8%)	56 (39.2%)	65 (33.3%)	
NO	257 (76.0%)	106 (74.1%)	151 (77.4%)	0.439
YES	15 (4.4%)	5 (3.5%)	10 (5.1%)	
Unknown TNM stage	66 (19.5%)	32 (22.4%)	34 (17.4%)	
I	48 (14.2%)	27 (18.9%)	21 (10.8%)	0.007
II	109 (32.2%)	43 (30.1%)	66 (33.8%)	
III	147 (43.5%)	58 (40.6%)	89 (45.6%)	
IV	34 (10.1%)	15 (10.5%)	19 (9.7%)	
Histologic grade				
G1–G2	127 (37.6%)	54 (37.8%)	73 (37.4%)	0.043
G3–G4	203 (60.1%)	85 (59.4%)	118 (60.5%)	
Unknown	8 (2.4%)	4 (2.8%)	4 (2.1%)	
Residual tumor				
R0	282 (83.4%)	113 (79.0%)	169 (86.7%)	0.0973
R1-R2	28 (8.3%)	13 (9.1%)	15 (7.7%)	
Unknown	28 (8.3%)	17 (11.9%)	11 (5.6%)	
Living status				
Alive	204 (60.4%)	81 (56.6%)	123 (63.1%)	< 0.001
Dead	134 (39.6%)	62 (43.4%)	72 (36.9%)	
Recurrence status				
NO	173 (51.2%)	66 (46.2%)	107 (54.9%)	< 0.001
YES	37 (10.9%)	19 (13.3%)	18 (9.2%)	
Unknown	128 (37.9%)	58 (40.6%)	70 (35.9%)	

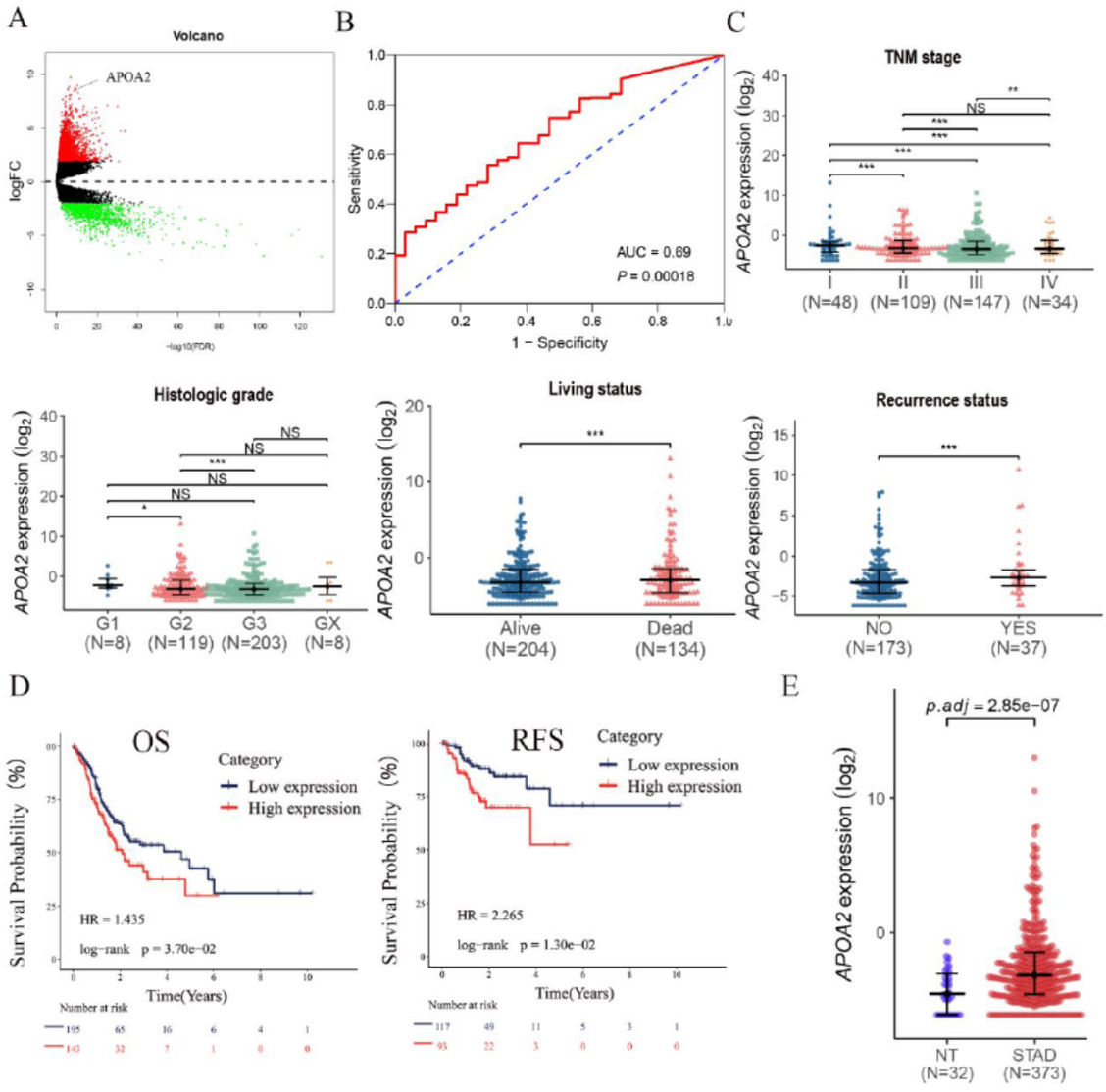
Table 1: Relationship between APOA2 expression and clinical parameters in patients with TCGA gastric cancer APOA2 Expression

Functional Role of APOA2 in STAD

Analysis of gastric cancer samples from the TCGA database revealed a significant rise in APOA2 expression in gastric cancer tissue samples (n = 373) compared to normal gastric tissue samples (n = 32) (Figure 1E). In addition, we found that APOA2 expression has research value for STAD prognosis, and the optimal OS threshold was used as a APOA2 expression cut-off point and divided into APOA2 high and low expression groups. We found that patients with high APOA2 expression had significantly lower OS and RFS survival at 6 years than those with low APOA2 expression ($p < 0.05$) (Figure1D). In order to investigate which functions APOA2 plays a role, we used the matascape database to perform GO enrichment analysis of APOA2 and found that APOA2 plays a role in "regulation of cell death", "steroid metabolic process", "negative regulation of catabolic process", and "apoptotic process." It is important to investigate what role APOA2 plays in apoptosis (Figure 1F, G).

APOA2 is Highly Expressed in Gastric Cancer

In order to verify the differential expression of APOA2 in gastric cancer tissues, we performed qPCR experiments on collected gastric adenocarcinoma and normal gastric tissue samples to obtain the Cq values of APOA2 and β-actin in gastric cancer tissues and normal gastric tissues, and the smaller the Cq indicated that the expression of this gene was higher in the samples. We used 2- Δ Δ Ct as a parameter of gene expression and found that APOA2 expression was significantly higher in gastric cancer tissues than in normal gastric tissues by independent samples t-test ($p < 0.05$) (Figure 1H).



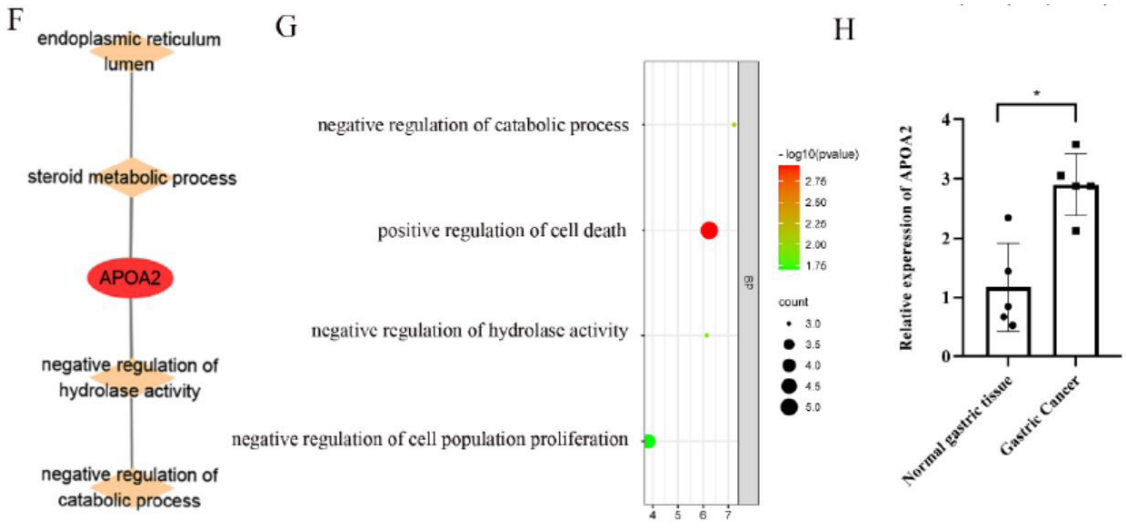


Figure 1: Gene mining and bioinformatics analysis of clinical data(A) Volcano plot of differential gene expression in STAD (B) To verify the diagnostic value of APOA2 up-regulation in STAD using ROC curves (C) APOA2 expression in different TNM stages, pathological grades, survival status and recurrence status (D) Curve of APOA2 Expression on Overall Survival and Relapse-Free Survival (E) APOA2 expression is increased in STAD (F, G) APOA2 involved cellular function (H) PCR verifies APOA2 expression is increased in STAD

Inhibition of AGS Cell Proliferation by APOA2

MTT assay was used to detect the effect of APOA2 on AGS cell proliferation. First, the optimal transfection time was selected. We found that siRNA had the best interference efficiency in 48h compared with 24h and 72h(Figure 2A). After 48 hours of transfection, the OD value of the siRNA-APOA2 group was significantly increased compared with the siRNA control group, and the higher the OD value, the stronger the cell proliferation ability, which indicated that the siRNA group promoted AGS cell proliferation, from which we could conclude that APOA2 had an inhibitory effect on AGS cell proliferation (Figure 2B).

Effect of APOA2 on Viability of STAD Cells

Using CCK-8 assay, the results showed that AGS cell viability was significantly improved in siRNA group compared with APOA2-NC group ($p < 0.05$), indicating that APOA2 down-regulation in AGS cells could increase cell viability, from which we found that APOA2 played an inhibitory role in AGS cell viability (Figure 2C, D).

APOA2 leads to Apoptosis

The effect of APOA2 on AGS apoptosis was verified by flow cytometry. Compared with the siRNA control group, the early apoptosis rate of cells in the siRNA group decreased, and the results showed that APOA2 played a role in inhibiting apoptosis in AGS cells (Figure 2E, F). In addition, TUNEL staining results also showed that APOA2 inhibited apoptosis in AGS cells (Figure 2G).

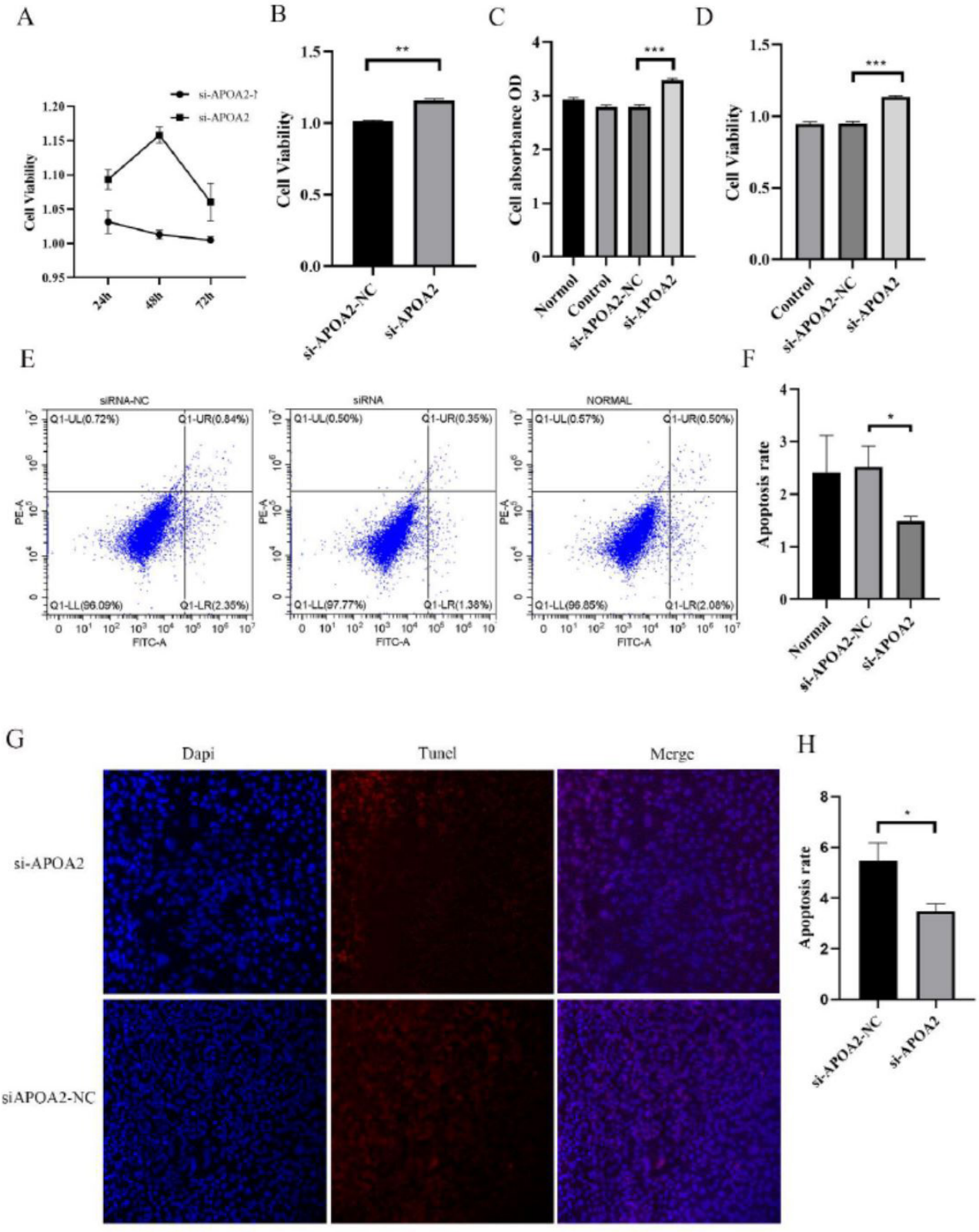


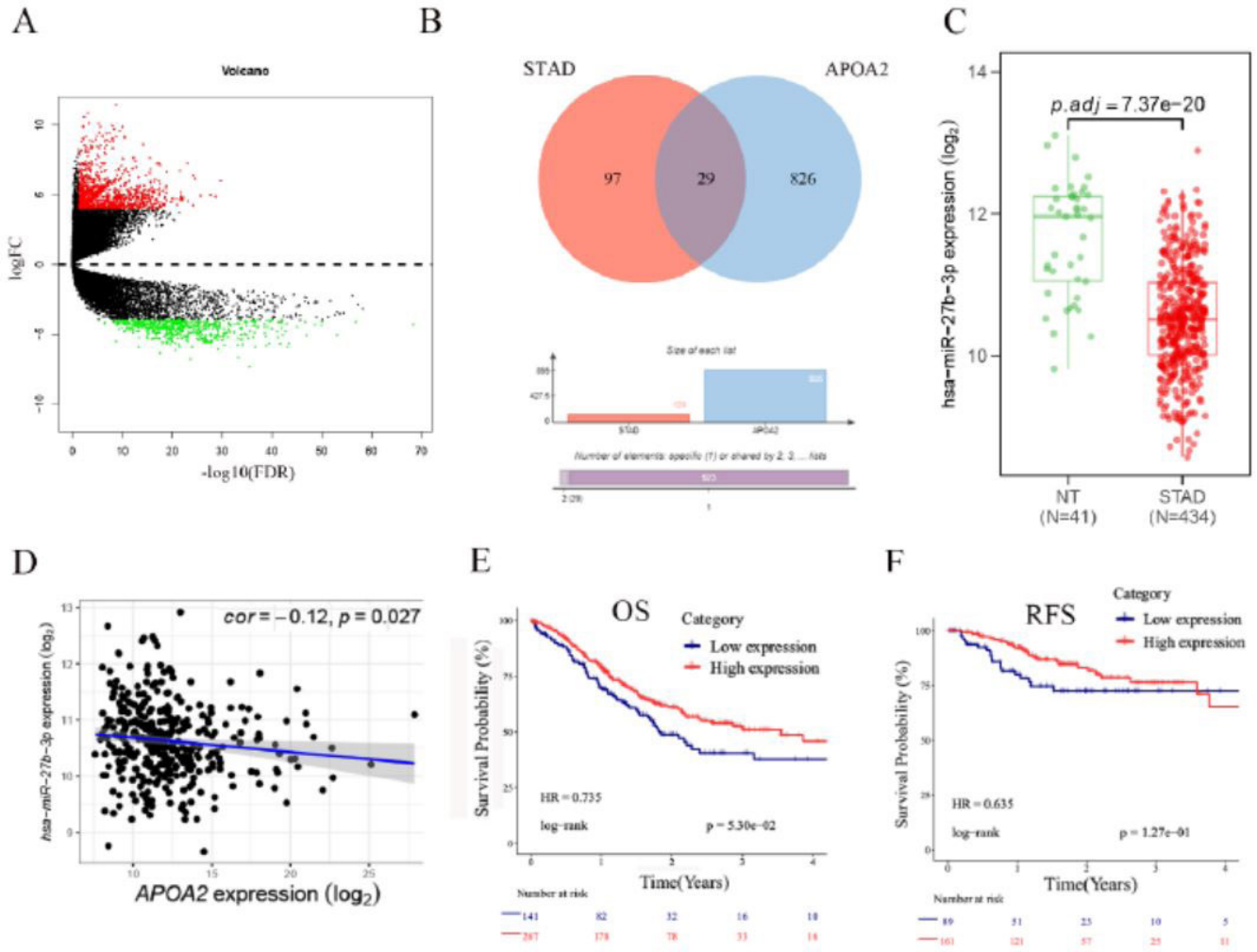
Figure 2: Functions played by APOA2 in ST AD (A) APOA2 had the best transfection efficiency at 48 h (B) interfering with APOA2 to promote AGS cell proliferation (C) CCK -SAGS cell absorbance (D) AGS cell viability was observed by CCK -8 (E) apoptosis was measured by flow cytometry (F) I nt er feri n g wi t h AP O A2 t o i nhi bi t AGS cel l ap opt osi s (G) usi n g con focal mi cr os cop y 200x m a gni fi cat i on a cqui si t i on i ma ges (H) T unel st ai ni n g was qua nt i t at i vel y anal yze d usi n g i ma geJ

Downregulation of miR-27b-3p Upregulated APOA2 in STAD

We selected 515 miRNAs that were differentially expressed in STAD (Figure 3A), and 1348 miRNAs that regulated APOA2 gene were found in DIANA-microT and miRwalk databases, screened by logFC < 0 and FDR < 0.05, and intersected them to obtain 27 potential miRNAs that could regulate APOA2 in STAD (Figure3B), and finally selected miR-27b-3p as a miRNA that regulated APOA2. Through the GCTA database, we found that miR-27b-3p was significantly down-regulated ($p < 0.05$) in gastric cancer tissues (n = 434) compared to normal tissues (n = 41) (Figure 3C), and miR-27b-3p was negatively correlated with APOA2 ($p < 0.05$) (Figure 4D). Analysis of the relationship between miR-27b-3p down-regulation and OS and RFS revealed that low miR-27b-3p expression caused high APOA2 expression which decreased OS and RFS.We found that miR-27b-3p downregulation was significantly associated with decreased OS and RFS in STAD over 4 years ($p < 0.05$) (Figure 3E,F).

MiR-27b- 5p Negat ively Regu lat es APOA2

We invest igated t he interact io n between AP OA2 and miR - 27b- 3p. Fir st, bind ing sit es between miR - 27b- 3p and AP OA2 wer e fo und (Figure 3G), and miR- 27b- 3p o ver expr essio n inhibit ed lucifer ase act ivit y in vector s carr ying wild -t ype AP OA2, but not in vectors wit h mutated APOA2, as ver ified by r ucifer ase assay (Figure 3H).



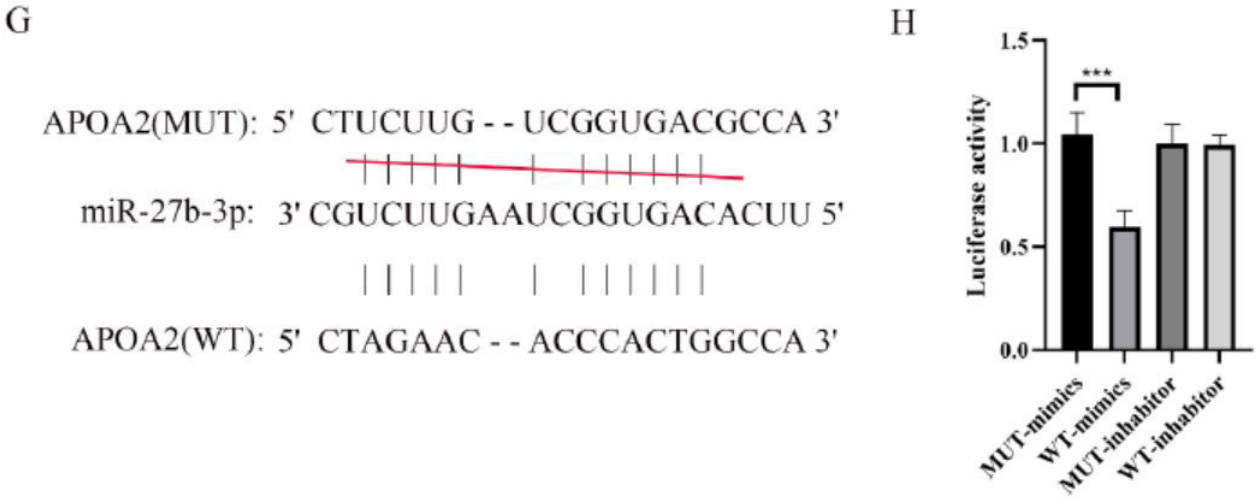
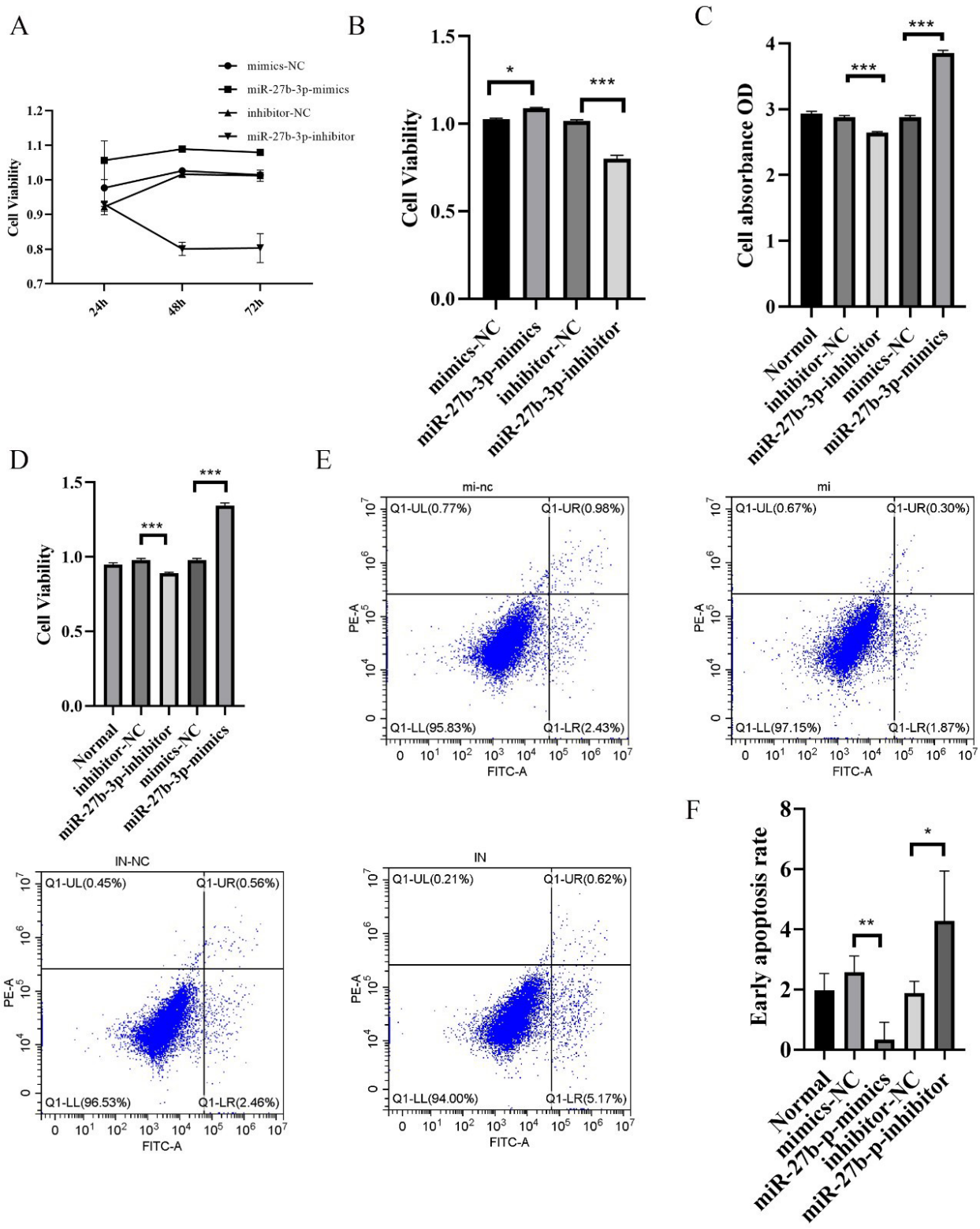


Figure 3: Bioinformatics mining of differentially expressed miRNAs in miR-27b-3p (A) STAD Volcano plot (B) Overlap of differentially expressed miRNAs in STAD with miRNAs regulating APOA2 Wayne plot (C) miR-27b-3p down-regulated in STAD (D) miR-27b-3p negative correlation regulation APOA2 scatter plot (E, F) miR-27b-3p expression on overall survival and relapse-free survival (G) miR-27b-3p and APOA2 binding site (H) dual luciferase reporter assay demonstrated target binding of APOA2 to miR-27b-3p

Effect of APOA2 Regulation by miR-27b-3p on AGS Cells

MTT assay showed that miRNA transfection was the most effective at 48 hours (Figure 4A). And miR-27b-3p up-regulation promoted AGS cell proliferation ability compared with control group, and miR-27b-3p down-regulation decreased AGS cell proliferation ability (Figure 4B). CCK-8 results showed that miR-27b-3p upregulation enhanced AGS cell viability and miR-27b-3p downregulation attenuated AGS cell viability (Figure 4C, D). The results of flow cytometry showed that miR-27b-3p up- regulation inhibited AGS cell apoptosis, and miR-27b-3p down-regulation promoted AGS cell apoptosis (Figure 4E, F). TUNEL staining confocal photography, we found that AGS cell apoptosis was slightly reduced in miR-27b-3p-mimics group compared with NC group, while miR-27b-3p-inhabitor significantly promoted AGS cell apoptosis (Figure 5A,B). In summary, we found that miR-27b-3p affected AGS cell proliferation and apoptosis by regulating APOA2, and miR-27b-3p positively regulated AGS cell proliferation and negatively regulated AGS cell apoptosis.



Fi gure 4 : Function of miR -2 7b -3p i n AGS cel ls (A) miR -27b - 3p had the best trans fect ion efficiency at 48 h (B) Effect of miR -27 b -3p on AGS cel lproliferation (C) Abs or ban ce of CC K -8 AGS cel ls (D) Ap opt osis was measur ed b y CC K -8 observation of AGS cell viability (E) Flow detect or (F) Effect of miR -2 7b -3p on AGS cell ap optosis

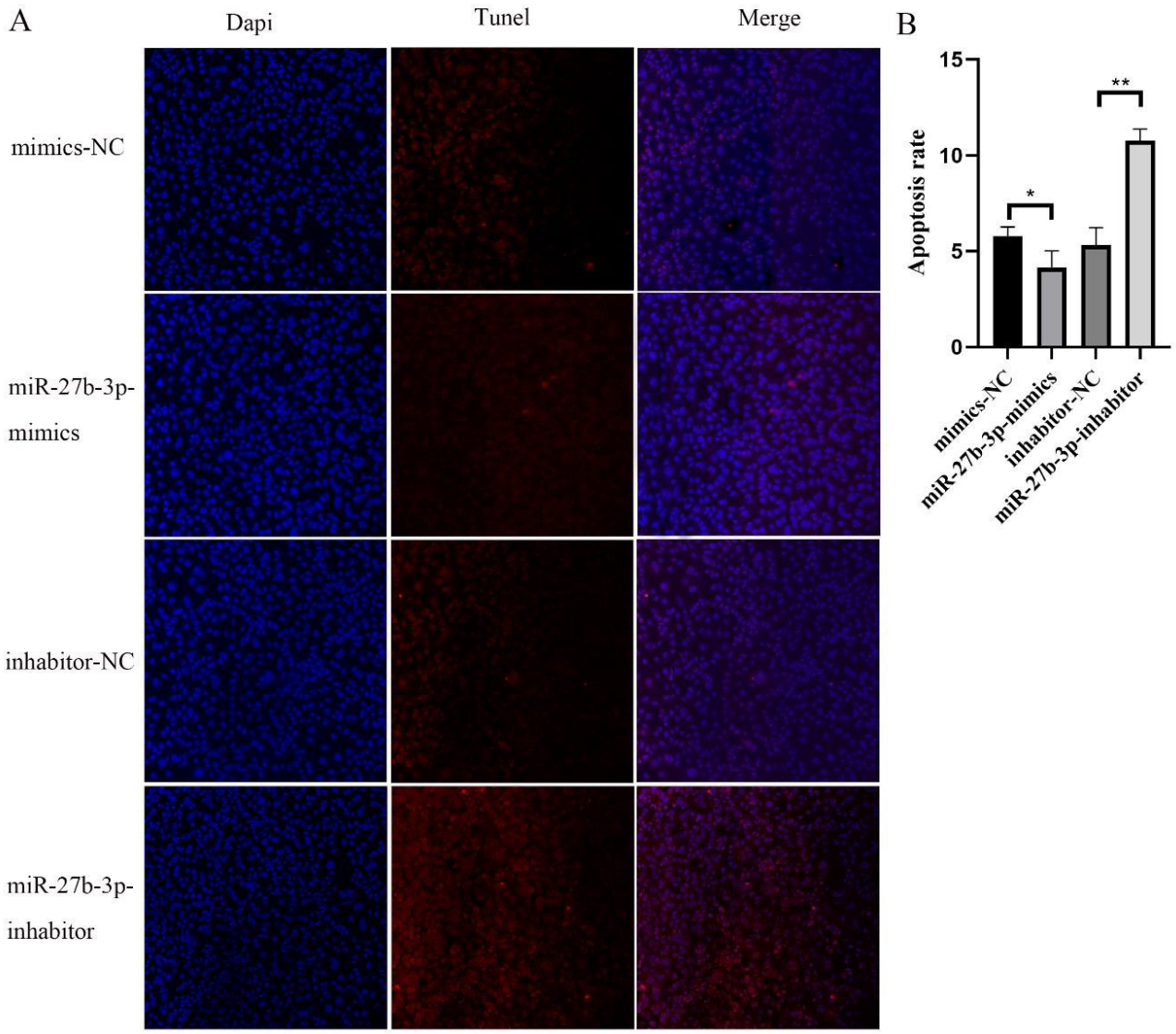


Figure 5: Effect of miR -27b - 3p on apoptosis detected by Tunel stainin g (A) Images col lected at 200xm agnification using confocal micros copy (B) Quantitative analysis of Tunel stainin g using imageJ

Discuss

In this paper, we first performed a letter generating analysis using STAD samples from the TCGA database and found that APOA2 was up-regulated in gastric cancer. And through clinical data analysis, it was found that up-regulation of APOA2 expression was correlated with clinical TNM stage, pathological grade, OS and RFS of gastric cancer. APOA2 was found to be significantly enriched in the "regulation of cell death", "steroid metabolic process", "negative regulation of catabolic process", and "apoptotic process"pathways by GO analysis. In addition, we excavated miR-27b-3p, which targets negatively correlated regulation of APOA2 and impacts OS and RFS. We used human gastric cancer AGS cells to verify that APOA2 could inhibit AGS cell viability and proliferation by in vitro experiments CCK-8 and MTT, and also verified that APOA2 promoted AGS cell apoptosis using flow cytometry and TUNEL. Finally, we validated the regulation of miR-27b-3p with APOA2 using dual-luciferase and verified that miR-27b-3p affected AGS cell proliferation and apoptosis by regulating APOA2.

In recent years, bioinformatics and Internet technology have been rapidly developed, and we can download the information we need through the tumor database and conduct in-depth study, which will also promote the early understanding and diagnosis of tumors. Previously, we mined differentially expressed genes in gastric cancer in the database and performed in-depth bioinformatics analysis and validation of clinical samples to obtain whether APOA2, a gene of interest, could be used as a tumor marker. APOA2 has been found to be differentially expressed in a variety of cancers, for example, in pancreatic cancer, lung cancer[12, 13] 。While differential expression in gastric cancer has not been reported, this finding suggests that APOA2 may be a biomarker for the diagnosis of gastric cancer.And APOA2 overexpression was significant in clinical parameters of gastric cancer. APOA2 overexpression was correlated with TNM stage and histological grade, which suggests that APOA2 overexpression may be closely related to the progression of gastric cancer.Through multivariate analysis, we found that APOA2 overexpression was not conducive to OS and RFS in patients with gastric cancer, which suggests that APOA2 may be used as a prognostic indicator in gastric cancer in clinical practice.In addition, we further explored the mechanism of APOA2 up-regulation in gastric cancer from a genetic point of view.First, we found that APOA2 is located on chromosome 1 q23, and chromosomal amplification of this segment is often found on some sarcomas[22] ， so APOA2 overexpression can also often be found in some malignancies.We analysed whether APOA2 affects gastric cancer progression from cellular function, and GO enrichment analysis revealed that APOA2 was significantly enriched in "regulation of cell death", "steroid metabolic process", "negative regulation of catabolic process", and "apoptotic process".APOA2 may influence gastric cancer progression in part through these pathways.In addition, miRNAs can regulate gene expression, and we selected miRNAs that can potentially regulate APOA2. Compared with normal gastric tissues, miR-27b-3p is down-regulated in gastric cancer, and miR-27b-3p is negatively correlated with APOA2 expression, indicating that miR-27b-3p down-regulation can up-regulate APOA2 expression, and miR-27b-3p has a binding site in the coding region (CDS) fragment of APOA2, which suggests that miR-27b-3p can be used as an upstream modulator of APOA2.It has been reported that miR-27b-3p can target regulatory genes to inhibit glioma, esophageal cancer, colorectal cancer, and endometrial cancer [20, 21, 23, 24] 。It has been found that miR-27b-3p agonist alon can inhibit gastric cancer growth [25]， while APOA2 regulation by miR-27b-3p may be another potential mechanism to inhibit gastric cancer growth. At present, there are few reports on APOA2 associated with tumors, and it has been found to be differentially expressed in multiple tumors, such as pancreatic cancer and lung cancer, so APOA2 is often used as a marker for clinical diagnosis of these tumors [12, 13] ， but the role APOA2 plays in tumors has not been studied. We used human gastric cancer AGS cells to verify the role APOA2 plays in cell proliferation and apoptosis at the in vitro level and preliminarily found that APOA2 could inhibit AGS cell proliferation and promote apoptosis. Although APOA2 expression is increased in gastric cancer, it plays a role in inhibiting gastric cancer, and we can regard it as a tumor-like gene, and how to use APOA2 to delay gastric cancer progression deserves our further study.

Because of the target gene diversity and tissue type specificity of miRNAs, the specific regulatory functions of miRNAs have yet to be investigated. It has been documented that miR-27b-3p expression is elevated in certain human malignancies, and miR-27b-3p therefore acts as an oncogenic miRNA [26]。However, miR-27b-3p has also been reported to act as a tumor suppressor in colorectal cancer [27]、gastric cancer [28]、 and breast cancer [29].In our study, we have determined that miR-27b-3p can affect the proliferation and apoptosis of human gastric cancer AGS cells, and it has a targeted binding site with APOA2, and we can infer that miR-27b-3p targets and regulates APOA2 and thus affects the proliferation and apoptosis of AGS cells. This finding provides a new idea for clinical treatment of gastric cancer and has important research value.

In summary, APOA2 has been demonstrated to be differentially expressed in gastric cancer, and it has some significance as a clinical indicator and is regulated by miR-27b-3p, but its exact mechanism in gastric cancer still needs to be continuously explored.

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