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Research

MicroRNA-34a negatively regulates Netrin1 and mediates MEK/ERK pathway to regulate chemosensitivity of gastric cancer cells

Haiping Liu[1] · **Limin Wang**[1]

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Abstract

Objective To explore the mechanism of action of MicroRNAs-34a (miR-34a) and Eurite growth guiding factor 1 (Netrin1) in cisplatin resistance in gastric cancer (GC), providing new clues for overcoming tumor resistance and optimizing anti-tumor therapy for GC.

Methods The Cancer Genome Atlas (TCGA), Differentially Expressed MicroRNAs (miRNAs) in human cancers (dbDEMC), and Starbase online databases were used to analyze the correlation between miR-34a and Netrin-1 and prognosis in GC, and to predict and verify the targeted binding of miR-34a to Netrin-1. The experimental methods including Cell transfection, real-time polymerase chain reaction (RT-PCR), Cell-Counting-Kit-8 (CCK8) assay, flow cytometry, wound scratch assay, transwell assay, and western blotting were used to investigate the effects of miR-34a and Netrin1 on chemotherapy resistance and biological characteristics in cisplatin-resistant GC cells (HGC27/DDP), and to analyze the molecular mechanism of cisplatin resistance.

Results miR-34a expression was downregulated in gastric cancer clinical samples and cisplatin-resistant cells, while Netrin1 was upregulated, and was related to overall survival (OS). Upregulation of miR-34a can significantly reduce the IC_{50} value of cisplatin(0.65 vs 1.6 ng/mL) and Multidrug Resistance 1 (MDR-1) protein level, inhibit the proliferation activity, reduce the expression levels of proliferating cell nuclear antigen (PCNA) and ki-67 protein, and induce the increase of apoptosis rate and the enhancement of cycle arrest. Upregulation of miR-34a can also significantly reduce the expression level of Matrix metalloproteinase 9 (MMP9) protein, promote the expression of E-cadherin protein, reduce the wound healing rate and invasion number to inhibit migration and invasion ability in drug-resistant gastric cancer cells. Moreover, overexpression of Netrin1 on the basis of upregulation of miR-34a can weaken the above changes caused by upregulation of miR-34a. In addition, upregulation of miR-34a can significantly inhibit the Mitogen-activated protein kinase kinase (MEK) / Extracellular regulated protein kinases (ERK) pathway, while overexpression of Netrin1 can activate the MEK/ERK pathway, and inhibition of MEK/ERK pathway can effectively counteract the protein expression of Netrin1, and reverse changes in the expression of cisplatin IC_{50} and MDR-1 proteins caused by co-upregulation of miR-34a/Netrin1 in HGC27/DDP, as well as changes in proliferation, apoptosis, migration and invasion. In addition, upregulation of miR-34a can significantly inhibit the MEK/ERK pathway, while overexpression of Netrin1 can activate the MEK/ERK pathway. If the MEK/ERK pathway was inhibited, it can effectively counteract the protein overexpression of Netrin1, and reverse the changes in the expression of cisplatin IC_{50} and MDR-1 proteins in HGC27/DDP induced by co-upregulation of miR-34a / Netrin1, as well as changes in proliferation, apoptosis, migration and invasion.

Conclusion miR-34a targets and negatively regulates Netrin1 to mediate the proliferation, apoptosis, apoptosis, migration, and invasion of drug-resistant gastric cancer cells via the MEK/ERK pathway, and change the chemosensitivity in

✉ Limin Wang, minhaii@126.com | [1]Key Laboratory of Microecology-Immune Regulatory Network and Related Diseases, School of Basic Medicine, Jiamusi University, 348 Dexiang Street, Xiangyang District, Jiamusi City 154000, Heilongjiang Province, People's Republic of China.

GC cells. miR-34a/Netrin1/MEK/ERK axis may serve as a novel therapeutic target for chemoresistance in GC, it is of great significance for overcoming drug resistance and developing new therapeutic strategies for GC.

Keywords MicroRNA-34a · Netrin1 · MEK/ERK pathway · Chemoresistance · Gastric cancer

1 Introduction

Gastric cancer (GC), one of the most common malignant tumors of the digestive tract, is a serious threat to human health. GC incidence rates have decreased in the last few decades in most parts of the world, but it is still the fourth most common cancer and the second most common cause of cancer-related death. Every year, approximately 990,000 people are diagnosed with GC worldwide, of whom approximately738,000 die, Among them, more than 40% of new incidents occur in China, and the 5-year overall survival rate of patients with advanced stage GC is less than 10% [1, 2]. Currently, chemotherapy is a vital treatment method to improve the survival rates of patients with GC, especially those with advanced GC, and platinum-based chemotherapy is the first-line treatment for GC. However, most GC patients develop resistance to chemotherapy drugs during treatment, which greatly increases the difficulty of anti-tumor [3]. Therefore, it is necessary to continue to explore the mechanism of GC chemoresistance, search for GC therapeutic targets, and develop effective methods to overcome chemotherapy resistance.

MicroRNA-34a(miR-34a) is induced by the tumor suppressor gene p53 and exhibits anti-tumor properties [4]. In recent years, studies have shown that miR-34a may be involved in the chemoresistance of various malignant tumors. For example, in breast cancer, miR-34a changes the sensitivity of breast cancer patients to Sunitinib by regulating the Wnt/ β-catenin signaling pathway [5], and also changes the resistance of breast cancer cells to doxorubicin by regulating Notch homolog 1 [6]. More, miR-34a can also form a signal axis with Interleukin-6 (IL-6) / Interleukin 6 Receptor (IL-6R) to promote triple-negative breast cancer epithelial-mesenchymal transition (EMT) process, which affects prognosis [7]. In colorectal cancer, targeting the miR-34a / leucine-rich PPR-motif containing (LRPPRC) / MDR1 axis reduces chemoresistance inducted by P53 inactivation [8]. In ovarian cancer, the miR-34a / Programmed Death-Ligand 1 (PD-L1) axis regulates cisplatin chemotherapy resistance of ovarian cancer cells [9]. In gastric cancer, Deng XJ et al. found that miR-34a regulates the Phosphatidylinositide 3-kinases (PI3K) / Serine/Threonine Protein Kinase B (AKT) / survivin pathway to regulate gastric cancer cell death induced by cisplatin [10]. A review study based on data mining showed that miR-34a may be involved in the pathways of gastric cancer cell stemness, EMT and drug resistance. However, the progression and drug resistance mechanism of gastric cancer are complex, and relatively few relevant studies have been conducted. there should be additional experimentation to validate these results [11]. Neurite growth guiding factor 1 (Netrin1) is the most characteristic molecule in the netrin family, which is involved in regulating various biological processes such as cell proliferation, migration, and differentiation, and has been confirmed to be related to the invasion and metastasis of malignant tumors [12]. Ducarouge B et al. showed that Netrin1 blockade inhibits cancer stemnes of tumor cells, and alleviates resistance to chemotherapy [13], Cassier PA et al. showed that Netrin1 blockade inhibits tumour growth and EMT features in endometrial cancer [14]. Bellina M et al. pointed out that Netrin-1 may be a potential molecular marker and therapeutic target for various malignant tumors [15]. In recent years, it has been found that Netrin1 can be used as a target gene of miRNA to participate in the mechanism of tumor chemotherapy resistance such as miR-214 reduces cisplatin resistance in bladder cancer cells by targeting netrin-1 [16]. In human GC, Netrin1 has a higher level than the para-carcinoma tissueand is related to tumor invasion and metastasis, and low levels of Netrin-1 contribute to prolonged overall survival [17]. Mechanistically, a few studies have shown that Netrin-1 promoted synergistically the proliferation and invasion of GC cells by regulating signaling pathways, such as the PI3K/AKT or ERK/MAPK pathways [18, 19].This reminds us that regarding Netrin1 may act as a target of miR-34a to regulate related signaling pathways mediating chemotherapy resistance in GC. MEK/ERK pathway is a typical tumor signaling pathway, which can be activated by various pathways to mediate tumor cell proliferation, apoptosis and other biological functions [20]. Previous studies have shown that Dual Specificity Phosphatase 2 (DUSP2) can down-regulate the MEK/ERK signaling pathway through Recombinant Protein Tyrosine Phosphatase, Non Receptor Type 7 (PTPN7) and affect the prognosis of bladder cancer [21], miR-206/E26 transformation-specific-1 (ETS1) promotes ovarian cancer progression through the MEK/ERK pathway [22], miR-770-5p/TRIM29 promotes gemcitabine resistance in patients with pancreatic ductal adenocarcinoma through the MEK/ERK pathway [23], miR-939/solute carrier family 34 (sodium phosphate), member 2 (SLC34A2) regulates chemotherapy resistance and metastasis of gastric cancer through Raf/MEK/ERK pathway [24]. This suggests that miRNA binding target genes may be involved in tumor chemotherapy resistance through some signaling pathway. Combined with the above

studies, we hypothesized that mir-34a/netrin1/MEK/ERK may be related to the mechanism of drug resistance in gastric cancer, which should be confirmed experimentally.

In this study, we analyzed the expression of miR-34a and Netrin1 in GC and their relationship with prognosis using online bioinformatic tools. The targeted binding relationship between miR-34a and Netrin1 was predicted and validated, and the role of miR-34a and Netrin1 in the development of cisplatin (DDP) resistance in GC was investigated in vitro. The results showed that the upregulation of miR-34a inhibited the proliferation, migration, and invasion of GC cells and induced apoptosis and cycle arrest, thereby improving the chemosensitivity of cisplatin. Mechanistically, miR-34a negatively regulates Netrin1(a direct target of miR-34a) to activate the MEK/ERK pathway, thereby affecting drug-resistant cell proliferation, apoptosis, migration, and invasion (The experimental design flow chart of this study is shown in Fig. 1). Thus, the miR-34a / Netrin1 / MEK/ERK axis could be a novel therapeutic target for chemoresistance in GC. This provides a new clue for anti-tumor treatment and overcoming chemoresistance.

2 Materials and methods

2.1 Cell lines and reagents

HGC27 (undifferentiated) was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences, and HGC27/DDP (Human gastric cancer cisplatin resistant cells) was established by our research group (Provincial Key Laboratory of School of Basic Medicine, Jimusi University). RPMI 1640 medium (including Penicillin and Streptomycin), inactivated fetal bovine serum (FBS), 0.25%Trypsin–EDTA (Gibco, Carlsbad, CA), Lipofectamine TM 3000 transfection reagent (Thermo, Waltham, MA), Netrin-1 WT, Netrin-1 MUT, pcDNA3.1-Netrin1 recombinant plasmids, and miR-34a mimic were synthesized by Beijing Krisbo Biotechnology Co., Ltd.. DDP, PD98059 (MCE, Beijing, China). The antibodies used were anti-Netrin1, anti-PCNA, anti-Bax, anti-Bcl-2, anti-MEK1/2, anti-p-MEK1/2, anti-ERK1/2, anti-p-ERK1/2 (Abcam, Cambridge, UK), anti-MMP9, anti-E-cadherin antibodies (Invitrogen, San Diego, CA); Annexin V-FITC/PI double staining apoptosis kit, PI single staining cell cycle detection kit, double-luciferase reporter gene detection kit (Beyotime, Beijing, China); CCK8 kit, RT-PCR kit, BCA protein detection kit (Solarbio, Beijing, China).

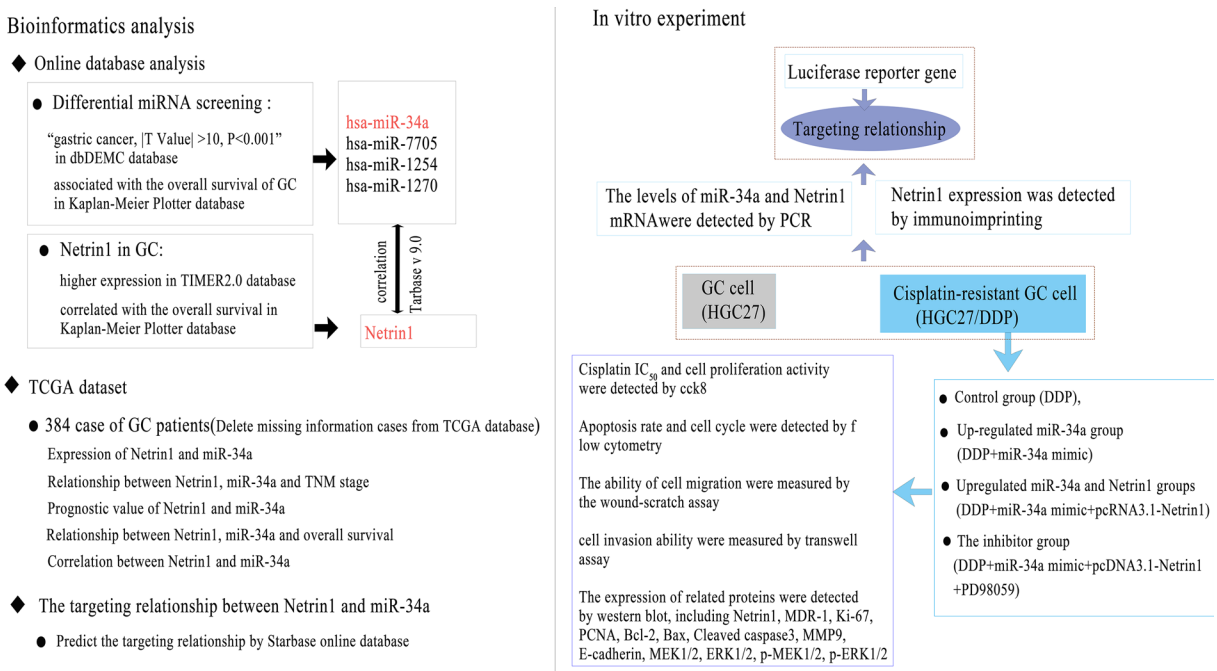

Fig. 1 The experimental design flow chart of this study

2.2 Bioinformatics analysis

2.2.1 Screening of different genes in gastric cancer

Using the human tumor differentially expressed MiRNAs database (dbDEMC) [25] (https://www.biosino.org/dbDEMC) was used to screen difference of miRNAs in GC. Search "gastric cancer" according to cancer type, download the "Differentially Expressed miRNAs List", and screen out the miRNAs with "$|T$ Value$|> 10$, $P < 0.001$". Kaplan–Meier Plotter online database [26] (https://kmplot.com/) was used to analyze the relationship between the screened miRNAs and the prognosis of GC. That is, the miRNAs screened from dbDEMC were respectively searched in the Pan-cancer miRNA interface, and "Stomach adenocarcinoma" is selected to obtain the survival curve analysis results of miRNAs in GC, and further screen out the miRNAs that is related to the overall survival of GC. Then, Using TIMER2.0 database [27] (http://timer.cistrome.org/) to obtain Netrin 1 expression patterns in tumors to confirm Netrin1 difference expression in GC, Then, The relationship between Netrin1 and overall survival of GC was obtained from the Kaplan–Meier Plotter online database. Finally, The tarbase v 9.0 online database (https://dianalab.e-ce.uth.gr/tarbasev9/) was used to predict the interaction between netrin-1 and miRNAs screened above, finding that only netrin-1 interacted with miR-34a.

2.2.2 Source and analysis of clinical datasets

Based on the Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/), the relevant clinical data of GC patients and expressions of miR-34a and Netrin-1 were downloaded, and data of patients with invalid or missing information were deleted to finally obtain the dataset of this study. including gender, age, gastric cancer type, pathological features, survival, and miR-34a and Netrin-1 expression level. The expression difference of miR-34a and Netrin-1 in gastric cancer was observed, and the relationship between miR-34a, Netrin 1 and Tumor Node Metastasis (TNM) stage of gastric cancer was examined by analysis of variance (ANOVA). Receiver operating characteristic curve(ROC) was used to analyze their predictive value to overall survival in gastric cancer, and Kaplan–Meier survival analysis was used to analyze their relationship with overall survival rate. Then, the correlation between miR-34a and Netrin-1 was analyzed by Pearson analysis.

Prediction of the targeting relationship between miR-34a and Netrin-1 [28]

The starbase 3.0 online database (https://starbase.sysu.edu.cn/) was used to retrieval miR—34a target genes, and obtain the targeted binding sites of miR—34a and Netrin 1. After that, target validation was performed according to the instructions of the double luciferase reporter gene detection kit.

2.3 Cell culture and transfection

HGC27/DDP cells were cultured in RPMI 1640 medium containing 10% FBS at 5% CO2, 37℃, and 90% humidity. When the degree of cell fusion reached 80–90%, according to the 1:3 passage culture. HGC27/DDP cells at the logarithmic growth stage were inoculated into 96-well plates at a density of 4×10^3 cells/well and cultured for 24 h. The cells were named as DDP group, DDP + miR-34a mimic group, DDP + miR-34a mimic + pcDNA3.1-Netrin1 group, and DDP + miR-34a mimic + pcDNA3.1-Netrin1 + PD98059 group. Corresponding mimics/recombinant plasmids were added to the above groups respectively, A mixed solution composed of mimic / recombinant plasmids (including mimic-NC, miR-34a mimic, Netrin 1 WT, Netrin 1 MUT, pcDNA3.1-Netrin1) and Lipofectamine TM 3000 was added accordingly. After 6h of continuous culture, the complete medium was replaced and the culture was continued. After transfection for 24 h, the expression levels of miR-34a and Netrin1 were detected by RT-PCR to confirm transfection success.

2.4 Real-time PCR

Total RNA extraction, reverse transcription, and PCR amplification were performed in strict accordance with the manufacturers' instructions. The cycle parameters of PCR amplification were as follows: 1 cycle with a pre-incubation step (95℃ for 5 min), 40 cycles with an amplification step(95℃ for 30 s, 60℃ for 45 s, 72℃ for 20 s). The expression of the

miR-34a gene was based on U6, the expression of Netrin 1 gene was based on β-actin, and the relative quantification of mRNA was calculated with $2^{-\Delta\Delta Ct}$. The primer sequence used was as follows: miR-34a forward.

5′-CCAGCTGTGAGTAATTCTTTGGCA-3′ reverse 5′-AATGTGCAGCACTTCTAGGGC-3′; U6, forward 5′-GCTTCGGCAGCACATATACTAAAAT-3′ reverse 5′-CGCTTCACGAATTTGCGTGTCAT-3′; Netrin 1, forward 5′-TTCCTCACCGACCTCAACAA-3′ reverse 5′-GTTGTACATCTTGCGGCACT-3′; β-actin, forward 5′-AGAAAATCTGGCACCACACC-3′ reverse 5′-TAGCACAGCCTGGATAGCAA-3.′

2.5 CCK8 assay

Cells transfected for 24 h were inoculated into 96-well plates at 2×10^3 cells and six parallel cells in each group. DDP, DDP + miR-34a mimic, and DDP + miR-34a mimic + pcDNA3.1-Netrin1 groups were replaced with complete medium. The DDP + miR-34a mimic + pcDNA3.1-Netrin1 + PD98059 group was cultured in a medium containing PD98059. Cells from each group cultured for 24 h were treated with cisplatin (DDP) concentrations of 2, 1.8, 1.6, 1.4, 1.2, 1, 0.8, 0.6, 0.4, 0.2, 0.1ng/mL, respectively. After 24 h, 10μL of cell-counting-kit-8 (CCK8) solution was added to the medium (100 μL per well) and incubated in the incubator for 2h. In addition, cells from each group at 0h, 24 h, 48 h, 72 h and 96 h were added to CCK-8 solution and incubated for 2 h at 37 °C, 5%CO2. Optical density (OD) at 490 nm was measured using a microplate reader (Thermo, Waltham, MA, USA). A larger OD indicates a stronger cell proliferation ability.

2.6 Flow cytometry

Cells in each group were collected after treatment were taken respectively (final concentration of cells ≥ 1×10^6/mL). Apoptosis was detected according to the instructions of the Annexin V-FITC/PI Apoptosis Detection Kit and PI Cell Cycle Detection Kit, respectively. All samples were examined using a flow cytometer (Becton Dickinson, Franklin, NJ), and FlowJo software was used for data analysis.

2.7 Wound-scratch assay

Cells of each groups After treatment, 5×10^5 cells were inoculated into 6-well plates marked with parallel lines of the marker, and three parallel cells were set in each group. When the cells were attached to the bottom of the culture hole, a scratch with a 20 μL gun tip along the edge of the ruler and hanging down to the hole plate and marker line. After removing excess cells, serum-free medium was replaced. The cell samples were taken at 0, 24, and 48 h, and observed and photographed under a 100-fold microscope. The changes in scratch area were analyzed with ImageJ software (v1.8.0 Chinese version), and the wound healing percentage in each group was calculated according to the formula "(initial area—area at a certain point in time)/initial area healing × 100%."

2.8 Transwell assay

Cells of each groups. After treatment, 2×10^4 cells were inoculated into the upper layer of a transwell chamber covered with liquid Matrigel matrix gel and FBS-free medium, and the lower chamber was added with 20%FBS medium 500 μL. After culturing in an incubator for 24 h, the upper chamber of the Transwell chamber was removed, and the matrix glue and non-migrating cells in the upper chamber were wiped away, fixed with formaldehyde for 30 min, and stained with 0.1% crystal violet for 20 min. The number of invading cells in each group was counted.

2.9 Western blot

After treatment, cells from each group were lysed using RIPA lysis buffer to extract the total protein. The total protein concentration was determined according to the instructions of the BCA Protein Detection Kit. Protein samples and molecular weight markers were added to separate wells, separated by SDS-PAGE, transferred to PVDF membranes, and blocked in 5% dry milk in Tris-buffered saline with Tween 20 (TBST, PLYGEN, Beijing, China). Next, the membranes were placed in a diluent of primary antibodies and incubated at 4℃ overnight, after that incubated the secondary antibodies and the membrane for 2 h at room temperature on a shaker. Target proteins were detected by enhanced chemiluminescence (ECL), and ImageJ software (v1.8.0 Chinese version) was used to quantitatively analyze the relative expression of target proteins.

Fig. 2 Screening of different genes in GC by bioinformatics. **A** Differential expression of miRNA in gastric cancer based on dbDEMC database. **B** Expression profile of Netrin1 in different tumors from TIMER2.0 database. **C** Relationship between miRNA and overall survival in GC patients by Kaplan–Meier Plotter database. **D** Relationship between Netrin1 and overall survival in GC patients by Kaplan–Meier Plotter database. TPM: Transcripts Per Million

2.10 Statistical analyses

Data were analyzed using SPSS26.0 and GraphPad Prism8.0. Transcripts Per Million (TPM) indicating miR-34a and Netrin1 gene expression from the online database, and the predictive value of miR-4a and Netrin1 on the prognosis of GC were analyzed by ROC. The Kaplan–Meier method was used to analyze the correlation between miR-34a and Netrin1 and the prognosis of GC. The experimental data are presented as mean ± standard deviation (SD). An independent sample t-test was used to compare the measurement data between the two groups, and ANOVA was used to compare the multi-component data, and the correlation between miR-34a and Netrin-1 was analyzed by Pearson analysis. $P < 0.05$ indicated statistical significance.

3 Results

3.1 Expression of miR-34a and Netrin1 were abnormal in GC and correlated with prognosis

A total of 23 miRNA results were obtained by searching "miRNA, gastric cancer, |T Value|> 10, $P < 0.001$" in dbDEMC database, including hsa-miR-543, hsa-miR-561-5p, hsa-miR-7705, hsa-miR-6854-5p, hsa-miR-125b-1-3p, hsa-miR-378d, hsa-miR-1226-3p, hsa-miR-30c-1-3p, hsa-miR-3610, hsa-miR-1262, hsa-miR-1258, hsa-miR-1270, hsa-miR-224-3p, hsa-miR-23B-5p, hsa-miR-1254, hsa-miR-6755-5p, hsa-miR-34a, hsa-miR-652-5p, hsa-miR-2110, hsa-miR-676-3p, hsa-miR-942-3p, hsa-miR-190a-3p, hsa-miR-411-3p (Fig. 2A). Netrin-1 expression patterns in different tumors were is obtained by TIMER2.0 database, showing Netrin-1 in the amount of expression in gastric cancer is significantly higher than normal group ($P < 0.001$) (Fig. 2B). In Kaplan–Meier Plotter database, we found that 9 of the above 23 different mirnas were effectively retrieved, including hsa-miR-7705, hsa-miR-1270, hsa-miR-1254, hsa-miR-34a, hsa-miR-543, hsa-miR-378d, hsa-miR-1262, hsa-miR-3610, hsa-miR-1258. Among them, hsa-miR-34a, hsa-miR-7705, hsa-miR-1254, hsa-miR-1270 were correlated with the overall survival of patients with gastric cancer ($P < 0.05$) (Fig. 2C), and Netrin-1 was also significantly correlated with the overall survival of gastric cancer (Fig. 2D). Tarbase v 9.0 was used to predict the relationship between Netrin-1 and hsa-miR-34a, hsa-miR-7705, hsa-miR-1254 and hsa-miR-1270, finding that Netrin-1 only had interaction with hsa-miR-34a.

In addition, we deleted the cases with missing information from the dataset downloaded from the TCGA database, and finally 384 cases of GC patients (general information is shown in Table 1) were entered into the study, we found that miR-34a expression was downregulated (Fig. 3A) and Netrin1 was upregulated (Fig. 3B) in GC, and the expression level of miR-34a and Netrin1 were correlated with the TNM stage of GC (Fig. 3C, D), and moreover, the expression levels of miR-34a (AUC 0.746, 95%CI 0.696–0.795) and Netrin1 (AUC 0.729, 95%CI 0.678–0.781) had a certain predictive value for patient prognosis, and the optimal cut-off values were 0.37 and 0.89, respectively (Fig. 3E). We divided the patients into high- and low-level groups according to the optimal cutoff values of miR-34a and Netrin1, respectively. Kaplan–Meier analysis showed that the overall survival (OS) of the patients in the high-level miR-34a and low-level Netrin1 groups was longer. (Fig. 3G, H). These findings again verified that low levels of miR-34a and high levels of Netrin1 were associated with poor prognosis in patients with GC. It is worth noting that there is a correlation between Netrin-1 and miR-34a (Fig. 3F), which is consistent with the predicted results of Tarbase v 9.0.

3.2 MiR-34a targets and negatively regulates Netrin-1 to mediate chemoresistance in GC cell

Chemoresistance is the main cause of chemotherapy failure and poor prognosis and is one of the core factors leading to the death of patients with tumors. Therefore, research on the mechanism of chemotherapy resistance is still a hot topic in the field of anti-tumor. Our study found that, compared with the HGC27 group, the mRNA expression of miR-34a in HGC27/DDP cells was decreased, while the mRNA and protein expression levels of Netrin1 were increased (Fig. 4A, B), indicating that miR-34a and Netrin1 may be related to GC cell chemoresistance. We transfected miR-34a mimic to

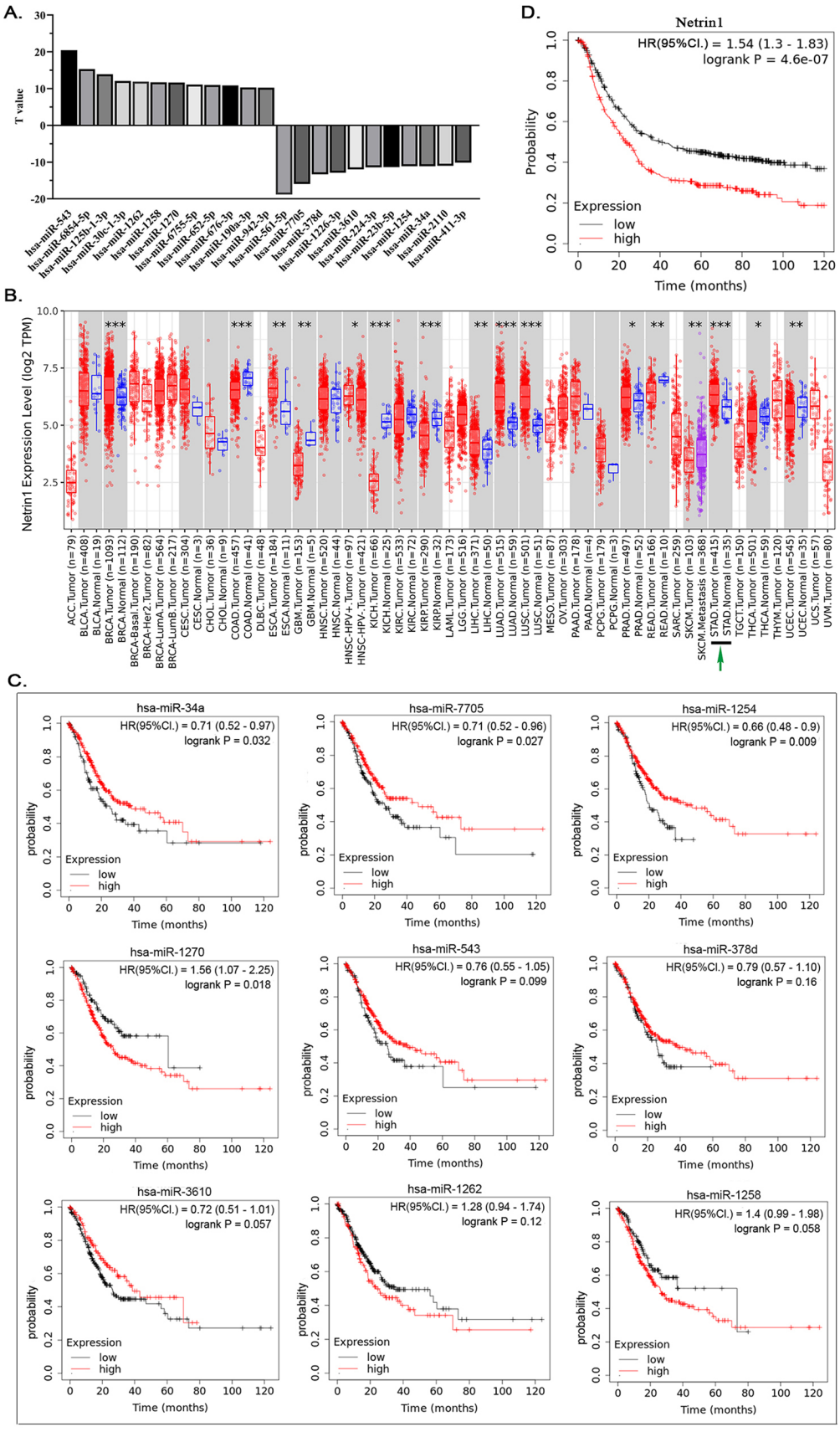
A.
T value
hsa-miR-543
hsa-miR-6854-5p
hsa-miR-125b-1-3p
hsa-miR-30c-1-3p
hsa-miR-1262
hsa-miR-1258
hsa-miR-1270
hsa-miR-6755-5p
hsa-miR-652-5p
hsa-miR-676-3p
hsa-miR-190a-3p
hsa-miR-942-3p
hsa-miR-561-5p
hsa-miR-7705
hsa-miR-378d
hsa-miR-1226-3p
hsa-miR-3610
hsa-miR-224-3p
hsa-miR-23b-5p
hsa-miR-1254
hsa-miR-34a
hsa-miR-2110
hsa-miR-411-3p
D.
Netrin1
HR(95%CI.) = 1.54 (1.3 - 1.83)
logrank P = 4.6e-07
Probability
Expression
low
high
Time (months)
B.
Netrin1 Expression Level (log2 TPM)
C.
hsa-miR-34a
HR(95%CI.) = 0.71 (0.52 - 0.97)
logrank P = 0.032
hsa-miR-7705
HR(95%CI.) = 0.71 (0.52 - 0.96)
logrank P = 0.027
hsa-miR-1254
HR(95%CI.) = 0.66 (0.48 - 0.9)
logrank P = 0.009
hsa-miR-1270
HR(95%CI.) = 1.56 (1.07 - 2.25)
logrank P = 0.018
hsa-miR-543
HR(95%CI.) = 0.76 (0.55 - 1.05)
logrank P = 0.099
hsa-miR-378d
HR(95%CI.) = 0.79 (0.57 - 1.10)
logrank P = 0.16
hsa-miR-3610
HR(95%CI.) = 0.72 (0.51 - 1.01)
logrank P = 0.057
hsa-miR-1262
HR(95%CI.) = 1.28 (0.94 - 1.74)
logrank P = 0.12
hsa-miR-1258
HR(95%CI.) = 1.4 (0.99 - 1.98)
logrank P = 0.058
probability
Expression
low
high
Time (months)

Table 1 Descriptive statistical results of the dataset of GC patients based on TCGA

Data name		N(384 cases)
Gender	Male	248
	Female	136
Age (year)	< 65	224
	≥ 65	160
GC types	Mucinous adenocarcinoma	80
	Canalicular adenoma	74
	Enteric adenocarcinoma	86
	Atypical adenocarcinoma	144
Distant metastasis occurs	Yes	34
	No	350
Local lymph node metastases	0	187
	≤ 7	106
	8 ~ 14	76
	≥ 15	15
Infiltration condition	The mucosa and submucosa	14
	The base and subserous membranes	76
	Penetrated the serosal layer	186
	Adjacent organs	108
TNM stage	I	44
	II	122
	III	168
	III	50
miR-34a	Low-level	147
	Hige-level	237
Netrin-1	Low-level	182
	Hige-level	202

further investigate the IC50 of cisplatin on HGC27/DDP cells and found that the IC50 of cisplatin after upregulating miR-34a (DDP + miR-34a group) was 0.65 ng/mL, which was lower than that of the DDP group (1.6 ng/mL). Based on the upregulation of miR-34a to overexpress Netrin1, IC50 of cisplatin recovered to 0.9ng/mL (Fig. 3C). More, we detected the expression level of MDR-1 protein by WB, and the results showed that the MDR-1 decreased after upregulation of miR-34a, however on that basis, the decrease of MDR-1 was compensated after overexpression of Netrin-1 after overexpression of Netrin1((Fig. 4D, E). This indicates that upregulation of miR-34a can significantly reduce cisplatin resistance, but overexpression of Netrin1 can offset the effect of miR-34a upregulation to a certain extent. This suggests that miR-34a and Netrin1 may play a negative regulatory role in cisplatin resistance. To clarify the relationship between miR-34a and Netrin1, we searched the Starbase online database and found a target binding site in the 3'UTR region of Netrin-1 to miR-34a (Fig. 4F). then, we verified this with a double luciferase reporter gene assay, and the results showed that the luciferase activity of cells in miR-34a mimic + Netrin-1WT group (0.51 ± 0.03) was significantly lower than that of cells in miR-34a mimic + Netrin-1MUT (1.01 ± 0.02) and mimic NC + Netrin-1WT (0.96 ± 0.06), the differences were statistically significant ($P < 0.05$) (Fig. 4G). This confirmed the existence of a targeted negative regulatory relationship between miR-34a and Netrin-1, laying a solid foundation for the discussion of the subsequent experimental mechanism. The above results also suggest us that miR-34a negatively regulates Netrin-1 to mediate cisplatin resistance in GC cells, but the specific mechanism needs to be further explored.

3.3 MiR-34a targets and negatively regulating Netrin-1 to induced the proliferation inhibtion, apotosis and cycle arrest of HGC27/DDP

As a commonly used anti-cancer chemotherapy drug, cisplatin can bind to DNA to prevent the replication and repair of DNA chains to induce cancer cells to enter the apoptosis process, accelerate the apoptosis process, and inhibit proliferation. Therefore, we investigated the effects of miR-34a negatively regulating Netrin-1 on the proliferation, apoptosis, and

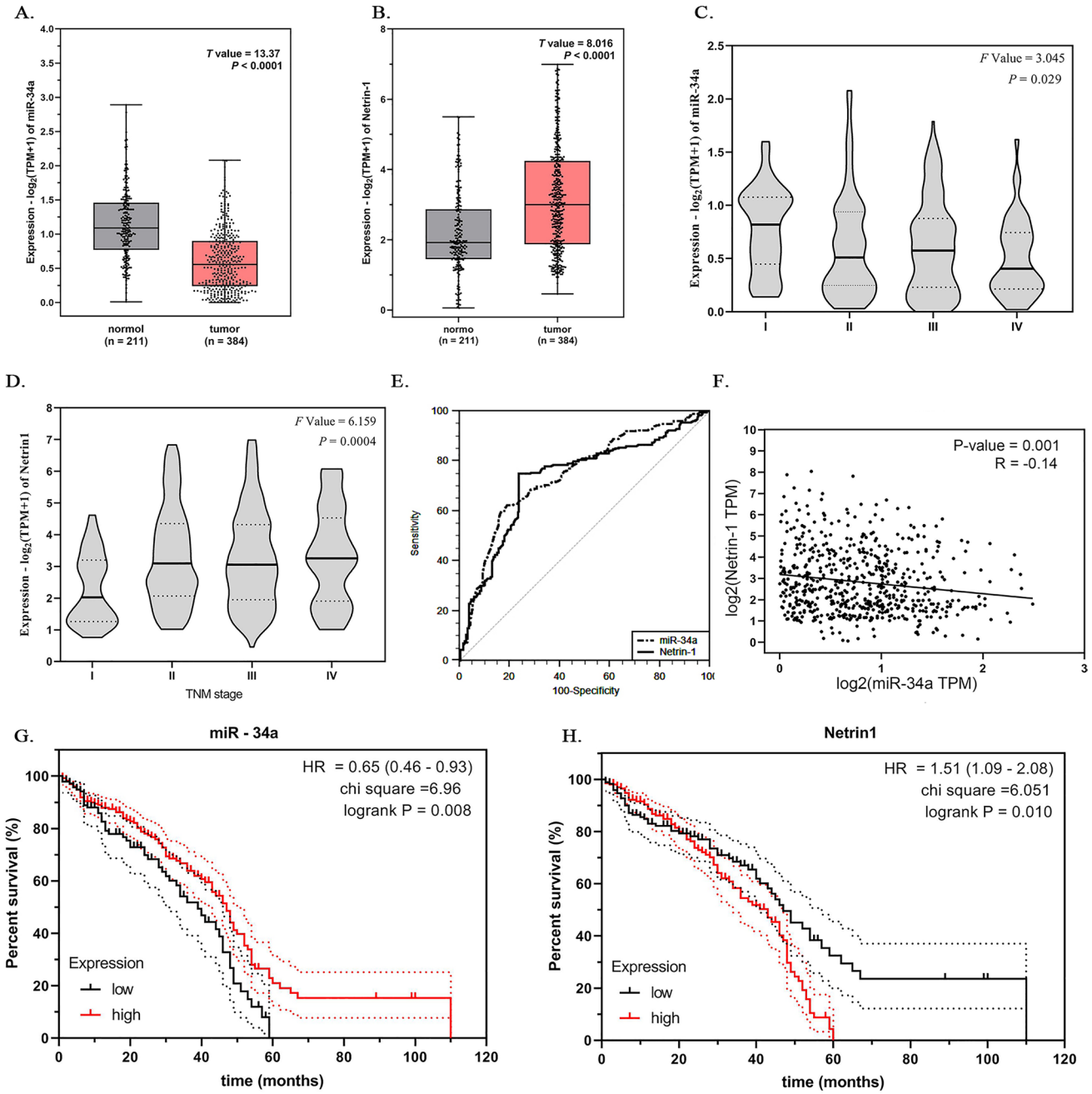

Fig. 3 The expression of miR-34a and Netrin1 and their correlation with prognosis based on TCGA database. **A** Expressin level of miR-34a **B** Expressin level of Netrin1. **C** Analysis of the difference of miR-34a in different TNM stages of GC. **D** Analysis of the difference of Netrin1 in different TNM stages of GC. **E** ROC curve analysis of miR-34a and Netrin1 on prognosis. **F** Correlation analysis of miR-34a and Netrin1. **G** Kaplan–Meier analysis results of different levels of miR-34a. (**H**) Kaplan–Meier analysis results of different levels of Netrin1. TPM: Transcripts Per Million

cycle arrest of HGC27/DDP cells. We treated cells with miR-34a mimic and pcDNA3.1-Netrin1, and the results showed that the cell proliferation rate, Ki-67, PCNA and Bcl-2protein expression level were significantly reduced, while the apoptosis rate, Cleaved-caspase3 and Bax expression level were increased, more over the proportion of S-phase cells was significantly increased while G1, G2-phase cells reduced promoting cycle arrest after upregulation of miR-34a. On that basis, the above phenomenon was reversed after overexpression of Netrin1 (Fig. 5). miR-34a can mediate the expression of related genes to inhibit proliferation, promote the apoptotic process and cycle arrest of HGC27/DDP cells by targeting and negatively regulating Netrin-1, thereby improving the cisplatin sensitivity of gastric cancer cells.

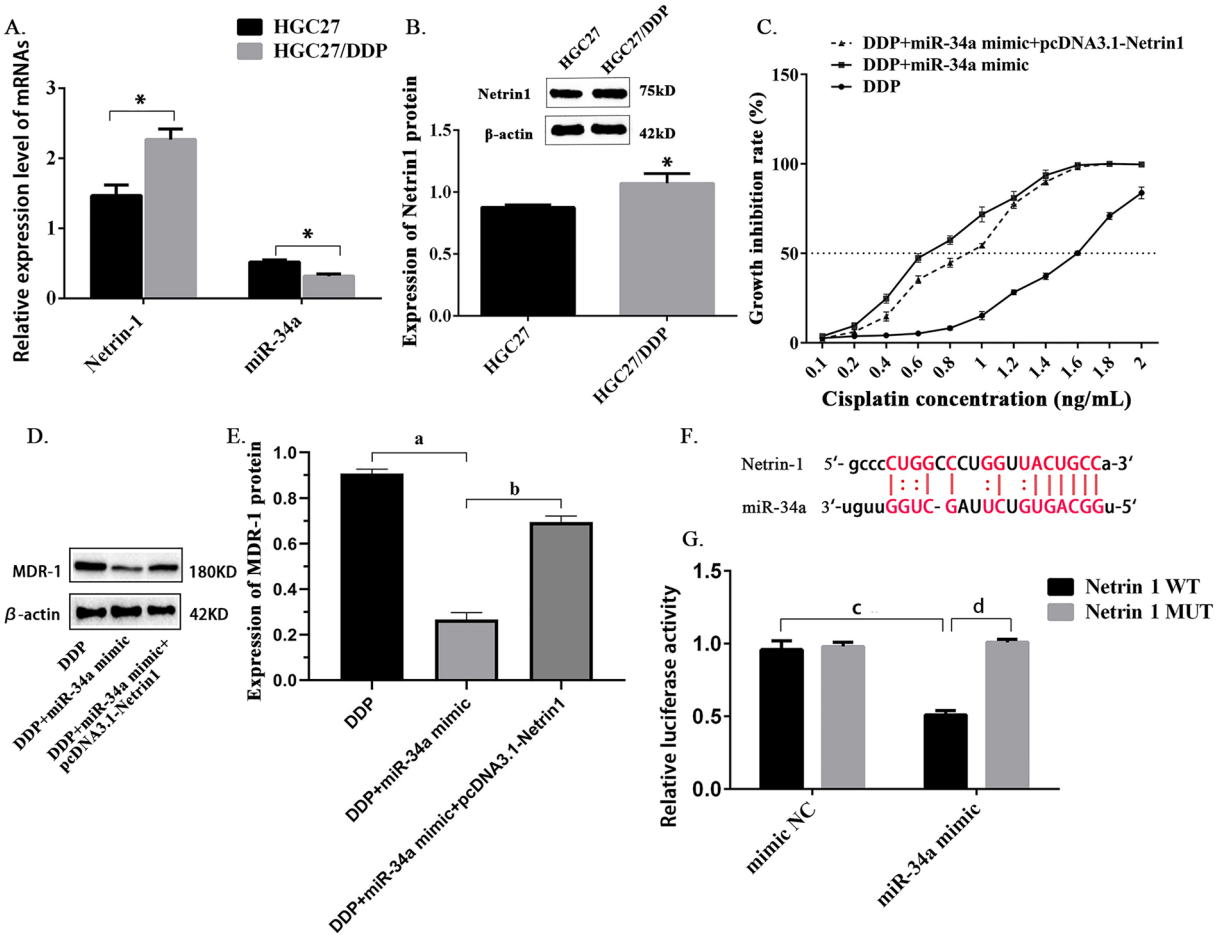

Fig. 4 The miR-34a targets Netrin-1 to mediate cisplatin resistance (n = 3). **A** Expressin level of miR-34a and Netrin1 mRNA in different cells. **B** Expressin level of Netrin1 from different cells. **C** IC50 of DDP in each group cells. **D** Expression of MDR-1 was detected by WB. **E** Comparison of MDR-1 expression in each group. **F** miR-34a and Netrin-1 target binding site from Starbase database. **G** The results of double luciferase reporter gene experiment. *, compared between HGC27 and HGC27/DDP groups was $P < 0.05$; a, compared between DDP and miR-34a mimic groups was $P < 0.05$; b, compared between miR-34a mimic and miR-34a mimic + pcDNA3.1-Netrin1 groups was $P < 0.05$; c, compared between mimi NC and miR-34a mimic groups was $P < 0.05$; d, compared between Netrin-1 WT and Netrin-1 MUT groups was $P < 0.05$

3.4 MiR-34a targets and negatively regulating Netrin-1 to inhibit the migration and invasion of HGC27/DDP

Migration and invasion are important characteristics of malignant tumor cells, and drug-resistant cells often show stronger invasion and metastatic abilities. Some studies have shown that upregulation of E-cadherin (a protein related to epithelial interstitial transformation) and downregulation of MMP9 can inhibit the migration and invasion ability of drug-resistant cells and collaboratively improve chemotherapy sensitivity [29, 30]. Thus, we detected the expression of MMP9 and E-cadherin by WB, and conducted wound-scratch tests and transwell assays to explore the regulatory mechanism of MiR-34a and Netrin-1 on the migration and invasion of HGC27/DDP cells. The results showed that after upregulation of miR-34a, the wound healing rate and number of invasive cells decreased, the level of MMP9 protein decreased, and E-cadherin increased. On this basis, the above phenomenon was offset to a certain extent after overexpression of Netrin1 (Fig. 6). This confirmed the role of miR-34a upregulation and negatively regulating Netrin-1 in inhibiting HGC27/DDP cell migration and invasion, and suggested that miR-34a can mediate the epithelial mesenchymal transformation process and inhibit cell invasion and metastasis by targeting and negative regulating Netrin-1, thereby reducing the activity of HGC27/DDP cells and synergistically increasing the cisplatin sensitivity of GC cells.

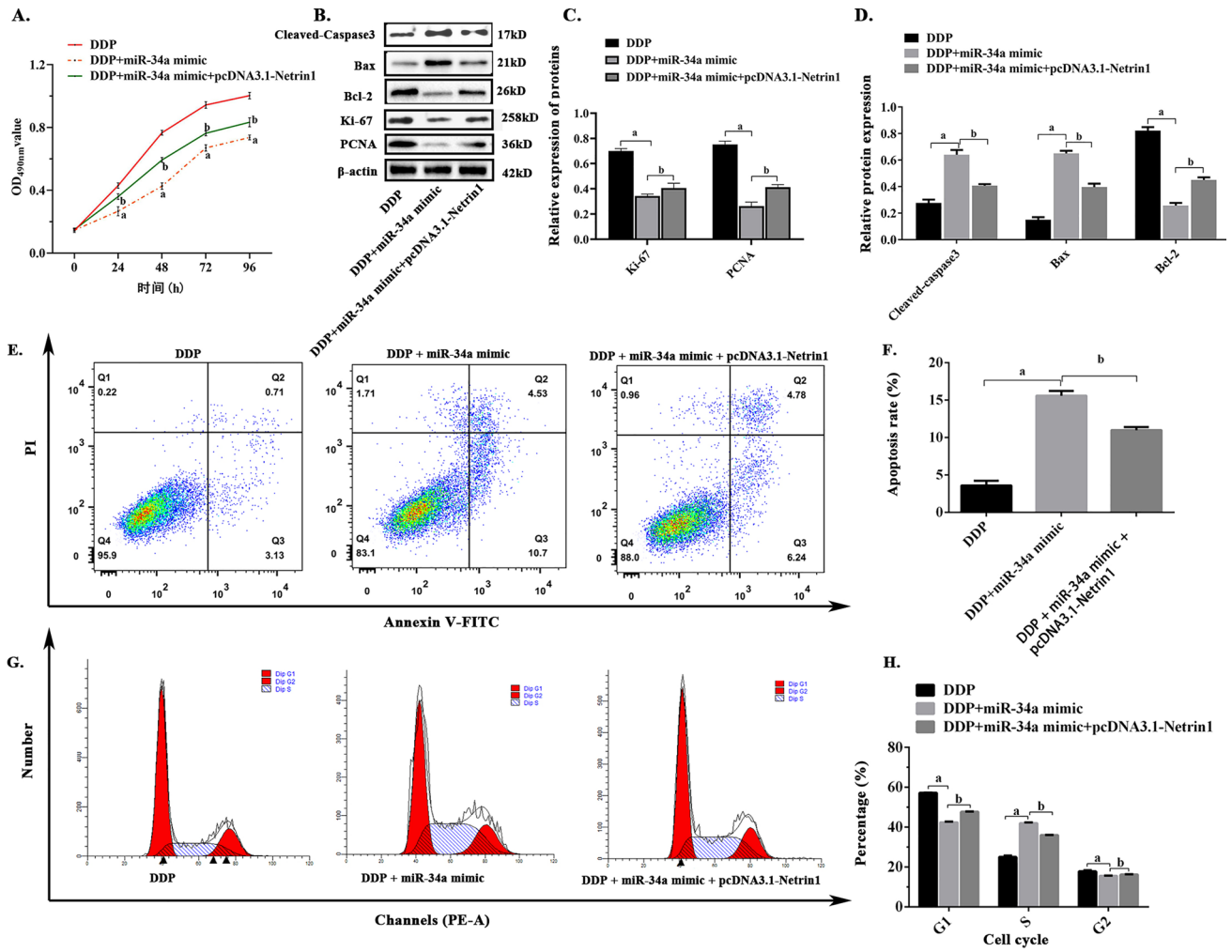

Fig. 5 miR-34a targets and negatively regulating Netrin-1 to induced the proliferation inhibtion, apotosis and cycle arrest of HGC27/DDP (n = 3). Proliferative ability of cells were detected by CCK8 in each group. **B** WB test results of proliferation and apoptosis related proteins. **C** Relative quantitative analysis of proliferation related proteins; **D** Relative quantitative analysis of apoptosis related proteins. **E** Flow diagrams of cell apoptosis in each group. **F** Comparison of apoptosis rate in diffirent groups. **G** Cell cycle diagrams of diffirent groups. **H** Comparison of cell cycle in different groups. a, compared between DDP and miR-34a mimic groups was $P < 0.05$; b, compared between miR-34a mimic and miR-34a mimic + pcDNA3.1-Netrin1 groups was $P < 0.05$

3.5 MEK/ERK signaling pathway is involved in the regulation of chemoresistance of GC by miR-34a targeting Netrin1

MEK/ERK signaling pathway is one of the widely activated MAPK pathways, and regulation of MEK/ERK signaling pathway had an impact on various biological characteristics of tumor cells such as proliferation and apoptosis [20, 31]. In recent years, some studies have shown that the MEK/ERK signaling pathway plays an important role in inducing tumor drug resistance [32]. For example, the MEK/ERK signaling pathway interacts with p53 to induce chemoresistance in prostate cancer cells [33]. However, the role of the MEK/ERK signaling pathway in the chemoresistance of GC requires further investigation. Our previous studies have confirmed that miR-34a targeting Netrin1 regulates the proliferation, apoptosis, migration, and invasion of HGC27/DDP cells to affect cisplatin resistance; however, whether the MEK/ERK pathway is involved in this process remains unclear. Therefore, we detected proteins related to the MEK/ERK pathway, and the results showed that MEK and ERK phosphorylation levels decreased after upregulation of miR-34a, whereas MEK and ERK phosphorylation levels recovered after overexpression of Netrin1 (Fig. 7A, B). These results indicate that miR-34a negatively regulates Netrin1 to inhibit activation of the MEK/ERK pathway. Next, we added the MEK1/2 inhibitor PD98059 based on the upregulation of miR-34a and Netrin1 together, and the results showed

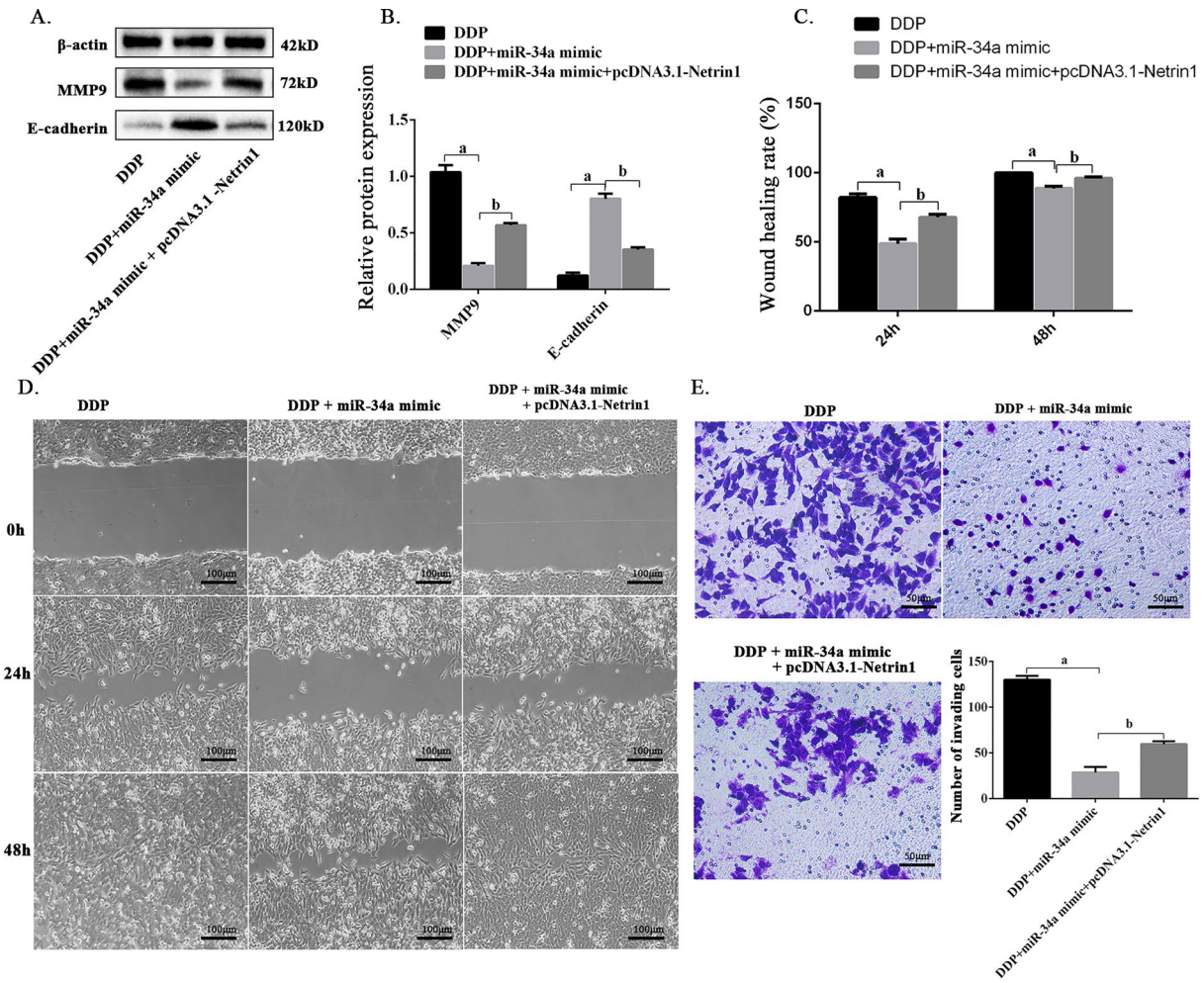

Fig. 6 miR-34a targets and negatively regulating Netrin-1 to inhibit the migration and invasion of HGC27/DDP (n = 3). **A** WB test results of MMP9 and E-cadherin. **B** Relative quantitative analysis of MMP9 and E-cadherin; **C** Comparison of wound healing rate. **D** Wound-Scratch test results. **E** Transwell assay results and comparative analysis. a, compared between DDP and miR-34a mimic groups was $P < 0.05$; b, compared between miR-34a mimic and miR-34a mimic + pcDNA3.1-Netrin1 groups was $P < 0.05$

that the phosphorylation levels of MEK and ERK were reduced, and the expression of Netrin1 protein was decreased (Fig. 7C, D), indicating that Netrin1 interacts with the MEK/ERK pathway. The above results suggest that the MEK/ERK signaling pathway is one of the downstream signaling pathways of miR-34a that targets Netrin1.

3.6 MiR-34a negatively regulates Netrin1-mediated MEK/ERK pathway to improve cisplatin sensitivity of HGC27/DDP

Our previous studies have confirmed that miR-34a negatively regulates Netrin1 to mediate the proliferation, apoptosis, migration, and invasion of GC cells and regulates cisplatin resistance, and that the MEK/ERK pathway is one of the downstream signaling pathways in which miR-34a targets Netrin1. It is speculated that miR-34a targeting the negative regulation of the Netrin1-mediated MEK/ERK pathway may be the molecular mechanism of drug resistance in GC. To verify this hypothesis, we treated cells with PD98059 based on the upregulation of miR-34a and Netrin1 to perform reversal experiments. The results showed that the IC50 value of cisplatin after treatment with PD98059 was approximately 0.6 ng/mL, which was significantly lower than that of the DDP + miR-34a mimic + pcDNA3.1-Netrin1 group (0.9 ng/mL), and in terms of protein level, MDR-1 was decreased significantly after the addition of PD98059, suggesting that cisplatin resistance was attenuated (Fig. 7E, F). Further on, we also investigated changes in proliferation, apoptosis, cycle, migration, and invasion ability of drug-resistant cells treated with PD98059. The results showed that the cell proliferation rate, migration, and invasion activity were decreased, the apoptosis rate was increased, S-phase cells was increased while G1,

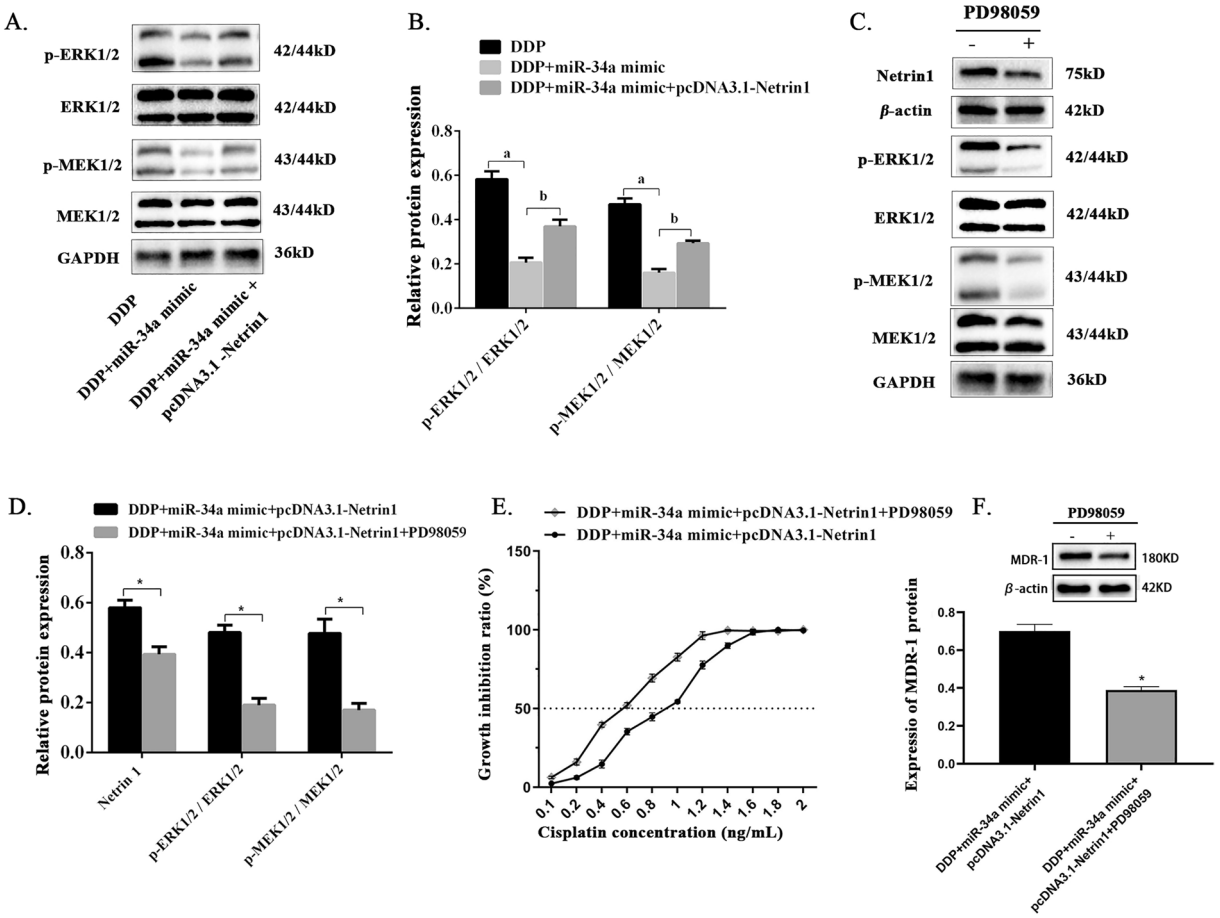

Fig. 7 The MEK/ERK signaling pathway is involved in the downstream mechanism of miR-34a targeting Netrin1 to regulate chemoresistance (n = 3). **A** Results of p-MEK and p-ERK by WB. **B** Relative quantitative analysis of p-MEK and p-ERK. **C** Results of Netrin1, p-MEK and p-ERK by WB in reversal experiment. **D** Relative quantitative analysis of Netrin1, p-MEK and p-ERK in reversal experiment. **E** Comparison of cisplatin IC50 in reversal experiment. **F** Expression and comparison of MDR-1 in each group. a, compared between DDP and miR-34a mimic groups was $P < 0.05$; b, compared between miR-34a mimic and miR-34a mimic + pcDNA3.1-Netrin1 groups was $P < 0.05$; *, compared between miR-34a mimic + pcDNA3.1-Netrin1 and miR-34a mimic + pcDNA3.1-Netrin1 + PD98059 groups was $P < 0.05$

G2-phase cells reduced, the phenomenon of cycle arrest was aggravated. At the protein level, the expression levels of Ki-67, PCNA, Bcl-2, and MMP9 decreased, and the levels of Cleaved-caspase3, Bax and E-cadherin increased (Fig. 8). This demonstrates that inhibition of the MEK/ERK pathway counteracts the enhancement of GC cell activity and cisplatin resistance caused by the overexpression of Netrin1. This study clarified the mechanism of chemoresistance in GC, where miR-34a targeted the Netrin1-mediated MEK/ERK pathway to regulate the proliferation, apoptosis, cycle, migration, and invasion of cancer cells, providing new clues for overcoming chemotherapy resistance.

4 Discussion

GC is one of the four malignant tumors with high incidence and mortality rates. The generation of chemoresistance not only increases the difficulty of GC treatment but is also a risk factor for disease progression and death of patients. Therefore, overcoming chemoresistance has become an important problem in the field of anti-tumor research. Cisplatin is the main chemotherapeutic drug, and cisplatin resistance is the direct cause of chemotherapy failure in GC, which is closely related to recurrence and low survival rate of patients [34]. Therefore, exploring the molecular mechanism of cisplatin resistance in gastric cancer is of great significance for overcoming cisplatin resistance and improving the survival rate of patients with GC.

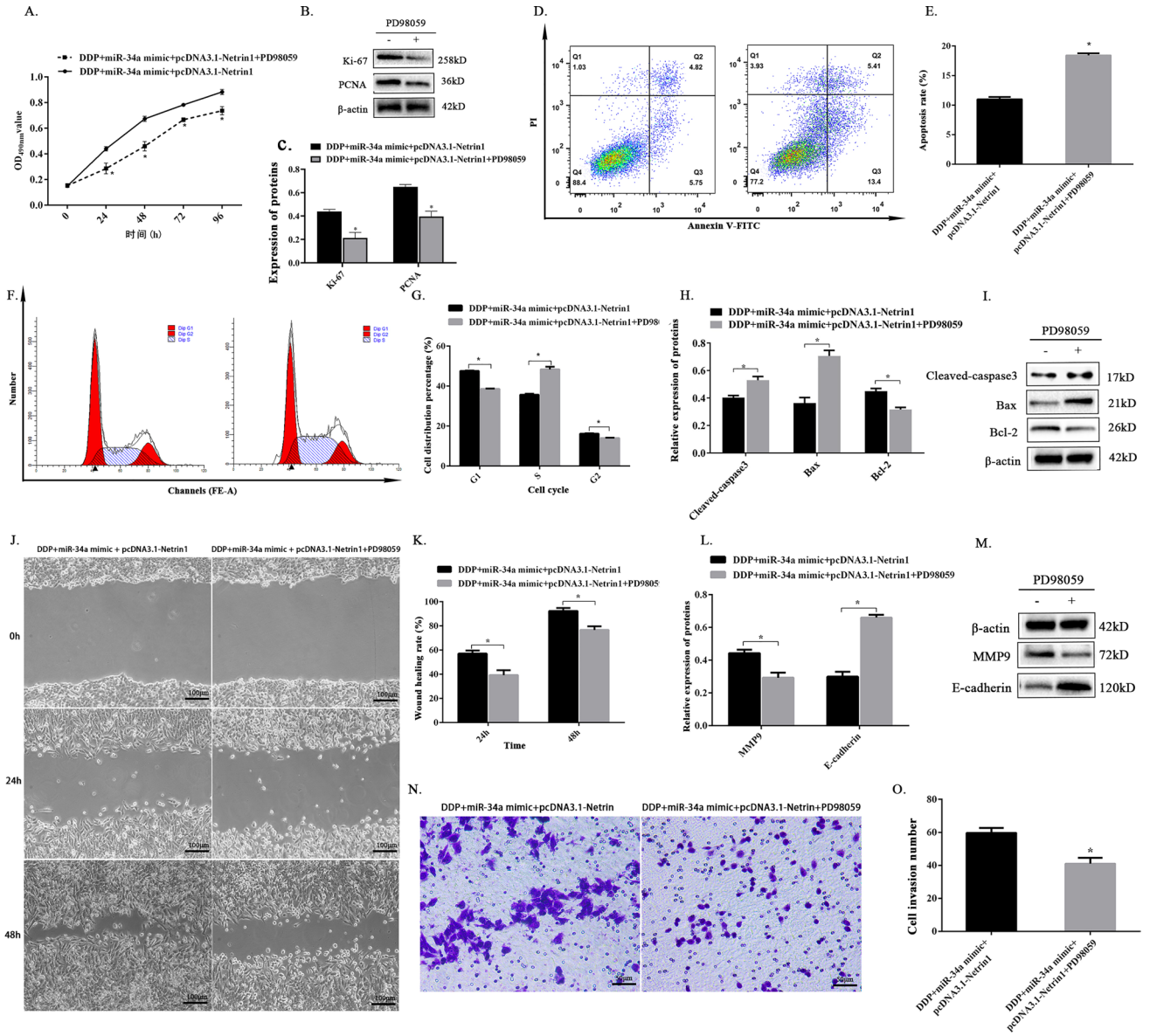

Fig. 8 Inhibition of MEK/ERK signaling pathway reverses HGC27/DDP cells activity and chemoresistance (n = 3). **A** Comparison of proliferation capacity. **B** Expression of Ki-67 and PCNA. **C** Comparison of Ki-67 and PCNA expression levels between two groups. **D** Flow diagrams of cell apoptosis. **E** Comparison of apoptosis rate between two groups. **F** Cell cycle diagrams. **G** Comparison of Cell cycle between two groups. **H** Relative quantitative analysis of Cleaved-caspase3, Bax and Bcl-2. **I** WB test results of Cleaved-caspase3, Bax and Bcl-2. **J** Wound-scratch test results. **K** Comparison of wound healing rate. **L** Relative quantitative analysis of MMP9 and E-cadherin. **M** WB test results of MMP9 and E-cadherin. **N** Results of transwell assay. **O** Comparison of cell invasion numbers. *, compared between miR-34a mimic + pcDNA3.1-Netrin1 and miR-34a mimic + pcDNA3.1-Netrin1 + PD98059 groups was $P < 0.05$

Many miRNAs are not only related to tumor progression and prognosis but also mediate the process of tumor chemotherapy resistance [35, 36]. As one of the miRNA subtypes, miR-34a is differentially expressed in multiple tumor tissues, such as breast, lung, and esophageal tissues, and the overall survival of cancer patients with miR-34a deletion or low expression is relatively short [37–39]. In GC, most studies have shown low expression of miR-34a, which correlates with prognosis [4, 40], but a few hold different views [41]. To further clarify the expression of miR-34a in GC and its correlation with prognosis, we used a bioinformatics database and found that miR-34a was expressed at low levels in GC, and the overall survival of patients with high levels of miR-34a was relatively prolonged. This suggests that miR-34a may be an important clue for improving the survival rate of GC patients. Because chemoresistance is one of the core factors threatening patient survival, it is unclear whether miR-34a levels affect cisplatin resistance in gastric cancer. Previous studies have confirmed that miR-34a levels affect the biological characteristics of proliferation, apoptosis, migration, and

invasion of tumor-resistant cells and may participate in the process of tumor drug resistance through complex potential regulatory mechanisms. For example, upregulation of miR-34a can significantly inhibit the proliferation, migration, and invasion and induce apoptosis of cisplatin-resistant cells to reverse cisplatin resistance in colorectal cancer by inhibiting the LRPPRC/MDR1 axis [8], mediate the expression of proteins related to proliferation, apoptosis, migration, and invasion of breast cancer cells to improve chemosensitivity by negatively regulating Notch1 [42], and restore cisplatin sensitivity in ovarian cancer cells by inhibiting the target gene of miR-34a to change proliferation, apoptosis, and cycle arrest [9]. Regarding the mechanism of drug resistance in GC, a few studies have found that upregulation of miR-34a on the one hand enhances cisplatin sensitivity of SGC7901/DDP cells by mediating cell proliferation and apoptosis; on the other hand, it can directly regulate the epithelial mesenchymal transformation process to reduce the invasion ability of SGC7901/DDP cells to synergistically enhance cisplatin sensitivity [43]. Further research is required to confirm this. Therefore, another GC cell line, HGC27, was used in our study to establish a GC cisplatin resistance cell model (HGC27/DDP), and we found that miR-34a mRNA levels in HGC27/DDP cells were significantly lower than those in HGC27 cells. After up-regulation of miR-34a, the IC50 of cisplatin decreased from 1.6ng/mL to 0.65ng/mL to HGC27/DDP cells, and the MDR-1 also decreased. It seems that the expression of miR-34a is related to cisplatin resistance in gastric cancer cells. We know that the change of cisplatin resistance in tumor cells involves the change of cell proliferation, apoptosis and other biological characteristics. Therefore, we investigated the effects of miR-34a on proliferation, apoptosis, migration, and invasion of HGC27/DDP cells. The results showed that after miR-34a upregulation, the proliferation rate, migration, and invasion ability of HGC27/DDP cells were significantly reduced, the apoptosis rate was increased, and cycle arrest was enhanced. At the protein level, Ki-67, PCNA, Bcl-2, and MMP9 levels decreased, and Cleaved-caspase3, Bax and E-cadherin levels increased significantly. These results suggest that miR-34a is involved in the cisplatin resistance process of GC, and its mechanism may directly or indirectly regulate the expression of related genes, mediate the proliferation, apoptosis, migration, invasion and cycle arrest of HGC27/DDP cells, ultimately altering cisplatin resistance. This finding is consistent with that of Zhang. It has been confirmed that miR-34a is related to cisplatin resistance in GC, but the specific molecular mechanism needs to be further explored.

Netrin1 is a secreted protein related to laminin and is involved in pathological processes, such as cancer, cardiovascular disease, and kidney disease [44]. Some studies have shown that Netrin1 is highly expressed in cancers, including liver cancer, cervical cancer and rectal cancer, and is associated with cancer pathology and prognosis [45, 46]. However, the expression of Netrin-1 in GC and its relationship with the pathology and prognosis have not been confirmed. In recent years, a few studies have found that Netrin-1 is highly expressed in GC tissues, which may be related to its progression and prognosis [47, 48]. In this study, we searched the TCGA database and found that the expression level of Netrin-1 in GC tissues was higher than that in normal tissues and correlated with the cycle of gastric cancer. The low level of Netrin-1 could effectively prolong the survival of GC patients, which is consistent with the results of previous studies. Recent studies indicate that Netrin1 is related to tumor resistance, which may be one of the reasons why Netrin1 can affect the prognosis of gastric cancer patients. In addition, Liu Jiao et al. found that miR-214 inhibits the activity of bladder cancer cells by targeting and negatively regulating Netrin-1. Thereby reducing the cisplatin resistance. This suggests that Netrin1 may interact with mRNA, such as miR-34a, to regulate cisplatin resistance. In order to verify the above possibilities, we searched the Starbase online database and found that there were targeting binding sites between Netrin1 and miR-34a, and verified this targeting relationship by a double luciferase reporter gene experiment, which pointed out the direction for the study of the mechanism of chemoresistance in GC. Thus, in the following experiments, we overexpressed Netrin1 on the basis of up-regulation of miR-34a, and found that the IC50 of cisplatin recovered from 0.65 ng/mL to 0.9 ng/mL,and the MDR-1 expression recovered to some extent, promotting the increase of cisplatin resistance. Moreover, compared with the miR-34a group alone, the proliferation rate, migration, and invasion ability of cells were significantly increased in the co-upregulated miR-34a and Netrin-1 groups, and the apoptosis rate was decreased and cell cycle arrest was relieved. At the protein level, the expression levels of Ki-67, PCNA, BCL-2, and MMP9 were increased, while the expression levels of Cleaved-caspase3, Bax and E-cadherin were decreased. It has been suggested that miR-34a negatively regulates Netrin-1 to inhibit the biological activity of HGC27/DDP cells by mediating the expression of related proteins, cell cycle arrest, and epithelial mesenchymal transformation, thereby reducing cisplatin resistance. This is in line with the currently recognized anti-tumor mechanism of miR-34a, which is "miR-34a directly regulates the expression of relevant target genes or indirectly affects the development and progression of tumors by mediating cycle arrest, cell activity, or affecting signaling pathways [49]." It is worth noting that all biological activities of cells are inseparable from signal transduction, and abnormal signal pathways may directly lead cells to enter abnormal processes, affecting the normal life activities of cells. Therefore, some signaling pathways may be involved in the process by miR-34a targeting Netrin-1 to regulate cisplatin resistance in GC, and further studies are needed to confirm this.

The MEK/ERK signaling pathway is one of the most widely activated MAPK pathways, and its upstream genes can mediate tumor cell proliferation, apoptosis, autophagy, and other biological functions by targeting MEK/ERK pathways and play an important role in inducing tumor chemoresistance [50]. For example, miRNAs regulate multiple signaling pathways, such as MEK/ERK, PI3K/PTEN/AKT through downstream targets to affect disease progression and chemoresistance in patients with liver cancer [51]; miR-21 induces oxitinib resistance in non-small cell lung cancer by regulating MEK/ERK [52]. This suggests that the MEK/ERK pathway is involved in miRNA-regulated tumor chemoresistance. In GC, activation of the MEK/ERK pathway promotes gastric cancer progression and chemoresistance and may play an important role in the development of cisplatin resistance in gastric cancer [53]. Based on these results, we speculated that the MEK/ERK pathway may be involved in the mechanism by miR-34a targeting Netrin-1 to regulate cisplatin resistance. Interestingly, our experimental results confirm this hypothesis. After upregulation of miR-34a, p-MEK/MEK and p-ERK/ERK decreased, while co-upregulation of miR-34a and Netrin1 again increased p-MEK/MEK and p-ERK/ERK. Interestingly, the expression of p-MEK/MEK, p-ERK/ERK, and Netrin1 proteins decreased after inhibition of the MEK/ERK pathway (PD98059 treated cells). This indicates that the MEK/ERK pathway may be one of the downstream signaling pathways of miR-34a targeting Netrin1. and then, we also found that compared with the co-up-regulation group of miR-34a and Netrin1, the IC50 value of cisplatin reversely decreased from about 0.9ng/mL to 0.6ng/mL and the MDR-1 expression decreased in reverse after inhibiting MEK/ERK pathway, and the cell biological characteristics (including proliferation, apoptosis, migration and invasion) and related protein expression levels were reversed. This suggests that inhibition of the MEK/ERK pathway counteracts the enhancement of GC cell activity and chemoresistance caused by Netrin1 overexpression. The mechanism of chemoresistance in gastric cancer was clarified: miR-34a targets Netrin1 to regulate the proliferation, apoptosis, migration, and invasion of cancer cells by mediating the MEK/ERK pathway, thereby improving chemosensitivity in GC.

In conclusion, miR-34a expression is downregulated in gastric cancer clinical samples and cisplatin-resistant cells, whereas Netrin1 is upregulated, and both are associated with prognosis. Moreover, miR-34a can target and negatively regulate Netrin1 to mediate proliferation, apoptosis, cycle, migration, and invasion of cells via the MEK/ERK pathway, thereby improving chemosensitivity in gastric cancer. MiR-34a/Netrin1/MEK/ERK axis may serve as a novel therapeutic target for chemoresistance in gastric cancer, and provides new clues for overcoming chemoresistance in clinical settings. This is of great significance for the prevention and treatment of multi-drug resistant tumors in the future and for improving the prognosis of tumor patients, It would be a new development in cancer biology. However, it is worth noting that there are some limitations in this study: first, the selection of cell lines in this study is single, and multiple cell lines will be selected for verification in future studies; Second, this study has not been verified in vivo, and it still needs to be confirmed by animal experiments.

Author contributions Haiping Li contributed to the conception and design of the work, and the experimental operation, acquisition, analysis, and interpretation of data for the work. Limin Wang guided article design and drafting, revised it critically for important intellectual content, and contributed to the final revision and supervision of the manuscript.

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Data availability The data used to support the fndings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and cosent to participate No ethical implications were involved in this study.

Informed consent It is not involved in this study.

Human and animal rights It is not involved in this study.

Patient consent statement It is not involved in this study.

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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