miR-135a promotes gastric cancer progression and resistance to oxaliplatin

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ABSTRACT

Resistance to oxaliplatin (OXA)-based chemotherapy regimens continues to be a major cause of gastric cancer (GC) recurrence and metastasis. We analyzed GC samples and matched non-tumorous control stomach tissues from 280 patients and found that miR-135a was overexpressed in GC samples relative to control tissues. Tumors with high miR-135a expression were more likely to have aggressive characteristics (high levels of carcino-embryonic antigen, vascular invasion, lymphatic metastasis, and poor differentiation) than those with low levels. Patients with greater tumoral expression of miR-135a had shorter overall survival times and times to disease recurrence. Furthermore, miR-135a, which promotes the proliferation and invasion of OXA-resistant GC cells, inhibited E2F transcription factor 1 (E2F1)-induced apoptosis by downregulating E2F1 and Death-associated protein kinase 2 (DAPK2) expression. Our results indicate that higher levels of miR-135a in GC are associated with shorter survival times and reduced times to disease recurrence. The mechanism whereby miR-135a promotes GC pathogenesis appears to be the suppression of E2F1 expression and Sp1/DAPK2 pathway signaling.

INTRODUCTION

The gastric cancer (GC) mortality rate has begun to decrease in Western countries, but is still increasing among Chinese patients. In fact, GC has become the third-ranking cause of cancer death in China [1]. During the early stages of GC, some patients can be treated effectively with radical resection and chemotherapy. For patients with advanced-stage GC, the prognosis is dismal because of the high rates of postsurgical recurrence and metastasis. Furthermore, most deaths from GC are caused by multi-drug resistance (MDR) [2, 3]. Generally, the molecular mechanism of MDR is complicated, but is thought to involve drug transport, drug pump-out, intracellular drug metabolism, DNA injury, DNA repair and apoptosis [4]. However, the most important elements that activate MDR in GC are still undefined.

miRNAs, a category of non-protein-coding RNAs, have been accepted as vital participants in various pathways, especially proliferation and apoptosis, and have been ascribed carcinogenic or cancer suppressive functions in many solid tumors [5–7]. Recently, it was proposed that abnormal expression of miRNAs stimulates the malignant transformation process of tumors [8], miRNAs belonging to the miR-5002, miR-4252, and miR-647 families were reported to be upregulated in GC patients and to increase lymphatic metastasis [9]. Interestingly, numerous studies have documented the involvement of miRNAs in MDR.
For example, miR200c attenuates P-gp-induced MDR and metastasis by inhibiting the JNK2/c-Jun signaling pathway in colorectal cancer [10]. Although elevated levels of miR-135a were previously reported to suppress the proliferation of GC cells [11], genes regulated by miR-135a might contribute to MDR in GC, and the detailed mechanisms underlying these associations have not been explored.

In this study, we observed aberrantly increased expression of miR-135a in GC cells/tissues, which correlated with malignant GC characteristics. Additionally, univariate and multivariate analyses indicated that miR-135a was a potential prognostic marker for worse outcomes after radical resection. Using miRNA arrays, we identified miRNAs that were differentially expressed between oxaliplatin (OXA)-resistant and OXA-sensitive GC cells, including genes in the E2F1/Death-associated protein kinase 2 (DAPK2) pathway. Moreover, we found that miR-135a was highly expressed in OXA-resistant GC cells and inhibited OXA-induced cytotoxicity and apoptosis, partly due to its upregulation by c-MYC.

RESULTS

miR-135 family expression is associated with malignant characteristics in patients with GC

To investigate the expression of miR-135 family members and their biological significance in OXA-resistant GC patients, we first examined the levels of miR-135 family members in 80 paired OXA-resistant GC samples by RT-PCR. miR-135a was upregulated in the majority of GC samples compared with the adjacent tissues, but no significant difference in miR-135b expression was observed between GC and adjacent samples, suggesting that upregulation of miR-135a might be involved in OXA resistance in GC (Figure 1A). We next performed qRT-PCR analyses using 280 paired GC samples. As shown in Figure 1B, miR-135a expression was significantly greater in tumor tissues than in the corresponding peritumoral tissues (relative expression of miR-135a: 0.389 ± 0.034 vs. 0.271 ± 0.102; P < 0.001). However, there were no significant differences in miR-135b expression between the tumor and peritumoral tissues of the 280 GC patients (relative expression of miR-135b: 0.319 ± 0.045 vs. 0.308 ± 0.037; P = 0.251).

In addition, the levels of miR-135a in tumor tissues were used to build a signature of prognosis in OXA-resistant GC patients (Supplementary Table S1 and S2). For each miRNA analysis, patients were classified into the higher miRNA expression group or the lower expression group, with the median value as the cutoff point. Kaplan-Meier curves demonstrated that patients with higher miR-135a expression had poorer overall survival and higher recurrence rates than those with lower expression (Figure 1C–1D, P < 0.05), whereas no substantial difference was observed based on miR-135b expression in the correlation analysis. As shown in Figure 1E, the multivariate analysis further indicated that higher miR-135a expression, together with vascular invasion, lymphatic metastasis, hepatic metastases, and pathological staging, was an important independent risk factor that reduced both the tumor-free and overall survival rates in OXA-resistant GC patients. As shown in Figure 1F, the upregulation of miR-135a in OXA-resistant GC tissues correlated significantly with several pathological staging levels (P < 0.05), vascular invasion (P < 0.05), lymphatic vessel metastasis (P < 0.05), and early recurrence (P < 0.05). Therefore, the expression of miR-135a can be used as an independent predictor of the prognosis of OXA-resistant GC.

Correlation of miR-135 family levels with drug resistance

In further investigating the clinical significance of miR-135 expression in the development and progression of GC, we wondered whether miR-135 itself might promote drug resistance. Previous studies have mainly focused on the adenomatous polyposis coli (APC) gene pathway in paclitaxel-induced apoptosis in tumor cells, while few have reported about OXA resistance in GC [12]. P-glycoprotein (P-gp) ejects intracellular drugs and reduces their cytotoxic accumulation, and thus can be used as a marker of MDR [13]. Therefore, we quantified the strength of the correlation between miR-135a and P-gp expression. As shown in Figure 2A, only miR-135a expression correlated with P-gp expression (P < 0.05), indicating that miR-135a may contribute to OXA resistance. We then examined the relationship between miR-135 family and P-gp gene expression in 80 GC samples by qRT-PCR. P-gp mRNA expression appeared to accord with miR-135a expression (P < 0.05). We then quantified the strength of the correlation between miR-135 family and P-gp expression in different OXA-resistant GC cells (SGC7901/OXA and MGC803/OXA). As shown in Figure 2B, only miR-135a expression positively correlated with P-gp expression (P < 0.05).

miR-135a overexpression reduces sensitivity to OXA, enhances cell proliferation and inhibits apoptosis in vitro

We next investigated the effects of up- or downregulating miR-135a expression in OXA-resistant GC cells. The expression of miR-135a in SGC7901/OXA and MGC803/OXA cells was successfully downregulated by transfection with a miR-135a inhibitor and upregulated by transfection with a miR-135a mimic, relative to its expression in cells transfected with the respective controls (Supplementary Figure S1). In view of the correlation between miR-135a expression and clinical drug resistance in GC patients, we assumed that the upregulation of
miR-135a was one of the risk factors for OXA resistance in GC. As OXA is known to kill GC cells by inducing apoptosis, we wondered whether overexpression of miR-135a would prevent OXA-induced apoptosis. Thus, after overexpressing miR-135a in SGC7901/OXA and MGC803/OXA cells by transfecting them with a miR-135a mimic, we treated the cells with increasing concentrations of OXA. Cell morphological changes were observed under the microscope, and markedly more viable cells were found in miR-135a mimic-treated cells than in control mimic-treated cells (Figure 3A). We then investigated whether overexpression or suppression of miR-135a would induce or inhibit proliferation in OXA-resistant GC cells (SGC7901/OXA and MGC803/OXA). As expected, overexpression of miR-135a in SGC7901/OXA cells increased the proliferation percentage of cells to 35.3% ± 2.7% compared with the control cells, which exhibited 22.5% ± 3.1%. On the other hand, inhibition of miR-135a expression in MGC803/OXA cells reduced the proliferation percentage of cells to 19.2% ± 3.1% compared with the control cells, which exhibited 24.3% ± 2.9%. (Figure 3B, \( P < 0.05 \)). Thus, lower expression of miR-135a reduced OXA-resistant GC cell proliferation.

In addition, to explore a possible association between the levels of miR-135a and the sensitivity of cells to OXA, we analyzed the sensitivity of GC cells to OXA based on their relative expression of miR-135a. As showed in Figure 3C, compared with the growth of control cells, the growth of OXA-resistant GC cells increased after miR-135a upregulation by the miR-135a mimic, but decreased after miR-135a downregulation by the miR-135a inhibitor. In summary, these results demonstrated that OXA-resistant GC cells proliferated in response to miR-135a expression.

We also performed Annexin V/propidium iodide (PI) assays to determine whether miR-135a expression was associated with apoptosis (Figure 3D). The lower-right quadrants represent early apoptotic cells, whereas the upper-right quadrants represent late apoptotic cells. Annexin V staining detected significantly fewer apoptotic cells in SGC7901/OXA cells transfected with the miR-135a mimic than in control cells (early apoptotic cells: 3.2% ± 1.1% vs. 8.1% ± 0.9%, \( P < 0.05 \); late apoptotic cells: 10.2% ± 4.7% vs. 18.3% ± 2.3%, \( P < 0.05 \)). On the other hand, the miR-135a inhibitor enhanced OXA-induced apoptosis in MGC803/OXA cells compared with control cells (early apoptotic cells: 11.1% ± 1.9% vs. 7.2%...
± 1.5%, \( P \leq 0.05 \); late apoptotic cells: 19.5% ± 2.2% vs. 12.3% ± 1.6%, \( P \leq 0.05 \). Thus, we found that miR-135a confers OXA resistance in GC cells by blocking OXA-induced apoptosis.

Poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme that is the substrate of caspase, is cleaved downstream of caspases during apoptosis. We detected the protein levels of cleaved and total PARP by Western blotting in miR-135a mimic-transfected SGC7901/OXA and MGC803/OXA cells and control cells after OXA treatment. The protein level of cleaved PARP was dramatically reduced in miR-135a mimic-transfected cells. After 24-h OXA treatment, the activity of caspase 3 was further detected with the Caspase-Glo® 3/7 assay system in miR-135a mimic-transfected SGC7901/OXA cells. Lower caspase 3 activity was found in miR-135a mimic-transfected SGC7901/OXA cells than in control cells (Figure 3E).

miR-135a inhibits the GC response to OXA in vivo

To verify that miR-135a suppresses the GC response to OXA, we transplanted SGC7901/OXA and MGC803/OXA tumors into mice (Figure 4A). As expected, miR-135a was stably downregulated in SGC7901/OXA and MGC803/OXA cells transfected with a miR-135a inhibitor and stably upregulated in cells transfected with a miR-135a mimic (Supplementary Figure S2). Notably, as shown in Figure 4B, the relative tumor burden was greater in mice inoculated with miR-135a mimic-transfected cells and was reduced in mice treated with miR-135a inhibitor-transfected cells (\( P \leq 0.05 \)). A TUNEL assay also demonstrated that miR-135a upregulation attenuated OXA-induced cell death in tumor tissues (Figure 4C, \( P \leq 0.05 \)). In addition, immunostaining and Western blotting revealed that VEGF expression was significantly greater in tumor tissues treated with the miR-135a mimic than in controls, suggesting that miR-135a promotes proliferation in vivo (Figure 4D). Consistent with the in vitro assay, miR-135a markedly reduced the protein levels of E2F transcription factor 1 (E2F1) and DAPK2 in the tumors (Figure 4E and Supplementary Figure S3). Lastly, P-gp expression in tumor tissues was assessed by Western blotting. miR-135a clearly promoted P-gp expression, suggesting that miR-135a upregulation induces OXA-based chemotherapy resistance in vivo, largely by enhancing the expression of proteins associated with tumor growth and drug resistance (Figure 4F).

Figure 1: (Continued) The miR-135 family is frequently upregulated in GC and is associated with poor prognosis. E. A multivariate analysis of the hazard ratios (HRs) revealed that the upregulation of miR-135a may be an independent prognostic factor for the overall survival (OS) and recurrence-free survival rates (based on the Cox multivariate proportional hazards regression model). The HRs are presented as the mean (95% confidence interval). The variables included in the multivariate analysis were selected based on the results of univariate analysis. F. Relative expression of miR-135a in 280 human GC samples with or without high levels of serum carcinoembryonic antigen (CEA), helicobacter pylori infection, pathological staging, vascular invasion, lymphatic vessel metastasis, and early recurrence. The data were from three independent experiments, \(* P \leq 0.05\).
miR-135a promotes OXA resistance by inhibiting E2F1 expression and the Sp1/DAPK2 signaling pathway

To explore the underlying molecular mechanisms of the miR-135a-induced increase in OXA resistance in GC, we had initially assessed whether miR-135a inhibited apoptosis, which was thought to be a key determinant of OXA resistance in GC cells, and above data indicated that miR-135a induced the protein expression of the drug efflux pump P-gp. It is known that the expression of APC can be downregulated by miR-135a (leading to paclitaxel resistance) [14]. Thus we wondered whether miR-135a might affect the expression of any transcription factors, and evaluated the expression of transcription factors by qRT-PCR in SGC7901/OXA and MGC803/OXA cells transfected with miR-135a. Among the transcriptional activators we examined, E2F1 and DAPK2 were the most strongly downregulated genes following miR-135a overexpression (Figure 5A). Western blotting confirmed that miR-135a significantly reduced the expression of E2F1 and DAPK2 (Figure 5B and 5C).

To further elucidate whether miR-135a promotes OXA resistance in GC cells by suppressing E2F1 and the DAPK2 signaling pathway, we transfected SGC7901/OXA and MGC803/OXA cells with E2F1 siRNA and pCDNA3.1-DAPK2. As shown in Figure 6A, qRT-PCR and Western blotting revealed that E2F1 siRNA enhanced P-gp expression, while DAPK2 overexpression reversed this effect, suggesting that DAPK2 is one of the most important downstream factors of E2F1. As increased expression of miR-135a contributes to OXA resistance, we performed qRT-PCR and Western blotting to evaluate whether there were synergistic effects of the miR-135a

Figure 2: Relationship between miR-135 family levels and P-gp expression in GC patients. A. The relationship between miR-135 family levels and P-gp expression in 80 GC samples was determined with linear regression models. B. The relationship between miR-135 family levels and P-gp mRNA expression in OXA-resistant GC cell lines. The data were from three independent experiments.
mimic and E2F1 siRNA or DAPK2 siRNA on P-gp expression. As expected, synergistic induction of P-gp expression was observed in cells treated with the miR-135a mimic and E2F1 siRNA (Figure 6B) or DAPK2 siRNA (Figure 6C). Since miR-135a downregulates DAPK2 and E2F1, which were demonstrated to be key promoters of drug resistance [15], these results suggested that miR-135a contributed to OXA resistance in GC cells via the E2F1/DAPK2 signaling pathway.

In addition, since it was previously reported that miR-135a suppresses tumor proliferation by downregulating c-MYC [16], we evaluated whether c-MYC was involved in the miR-135a-induced OXA resistance of GC cells. Thus, we quantified the strength of the correlation between c-MYC and miR-135a expression by qRT-PCR. As shown in Figure 7A, we found a positive correlation between c-MYC and miR-135a expression in SGC7901/OXA cells (P < 0.05), indicating that c-MYC might positively regulate miR-135a in the development of OXA resistance. To further verify this mechanism, we examined c-MYC and miR-135a expression in four GC cell lines by qRT-PCR and Western blotting. As shown in Figure 7B-7C, miR-135a expression positively correlated with c-MYC expression (P < 0.05), consistent with the SGC7901/OXA data (Figure 7D).

Then, we treated GC cells with H2O2 and cisplatin, which induce a senescence-like phenotype in cancer cells by activating c-MYC [17, 18]. c-MYC protein expression was significantly reduced, and miR-135a expression was also relatively reduced. However, in either c-MYC-mutant NCI-N87 cells or c-MYC-deficient SNU-5 cells, nothing changed after treatment with H2O2 or cisplatin.

**Figure 3:** miR-135a promotes OXA resistance in GC cells. **A.** Transfection of a miR-135a mimic (containing a segment of GFP) into OXA-resistant GC cell lines increased cellular viability, as revealed by fluorescence microscopy at 0, 5, 15, and 30 hours (×100). **B.** EdU staining indicating that SGC7901/OXA cell proliferation and the percentage of EdU-positive SGC7901/OXA cells increased when miR-135a was upregulated; likewise, MGC803/OXA cell proliferation and the percentage of EdU-positive MGC803/OXA cells decreased when miR-135a was downregulated. **C.** After 48 h of transfection with a miR-135a mimic or inhibitor, the growth rates of SGC7901/OXA and MGC803/OXA cells were measured with CCK8 assays. (Continued)
When endogenous c-MYC expression was knocked down by c-MYC siRNA in the two c-MYC-wild-type OXA-resistant GC cell lines, miR-135a expression was also attenuated (Figure 8A). Subsequently, Western blotting also confirmed that the levels of E2F1 and DAPK2 increased and P-gp levels decreased after treatment with c-MYC siRNA. These results demonstrated that the induction of OXA resistance by miR-135a in GC cells was probably stimulated by c-MYC (Figure 8B).

Further, qRT-PCR results suggested that the overexpression of miR-135a was able to reverse the suppression of P-gp caused by c-MYC siRNA, confirming that c-MYC promotes miR-135a-induced OXA resistance in GC cells (Figure 8C). Consistent with the results of the qRT-PCR, we found that the miR-135a mimic significantly increased the protein level of P-gp in OXA-resistant GC cells by immunofluorescence (Figure 8D). c-MYC activity was represented via direct repression of E2F1 [19], then, to investigate whether E2F1 could directly bind the c-MYC protein in GC cells, we co-transfected GC cells with green fluorescent protein (GFP) or GFP-E2F1 and c-MYC. Immunoprecipitation with a GFP antibody co-precipitated GFP-E2F1 with c-MYC (Figure 9A–9B).

DISCUSSION

OXA (OXA) is an effective chemotherapeutic agent for the treatment of GC. Despite the impressive clinical remission rate for GC, the majority of patients will eventually develop some degree of resistance...
to OXA-based chemotherapy regimens [20]. The mechanisms underlying OXA resistance in GC cells are not fully understood. Hence, this report introduces a novel mechanism whereby miR-135a induces OXA resistance in GC cells by suppressing the E2F1/DAPK2 pathway. In addition, our lentiviral data provide in vivo evidence that suppressing miR-135a can prolong OXA drug sensitivity in GC. Recently, elevated expression of miR-135a has been reported to promote carcinogenesis [21]; for instance, miR-135a was shown to be upregulated in human bladder cancer [22]. On the other hand, relatively low levels of miR-135a have been observed in several cancer types.

**Figure 4: Lentiviral administration of miR-135a suppresses OXA-resistant GC cell growth in vivo.** A. A suspension of SGC7901/OXA or MGC803/OXA cells was injected i.p. into mice. Virus or OXA (10 mg/kg) was administered i.p. every two or three days. Tumors were treated until they reached approximately five times their original volume. B. Green fluorescence photographs illustrating representative features and growth curves of SGC7901/OXA or MGC803/OXA tumors in nude mice after injection with LV-GFP-miR-135a mimic, LV-GFP-control mimic, LV-GFP-miR-135a inhibitor, or LV-GFP-control inhibitor. The relative tumor volume was evaluated at two-day intervals in comparison with day 0, when the virus treatment was performed. C. The percentage of apoptotic cells in GC tissue was analyzed by TUNEL. D. Immunohistochemistry staining for VEGF in tumor tissues from mice with subcutaneous OXA-resistant GC cell implantation. Cells with positive staining were counted from 10 different visual fields. Western blotting was performed with an antibody against total VEGF, and β-Actin was used as a loading control. E. After administration of the miR-135a-expressing lentivirus, the levels of E2F1 and DAPK2 protein in the implanted tumor tissue were analyzed by Western blotting. F. After administration of the miR-135a-expressing lentivirus, the levels of P-gp protein in the implanted tumor tissue were analyzed by Western blotting. The data were from three independent experiments, *P < 0.05.
Figure 5: miR-135a downregulates E2F1 and DAPK2 in OXA-resistant GC cells. A. Heat maps of gene expression changes after transfection with the miR-135a mimic, as revealed by qRT-PCR. The red, white, and blue right-hand panel indicates the log2 of expression ratios after miR-135a mimic transfection. White represents a 1:1 ratio, red indicates upregulation, and blue indicates downregulation. B, C. After 48 h of miR-135a mimic transfection, E2F1 and DAPK2 protein levels with OXA treatment for 0 and 15 minutes were analyzed by Western blotting with anti-E2F1, anti-DAPK2, and anti-β-actin antibodies.
miR-135a might also be differently expressed in the carcinogenesis of GCs caused by different mechanisms.

Discrepancies emerged when we focused on miR-135a expression in GC. In human GC, it was previously reported that miR-135a expression was downregulated in some human primary GC tissues [11]. Contradictorily, another study demonstrated with microarrays and qRT-PCR that miR-135a expression was upregulated in GC [9]. In accordance with this result, we detected significant upregulation of miR-135a in GC tissues. Furthermore, the upregulation of miR-135a correlated with tumor drug resistance features and a poor tumor-free survival rate.

Figure 6: miR-135a promotes OXA resistance in GC cells by suppressing the E2F1/DAPK2 signaling pathway. A. E2F1 siRNA enhanced P-gp expression, while DAPK2 overexpression reversed this effect, as revealed by qRT-PCR (right panels) and Western blotting (left panels). B. Similar effects of the miR-135a mimic and siRNA-E2F1 on P-gp expression, with possible synergistic effects when transfected together, as revealed by qRT-PCR (right panels) and Western blotting (left panels). C. Similar effects of the miR-135a mimic and siRNA-DAPK2 on P-gp expression, with possible synergistic effects when transfected together, as revealed by qRT-PCR (right panels) and Western blotting (left panels). The data were expressed as the mean ± S.E.M. of three independent experiments, *P < 0.05.
Figure 7: miR-135a promoting oxaliplatin resistance in gastric cancer cells is regulated by c-MYC. A. Relationship between miR-135a and c-MYC expression levels in SGC7901/OXA cells. B, C, D. Relationship between miR-135a and c-MYC expression levels in oxaliplatin resistance and normal gastric cancer cells. E, F. Expression analysis of c-MYC and miR-135a level changes in different gastric cancer cells after treatment of cisplatin and H2O2, respectively. The data were expressed as the means ± S.E.M. of three independent experiments. *P < 0.05.
The induction of apoptosis is one of the predominant mechanisms by which cancer chemotherapeutic agents such as OXA kill GC cells and colorectal cancer cells. The association between miR-135a activity and apoptosis has been studied previously. Inactivation of miR-135a was found to be an initiating event in malignant glioma, while enrichment of miR-135a stimulated STAT6, SMAD5 and BMPR2, leading to mitochondria-dependent apoptosis of malignant glioma. Moreover, miR-135a was shown to be involved in the p53-dependent senescence pathway [25]. Yamada et al. identified c-MYC as a target of miR-135a [16], which was consistent with our results. Recently, Choi et al. reported that miR-135a induced cytotoxic effects in mouse Sertoli cells with a concomitant increase in reactive oxygen species, and suggested this as a new mechanism of apoptosis by miR-135a [26].

Figure 8: miR-135a promoting oxaliplatin resistance in gastric cancer cells is regulated by E2F1/DAPK2/P-pg axis. A. Expression analysis of c-MYC and miR-135a level changes in different gastric cancer cells after siRNA-c-MYC transfection, protein expression level for 0, and 5 minutes was analyzed by western blot with anti-MYC, and anti-β-actin antibodies. B. After treatment of siRNA-c-MYC, c-MYC downstream factor protein extracts were analyzed by western blot with anti-MYC, anti-E2F1, anti-DAPK2, anti-P-pg and anti-β-actin antibodies. C. The effect of miR-135a overexpression to rescue the siRNA-c-MYC-mediated suppression of P-pg, as revealed by qRT-PCR. D. SGC7901/OXA and MGC803/OXA cells were stained for P-pg expression, representative images. The data were expressed as the means ± S.E.M. of three independent experiments. \*P < 0.05 and \**P < 0.01.
which is also stimulated by E2F1, induces apoptosis when it is upregulated by the KLF6/Sp1 pathway [30]. Combining our results with the existing knowledge [31], we speculate miR-135a acts as a bridge between E2F1 and p53-dependent pathways in apoptosis. This evidence suggested that miR-135a might suppress apoptosis in GC by downregulating the E2F1/DAPK2 pathway. C-MYC, another key gene in E2F1- and p53-dependent pathways, is frequently upregulated in tumor tissues, and has been described as a proto-oncogene in a wide range of cancers. More importantly, it is also known to activate ERK1/2-c-MYC-CXCR4 signaling and to promote drug resistance in cancer cells [32, 33]. Our results indicated that c-MYC-dependent expression of miR-135a promoted OXA resistance in SGC7901/OXA cells by inhibiting the E2F1/Sp1/DAPK2 signaling feedback pathway.

In sum, we have shown that miR-135a is upregulated in OXA-resistant GC cells in vivo and in vitro. The overexpression of miR-135a markedly inhibited OXA-induced apoptosis and increased the resistance of cells to OXA. Our results also demonstrated that E2F1 was directly suppressed by miR-135a. Based on the results, we regard E2F1 as an indicator of the OXA sensitivity of GC cells. Since miR-135a contributed to OXA resistance by suppressing E2F1 expression, therapies designed to downregulate miR-135a may help GC patients to overcome OXA resistance.

MATERIALS AND METHODS

Patients, cells and animals

The samples of 280 patients who had undergone curative gastrectomy were collected at the Tumor Hospital of Guangxi Medical University from September 2010 to January 2015. The study design was approved by the Guangxi Medical University ethical review board and was performed in agreement with the Helsinki Declaration. All participants were informed about the nature of the proposed treatment, and informed consent forms were signed individually. The study was performed at the Department of Gastrointestinal Surgery, Affiliated Tumor Hospital of Guangxi Medical University. All patient tissue samples were randomly selected for tissue microarray construction and analysis. Human GC cells (SNU-5 and NCI-N87) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SGC7901/OXA, MGC-803/OXA, SGC7901, and MGC-
803 cells had been previously stored by our laboratory [15, 34, 35]. All cells were cultured in DMEM/1640 medium (Invitrogen, Gaithersburg, USA) with 10% FCS (ShiJiQin Biotechnology, Hangzhou, China) and Levofloxacin (Qilu Pharm, Jinan, China). Animal experiments were approved by the Guangxi Medical University Animal Ethics Committee. All BALB/c five-wk-old male nude mice were provided by the Animal Center of Guangxi Medical University (Nanning, China).

miRNA preparation and transfection

The miRNA mimic, inhibitor, and siRNAs were synthesized by Genechem (Shanghai, China). The sequences were as follows - *miR-135a* mimic: 5’-UUGGCUUUAUCCUAUGUGAAGCAUGAAUAAAAAGCCCAUAU-3’, mimic control: 5’-CACAGUUUUUGUGUAGUGAAAC-3’, *miR-135a* inhibitor: 5’-UCACAUAGGAAUAAAAGCCCAUA-3’, inhibitor control: 5’-CACAGUUUUUGUGUAGUGAAAC-3’, The siRNA sequences were: *siRNA-E2F1*: 5’-CCUAGCCUUCUGAUGGAAAUUGGUAUAGUAGCAAA-3’, *siRNA-DAPK2*: 5’-CCUAGCCUUCUGAUGGAAAUUGGUAUAGUAGCAAA-3’, *siRNA-Sp1*: 5’-GGAACAGGAAAGGGGCGGAAUAAUUCGUUUAUUUUUUAUUUCCUAUGUGAAGCAUGAAUAAAAAGCCCAUAU-3’, and *siRNA-NC*: 5’-CCUAGCCUUCUGAUGGAAAUUGGUAUAGUAGCAAA-3’. The miRNA mimic, inhibitor, and siRNAs were provided by the Animal Center of Guangxi Medical University (Nanning, China).

Reverse-transcriptase polymerase chain reaction (qRT-PCR) and miRNA microarray analysis

Total RNA was isolated and purified from cells with a TRIzol Reagent Kit (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. In brief, the primary miRNA and mRNA analyses were carried out with a SYBR Green RT-PCR Kit and the ABI Prism system (Applied Biosystems, Carlsbad, CA). All mRNA and miRNA levels were quantified with the ΔΔCt method, and relative expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The primers were synthesized by Sangon Biotech (Shanghai, China). For miRNA microarray analysis, miRNAs were extracted from total RNA with a miRNA isolation kit (Takara, Dalian, China). The miRNA sequences were as follows - *siRNA-E2F1*: 5’-CCUAGCCUUCUGAUGGAAAUUGGUAUAGUAGCAAA-3’, *siRNA-DAPK2*: 5’-CCUAGCCUUCUGAUGGAAAUUGGUAUAGUAGCAAA-3’, *siRNA-Sp1*: 5’-GGAACAGGAAAGGGGCGGAAUAAUUCGUUUAUUUUUUAUUUCCUAUGUGAAGCAUGAAUAAAAAGCCCAUAU-3’, and *siRNA-NC*: 5’-CCUAGCCUUCUGAUGGAAAUUGGUAUAGUAGCAAA-3’. The miRNA sequences were - *miRNA-E2F1*: 5’-CCUAGCCUUCUGAUGGAAAUUGGUAUAGUAGCAAA-3’, *miRNA-DAPK2*: 5’-CCUAGCCUUCUGAUGGAAAUUGGUAUAGUAGCAAA-3’, *miRNA-Sp1*: 5’-GGAACAGGAAAGGGGCGGAAUAAUUCGUUUAUUUUUUAUUUCCUAUGUGAAGCAUGAAUAAAAAGCCCAUAU-3’, and *miRNA-NC*: 5’-CCUAGCCUUCUGAUGGAAAUUGGUAUAGUAGCAAA-3’. The miRNA mimic, inhibitor, and siRNAs were provided by the Animal Center of Guangxi Medical University (Nanning, China).

Western blotting

Equal amounts of total protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes (Millipore, Bedford, USA). The supernatant of the culture medium was collected and incubated with an HRP-conjugated anti-rabbit secondary antibody (1:1000) (Santa Cruz Biotec, USA) at 4°C for 2 h. All protein signals were analyzed with an ECL Kit (Pierce, Rockford, USA). β-actin or α-Tubulin expression was used as a loading control (antibodies from Santa Cruz Biotec, USA).

Luciferase reporter assay

For Luciferase assays, in short, wild-type Luc-E2F1, mutant Luc-E2F1, wild-type Luc-DAPK2 and mutant Luc-DAPK2 3’-UTR were synthesized by Sangon Biotech (Shanghai, China). Firefly luciferase and Renilla luciferase are regarded as tracking genes in plasmids. Cells were cultured in 12-well plates and transfected with 5 ng Luc-E2F1 / DAPK2 3’-UTR and 5 ng Renilla. Every analysis was performed three times.

Immunoprecipitation analysis

SGC7901/OXA-E2F1, MGC803/OXA-E2F1, SGC7901/OXA-GFP and MGC803/OXA-GFP cells were harvested from 1640 culture medium and then supplemented with an immunoprecipitation buffer (Sigma-Aldrich, USA). The supernatant of the culture medium was collected and immunoprecipitated with an anti-c-MYC or anti-GFP antibody and the supernatant was separated by SDS-PAGE. Protein expression was analyzed by immunoblotting.

Aptoptosis analysis by flow cytometry

Cells were suspended in 24-well plates at a density of 1 × 10⁶ cells per well and incubated with Annexin V-FITC at a density of 10μL/mL and 7-amino-actinomycin D at a density of 10μL/mL at 4°C for 0.5 h. Then, the flow cytometry was carried out with a FACSCalibur system (Becton–Dickinson, CA, USA). After the insoluble crystals were completely dissolved, the data from the FACSCalibur system were analyzed with MultiCycle Flow System Software (Phoenix, CA, USA), and every analysis was performed three times.
**In vivo analysis of the effect of miR-135a overexpression or suppression on OXA resistance in GC**

SGC7901/OXA and MGC803/OXA-induced tumorigenesis were accomplished essentially through tumor implantation in nude mice. Intraperitoneal (i.p.) injections of approximately 5 × 10^6 tumor cells with 100 µL PBS were performed at day 0 and daily during the first week, until the subcutaneous tumor diameter measured about 12 mm. Then, mice received intratumor injections at a titer of 5 × 10^8 TU LV-miR-135a mimic-GFP, LV-Control mimic-GFP, LV-miR-135a inhibitor-GFP, or LV-Control inhibitor-GFP (Sangon Biotech, Shanghai, China) every other day. For simulated treatment, mice were injected i.p. with OXA (Qilu Pharm, Shangdong, China) at a dose of 30 mg/kg in PBS after LV-miR-135a mimic-GFP, LV-Control mimic-GFP, LV-miR-135a inhibitor-GFP, and LV-Control inhibitor-GFP treatment. The tumor volume was measured every two days, and a growth curve was drawn based on the formula \( V = L^2 \times W / 2 \), where \( L \) = tumor length and \( W \) = tumor width, as previously described [34]. At 35 days, the animals were sacrificed after tumor injection.

For the determination of apoptosis rates (TUNEL assay), apoptotic cells from mouse tumor tissue were analyzed on an apoptosis detection kit (KEYGEN, Nanjing, China) according to the manufacturer’s instructions. Apoptotic cells were counted under an Inverted System Microscope (Kyle Zeiss, GER), and the apoptotic ratio was calculated as the number of apoptotic cells/total number of cells.

**Statistical analysis**

Origin software (Version 7.5, OriginLab, USA) was used to carry out statistical analysis. Calculations and graphics were performed in Prism 5 (Graph Pad, USA). Data are expressed as the mean ± standard deviation unless otherwise noted. The analysis of categorical data was performed with Fisher’s exact test, and continuous parametric data were assessed with an independent t test. Mean values were obtained from the results of three independent experiments, and \( P \)-values < 0.05 were considered as significant.

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**COMPETING INTERESTS**

The authors have declared that no competing interests exist.

**REFERENCES**

1. Yu S, Yang CS, Li J, You W, Chen J, Cao Y, Dong Z and Qiao Y. Cancer Prevention Research in China. Cancer Prev Res (Phila). 2015; 8:662-674.
2. Micevicius A, Ignatavicius P, Markelis R, Parseliunas A, Butkute D, Ktaliokus M, Endzinas Z, Maleckas A and Dambrauskas Z. Trends and results in treatment of gastric cancer over last two decades at single East European centre: a cohort study. BMC Surg. 2014; 14:98.
3. Shang Y, Feng B, Zhou L, Ren G, Zhang Z, Fan X, Sun Y, Luo G, Liang J, Wu K, Nie Y and Fan D. The miR27b-CCNG1-P53-miR-508-5p axis regulates multidrug resistance of gastric cancer. Oncotarget. 2016; 7:538-549. doi: 10.18632/oncotarget.6374.
4. Riquelme I, Letelier P, Riffio-Campos AL, Brebi P and Roa JC. Emerging Role of miRNAs in the Drug Resistance of Gastric Cancer. Int J Mol Sci. 2016; 17: E424.
5. Cheng Y, Jutooru I, Chalalapaka G, Corton JC and Safe S. The long non-coding RNA HOTTIP enhances pancreatic cancer cell proliferation, survival and migration. Oncotarget. 2015; 6: 10840-10852. doi: 10.18632/oncotarget.3450.
6. Chen Y, Huang J and Liu B. AutomiRDB: a web resource connecting microRNAs and autophagy in cancer. Apoptosis. 2015; 20: 1016-1017.
7. Zhang Y, Hu Y, Fang JY and Xu J. Gain-of-function miRNA signature by mutant p53 associates with poor cancer outcome. Oncotarget. 2016; 7:11056-11066. doi: 10.18632/oncotarget.7090.
8. Sayed D and Abdellatif M. MicroRNAs in development and disease. Physiol Rev. 2011; 91:827-887.
9. Yang B, Jing C, Wang J, Guo X, Chen Y, Xu R, Peng L, Liu J and Li L. Identification of microRNAs associated with lymphangiogenesis in human gastric cancer. Clin Transl Oncol. 2014; 16:374-379.
10. Sui H, Cai GX, Pan SF, Deng WL, Wang YW, Chen ZS, Cai SJ, Zhu HR and Li Q. miR200c attenuates P-gp-mediated MDR and metastasis by targeting JNK2/c-Jun signaling pathway in colorectal cancer. Mol Cancer Ther. 2014; 13:3137-3151.
11. Wu H, Huang M, Cao P, Wang T, Shu Y and Liu P. MiR-135a targets JAK2 and inhibits gastric cancer cell proliferation. Cancer Biol Ther. 2012; 13:281-288.
12. Zhou M, Liu Z, Zhao Y, Ding Y, Liu H, Xi Y, Xiong W, Li G, Lu J, Fodstad O, Riker AI and Tan M. MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through suppression of pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) expression. J Biol Chem. 2010; 285:21496-21507.
13. Callaghan R. Providing a molecular mechanism for P-glycoprotein; why would I bother? Biochem Soc Trans. 2015; 43:995-1002.
14. Holleman A, Chung I, Olsen RR, Kwak B, Mizokami A, Saijo N, Parissenti A, Duan Z, Voest EE and Zetter
BR. miR-135a contributes to paclitaxel resistance in tumor cells both in vitro and in vivo. Oncogene. 2011; 30:4386-4398.

15. Yan LH, Wang XT, Yang J, Kong FB, Lian C, Wei WY, Luo W, Xie YB and Xiao Q. Reversal of multidrug resistance in gastric cancer cells by E2F-1 downregulation in vitro and in vivo. J Cell Biochem. 2013. 2014 Jan;115:34-41.

16. Yamada Y, Hidaka H, Seki N, Yoshino H, Yamasaki T, Itsako T, Nakagawa M and Enokida H. Tumor-suppressive microRNA-135a inhibits cancer cell proliferation by targeting the c-MYC oncogene in renal cell carcinoma. Cancer Sci. 2013; 104:304-312.

17. Huang XS, Zhang Q, Zhu D, Fu X, Wang M, Zhang Q, Moriuchi T and Liu JH. ICE1 of Poncirus trifoliata functions in cold tolerance by modulating polyamine levels through interacting with arginine decarboxylase. J Exp Bot. 2015; 66:3259-3274.

18. Zhu X, Zhang K, Wang Q, Chen S, Gou Y, Cui Y and Li Q. Cisplatin-mediated c-myc overexpression and cytochrome c (cYT c) release result in the up-regulation of the death receptors DR4 and DR5 and the activation of caspase 3 and caspase 9, likely responsible for the TRAIL-sensitizing effect of cisplatin. Med Oncol. 2015; 32:133.

19. Tai MC, Kajino T, Nakatomi M, Arima C, Shimada Y, Suzuki M, Miyoshi H, Yatabe Y, Yanagisawa K and Takahashi T. miR-342-3p regulates MYC transcriptional activity via direct repression of E2F1 in human lung cancer. Carcinogenesis. 2015; 36:1464-1473.

20. Tsimberidou AM, Said R, Culotta K, Wistuba I, Jelinek J, Fu S, Falchook G, Naing A, Piha-Paul S, Zinner R, Siddik ZH, He G, Hess K, Stewart DJ, Kurzrock R and Issa JP. Phase I study of azacitidine and oxaliplatin in patients with advanced cancers that have relapsed or are refractory to any platinum therapy. Clin Epigenetics. 2015;7:29.

21. Leung CO, Deng W, Ye TM, Ngan HY, Tsao SW, Cheung AN, Pang RT and Yeung WS. miR-135a leads to cervical cancer cell transformation through regulation of beta-catenin via a SIAH1-dependent ubiquitin proteasomal pathway. Carcinogenesis. 2014;35:1931-1940.

22. Mao XP, Zhang LS, Huang B, Zhou SY, Liao J, Chen LW, Qiu SP and Chen JX. Mir-135a enhances cellular proliferation through post-transcriptionally regulating PHLPP2 and FOXO1 in human bladder cancer. J Transl Med. 2015; 13:86.

23. Tang W, Jiang Y, Mu X, Xu L, Cheng W and Wang X. MiR-135a functions as a tumor suppressor in epithelial ovarian cancer and regulates HOXA10 expression. Cell Signal. 2014;26:1420-1426.

24. Butz H, Liko I, Czirjak S, Igaz P, Korbonits M, Racz K and Patocs A. MicroRNA profile indicates downregulation of the TGFbeta pathway in sporadic non-functioning pituitary adenomas. Pituitary. 2011; 14:112-124.

25. Wu S, Lin Y, Xu D, Chen J, Shu M, Zhou Y, Zhu W, Su X, Zhou Y, Qiu P and Yan G. MiR-135a functions as a selective killer of malignant glioma. Oncogene. 2012; 31:3866-3874.

26. Choi JS, Oh JH, Choi MS, Park SM, Kang SJ, Oh MJ, Kim SJ, Hwang SY and Yoon S. miRNA regulation of cytotoxic effects in mouse Sertoli cells exposed to nonylphenol. Reprod Biol Endocrinol. 2011; 9:126.