European Review for Medical and Pharmacological Sciences 2020; 24: 12116-12123

MiR-1231 enhances docetaxel sensitivity to gallbladder carcinoma cells by downregulating FOXC2

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Abstract. – OBJECTIVE: To illustrate the role of microRNA-1231 (miR-1231) in regulating malignant proliferative potential and DTX sensitivity to gallbladder carcinoma (GBC) by regulating FOXC2 level.

PATIENTS AND METHODS: Expression levels of miR-1231 in GBC tissues and paracancerous ones were detected. The relationship between miR-1231 level and clinical parameters of GBC patients was analyzed. After overexpression of miR-1231, changes in proliferative and apoptotic potentials in GBC-SD and NOZ cells were examined by Cell Counting Kit-8 (CCK-8), colony formation assay and flow cytometry, respectively. Regulatory effects of miR-1231 on its downstream gene FOXC2 were determined by Luciferase assay. Finally, the role of miR-1231 in regulating DTX sensitivity to GBC cells was assessed.

RESULTS: MiR-1231 was downregulated in GBC tissues compared to paracancerous ones. GBC patients expressing lower level of miR-1231 had worse tumor staging and larger tumor size. Overexpression of miR-1231 attenuated proliferative potential, and induced apoptosis in GBC cells. FOXC2 was upregulated in GBC and negatively linked to miR-1231. Luciferase activity confirmed that FOXC2 was the target gene binding miR-1231. DTX treatment dose-dependently suppressed viability in GBC cells and overexpression of miR-1231 could enhance DTX sensitivity in GBC. Notably, overexpression of FOXC2 abolished regulatory effects of overexpressed miR-1231 on proliferative and apoptotic potentials in GBC cells.

CONCLUSIONS: MiR-1231 is downregulated in GBC species. Its level is closely linked to tumor staging and tumor size in GBC patients. By downregulating FOXC2, miR-1231 enhances DTX sensitivity to GBC cells and thus alleviates the malignant development of GBC.

Key Words:
MiR-1231, FOXC2, Gallbladder carcinoma, Docetaxel, Malignant development.

Introduction

In recent years, the incidence of malignant tumors has increased. Gallbladder carcinoma (GBC) is a highly prevalent tumor in the biliary system. The mortality of GBC ranks sixth in cancer death, posing a serious threat to affected people[1,2]. Detective rate of early stage GBC is relatively low and clinical efficacy is unsatisfactory[3,4]. The development of GBC involves multiple factors and pathways[5,6]. Nowadays, effective and specific biomarkers are highlighted in tumor treatment[7-9].

MicroRNAs (miRNAs) are extensively involved in tumor development, serving as oncogenes or tumor suppressors[10,11]. They are considered as potentials biomarkers used in the screening, treatment and prognosis prediction of tumors[12,13]. Since miRNAs are stably expressed in body fluids, detection of miRNA levels may contribute to clinical examinations of tumor diseases[14,15]. Moreover, they participate in cell growth, immune response, inflammatory response, and other pathological progressions[15,16]. MiR-1231 is found to be abnormally expressed in solid tumors as a tumor-suppressor gene[17,18].

In this paper, we collected GBC species and paracancerous ones to determine miR-1231 level. Subsequently, *in vitro* effects of miR-1231 on DTX sensitivity, proliferative, and apoptotic potentials in GBC cells were assessed.

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Patients and Methods

GBC Patients and Samples

A total of 43 GBC patients with complete clinical data were included. None of them had preoperative treatment. GBC tissues (0.8 cm×0.8 cm×0.6 cm) and paracancerous ones (at least 3 cm away from the tumor) were surgically resected and stored in liquid nitrogen. The International Guidelines followed to collect GBC patients were consistent with the Union for International Cancer Control Center. This investigation was approved by the Ethics Committee of Guangrao People's Hospital and was conducted after the informed consent of each subject.

Cell Culture

GBC cell lines (GBC-SD and NOZ) were purchased from Cell Bank (Shanghai, China). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and William's E Medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted using ethylenediaminetetraacetic acid (EDTA).

Transfection

Transfection plasmids were purchased from GenePharma (Shanghai, China). Cells were cultured to 50-70% confluence and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). They were collected 48 h later for the following use.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Assay

200 cells were inoculated in each well of a 6-well plate and cultured for 2 weeks. Medium was replaced once in the first week and twice in the second week. Visible colonies were washed in phosphate-buffered saline (PBS), fixed in 2 mL of methanol for 20 min, and dyed in 0.1% crystal violet for 20 min. Colonies were finally captured and counted.

Flow Cytometry

Cell suspension was prepared at 1×10^6 cells/mL. 10 μL of Annexin-V and 380 μL of loading buffer were added in 100 μL of suspension. Following 15-min incubation in the dark, cell apoptosis was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. miR-1231: forward: 5'-GUGUCUGGGCGGACAGCUGC-3'; reverse: 5'-GCAGCUGUCCGCCCAGACAC-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3'; reverse: 5'-AACGCTTCACGAATTTGCGT-3'; FOXC2: forward: 5'-CCTACCTGAGCGAGCAGAAT-3'; reverse: 5'-ACCTTGACGAAGCACTCGTT-3'; GAPDH: forward: 5'-CACCCACTCCTCCACCTTTGA-3'; reverse: 5'-ACCACCCTGTTGCTGTAGCCA-3'.

Western Blot

The cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Luciferase Assay

The cells were inoculated in a 24-well plate. They were co-transfected with NC mimic/miR-1231 mimic and FOXC2-WT/FOXC2-MUT, respectively. Cells were lysed for determining relative Luciferase activity 48 h later (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the *t*-test. Chi-square test was used for analyzing the relationship be-

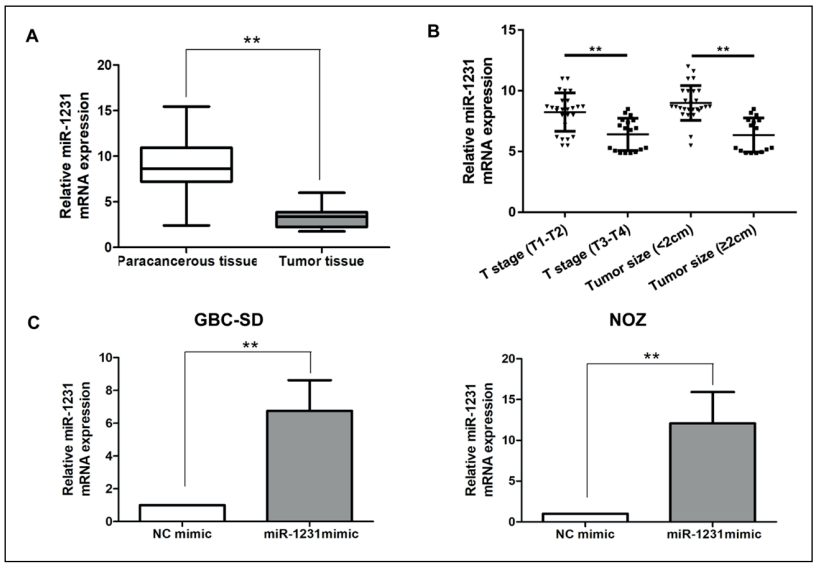

Figure 1. MiR-1231 was downregulated in GBC and linked to tumor staging and tumor size. **A,** Expression levels of miR-1231 in paracancerous tissues and GBC tissues. **B,** Expression levels of miR-1231 in GBC with different tumor staging or tumor sizes. **C,** Transfection efficacy of miR-1231 mimic in GBC-SD and NOZ cells. Data were expressed as mean±SD, $^{**}p<0.01$.

tween miR-1231 level and clinical data of GBC patients. Pearson correlation test was applied for evaluating the relationship between expression levels of miR-1231 and FOXC2 in GBC species. $p<0.05$ was considered as statistically significant.

Results

MiR-1231 Was Downregulated in GBC and Linked to Tumor Staging and Tumor Size

Compared with paracancerous tissues, miR-1231 was downregulated in GBC tissues (Figure 1A). Notably, miR-1231 level was lower in GBC patients with T3-T4 than those with T1-T2. MiR-1231 level was lower in GBC patients with a smaller tumor size (< 2 cm) than those with a larger one (≥ 2 cm) (Figure 1B). By analyzing clinical data of included GBC patients, it is found that miR-1231 level was significantly linked to tumor staging and tumor size in GBC patients, while it was not related to age, gender and distant metastasis rate (Table I). It is suggested that miR-1231 may be used to predict the malignant development of GBC.

MiR-1231 Alleviated Proliferative Potential and Induced Apoptosis in GBC

To explore the biological function of miR-1231 in GBC, we constructed miR-1231 mimic and

Table I. Association of miR-1231 expression with clinicopathologic characteristics of gallbladder cancer.

Parameters	No. of cases	miR-1231 expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.864
< 60	19	10	9	
≥ 60	24	12	12	
Gender				0.443
Male	22	10	12	
Female	21	12	9	
T stage				0.009
T1-T2	25	17	8	
T3-T4	18	5	13	
Tumor size (cm)				0.044
< 2	27	17	10	
≥ 2	16	5	11	
Distance metastasis				0.172
No	25	15	10	
Yes	18	7	11	

tested its transfection efficacy (Figure 1C). CCK-8 assay showed that overexpression of miR-1231 reduced viability in GBC-SD and NOZ cells (Figure 2A). Similarly, transfection of miR-1231 mimic reduced colony number in GBC cells, suggesting the inhibited proliferative potential (Figure 2B). In addition, apoptotic rate markedly increased in GBC cells overexpressing miR-1231 (Figure 2C).

MiR-1231 Specifically Bound FOXC2

Bioinformatics analysis uncovered binding sequences in the 3'UTR of miR-1231 and FOXC2. Based on these predicted sequences, we constructed wild-type and mutant-type Luciferase vectors targeting FOXC2. It is shown that overexpression of miR-1231 markedly quenched Luciferase activity in the wild-type vector of FOXC2, verifying the binding between miR-1231 and FOXC2 (Figure 3A). Furthermore, protein level of FOXC2 was downregulated in GBC-SD and NOZ cells overexpressing miR-1231 (Figure 3B). Compared with paracancerous tissues, FOXC2 was markedly upregulated in GBC tissues (Figure 3C). FOXC2 level was negatively correlated to miR-1231 level in GBC species (Figure 3D).

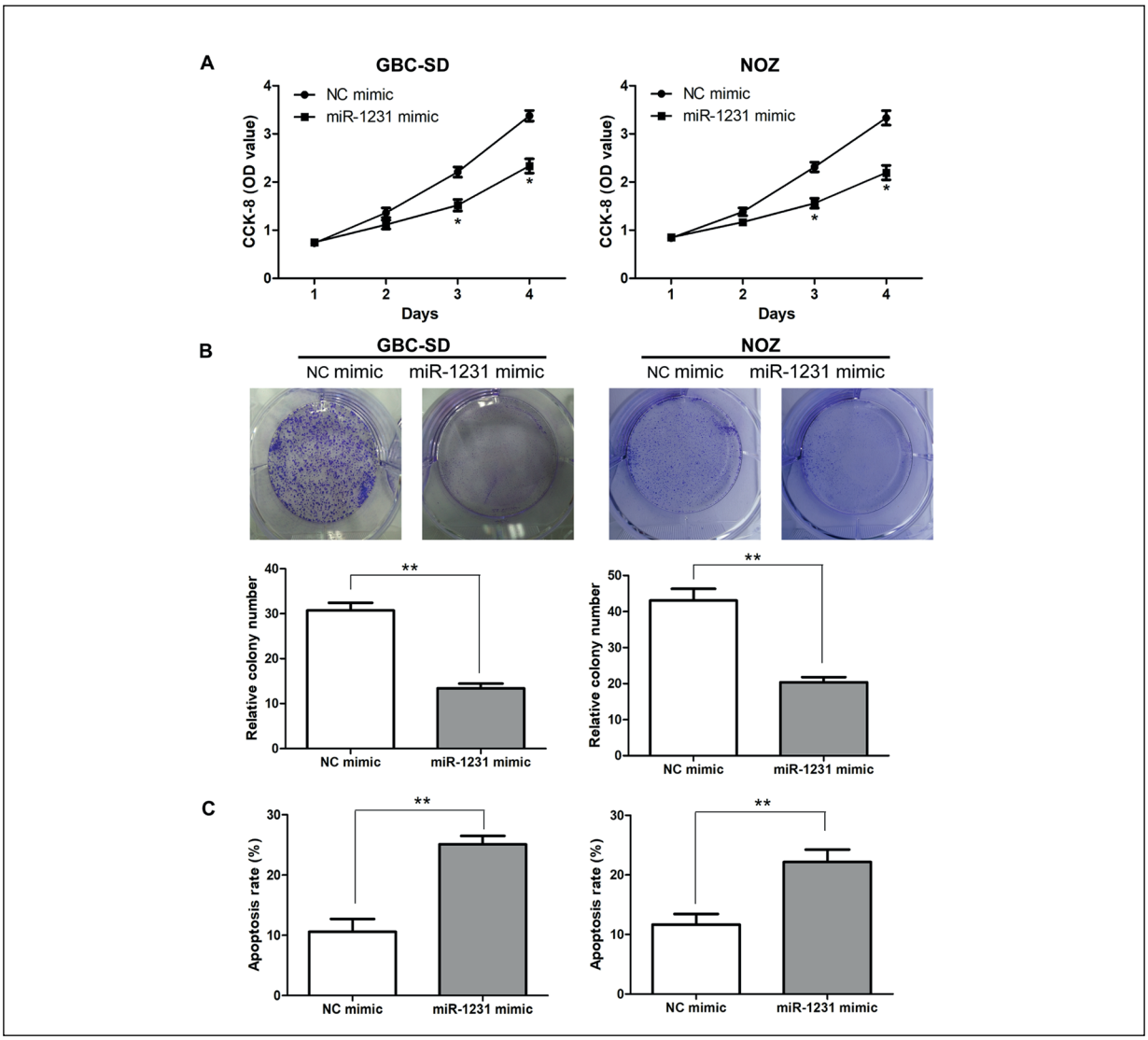

Figure 2. MiR-1231 alleviated proliferative potential and induced apoptosis in GBC. **A,** CCK-8 assay showed viability in GBC-SD and NOZ cells transfected with NC mimic or miR-1231 mimic. **B,** Colony formation assay showed colony number in GBC-SD and NOZ cells transfected with NC mimic or miR-1231 mimic (magnification: 10×). **C,** Flow cytometry showed apoptotic rate in GBC-SD and NOZ cells transfected with NC mimic or miR-1231 mimic. Data were expressed as mean±SD, *p<0.05, **p<0.01.

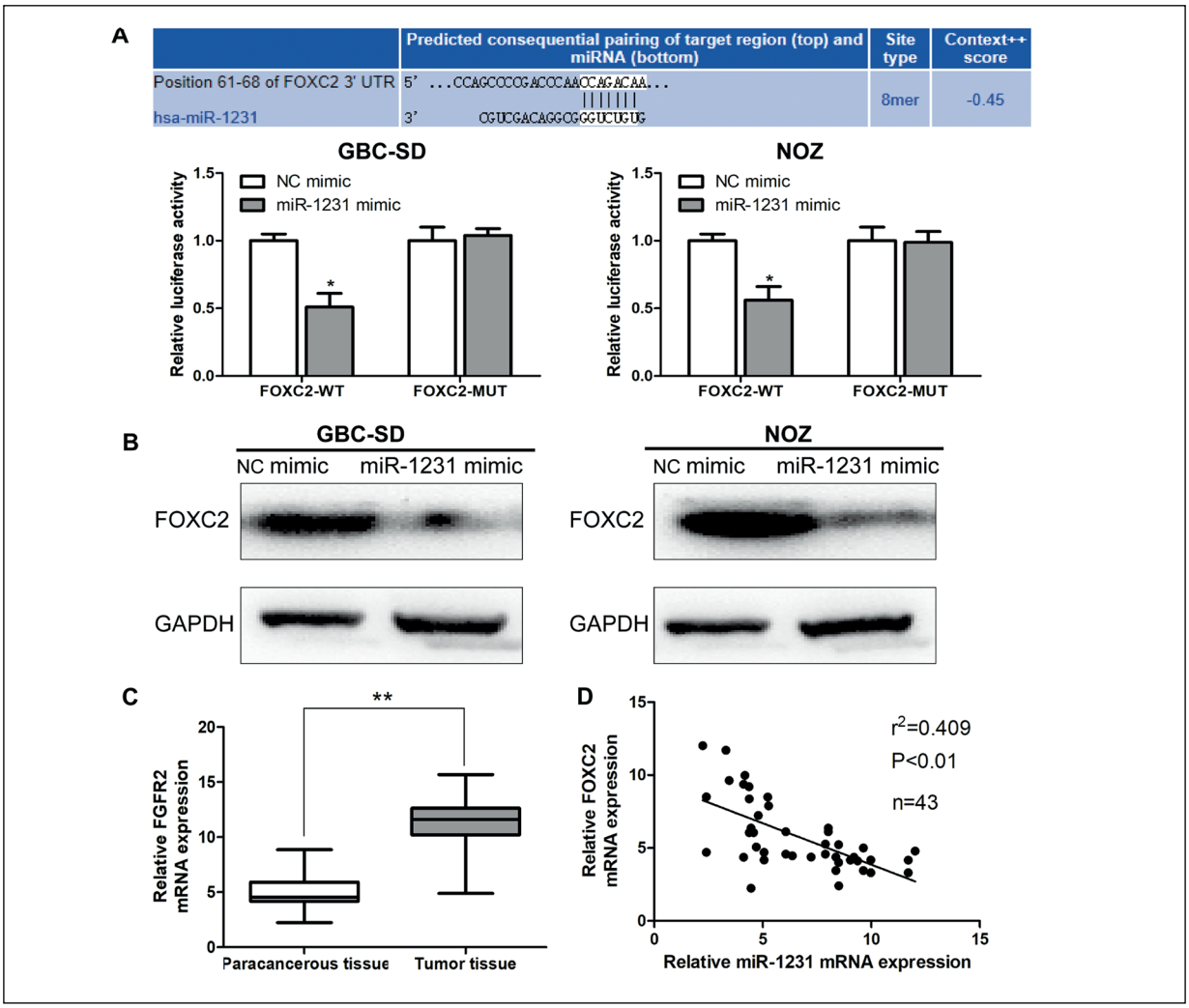

Figure 3. MiR-1231 specifically bound FOXC2. **A,** Luciferase activity in GBC-SD and NOZ cells co-transfected with NC mimic/miR-1231 mimic and FOXC2-WT/FOXC2-MUT. **B,** Protein level of FOXC2 in GBC-SD and NOZ cells transfected with NC mimic or miR-1231 mimic. **C,** Expression levels of FOXC2 in paracancerous tissues and GBC tissues. **D,** A negative correlation between expression levels of miR-1231 and FOXC2. Data were expressed as mean±SD, *p<0.05, **p<0.01.

MiR-1231 Enhanced DTX Sensitivity in GBC

GBC-SD and NOZ cells were induced with 10, 20, 50 or 100 μM docetaxel (DTX) for 24 h. It is shown that compared with those treated with dimethyl sulfoxide (DMSO) (negative control), treatment of 10 μM DTX or 20 μM did not affect drug sensitivity in GBC cells (Figure 4A). In particular, 10 μM DTX was the best drug-resistance concentration to GBC cells. Our findings uncovered that GBC cells were sensitive to 10 μM DTX induction after overexpression of miR-1231 (Figure 4B). In addition, cell static effects of miR-1231 on DTX-induced GBC cells were assessed. It is shown that viability in 10 μM DTX-induced GBC cells overexpressing miR-1231 was reduced (Figure 4C).

FOXC2 Abolished the Role of MiR-1231 in Cell Phenotypes and DTX Sensitivity in GBC

Overexpression of miR-1231 downregulated FOXC2 in GBC cells, which was further upregulated by transfection of pcDNA-FOXC2. In particular, transfection of pcDNA-FOXC2 also upregulated FOXC2 in DTX-induced GBC cells (Figure 5A). Overexpression of FOXC2 was able to reverse the regulatory effect of

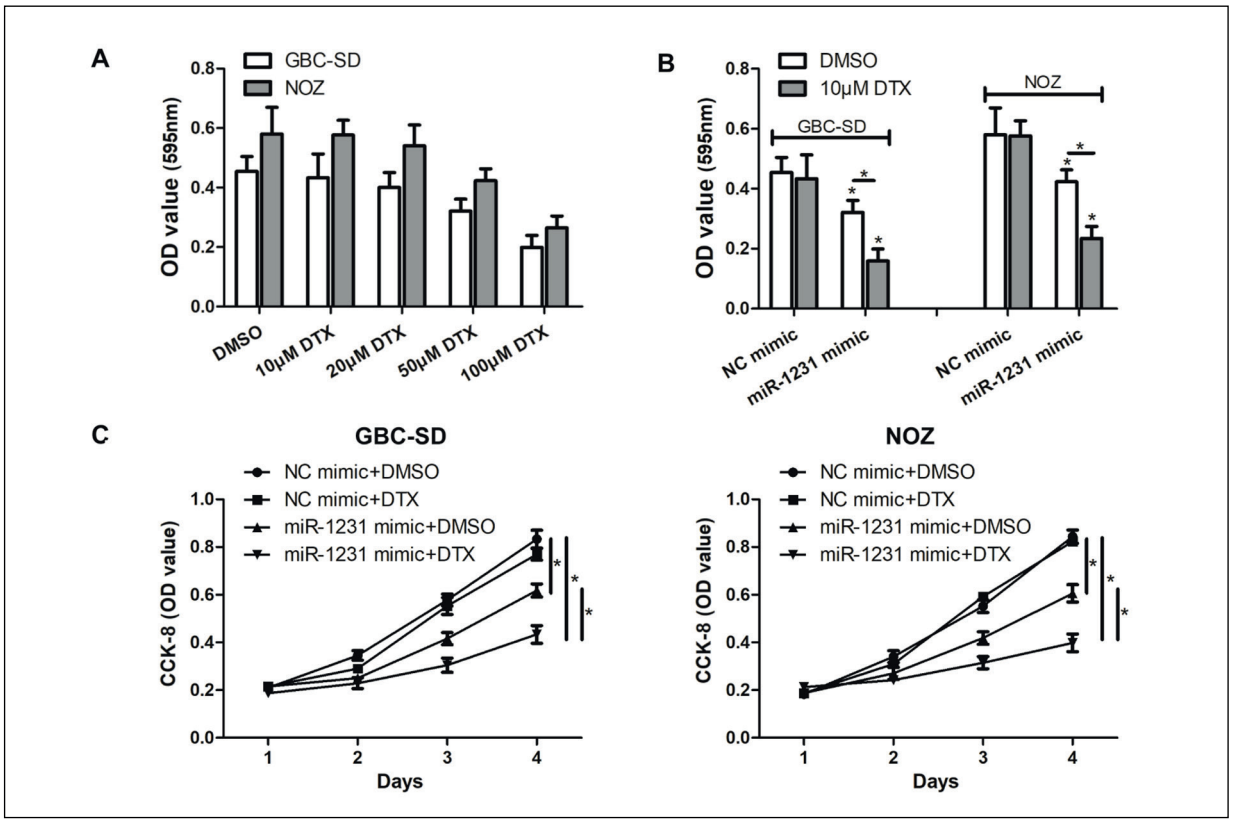

Figure 4. MiR-1231 enhanced DTX sensitivity in GBC. **A,** Drug sensitivity in GBC-SD and NOZ cells induced with DMOS, 10 μM, 20 μM, 50 μM or 100 μM DTX. **B,** Cytotoxicity in GBC-SD and NOZ cells induced with 10 μM DTX and transfected with NC mimic or miR-1231 mimic. **C,** CCK-8 assay showed viability in GBC-SD and NOZ cells induced with 10 μM DTX or DMSO and transfected with NC mimic or miR-1231 mimic. Data were expressed as mean±SD, *p<0.05.

overexpressed miR-1231 on DTX sensitivity (Figure 5B) and viability (Figure 5C) in GBC-SD and NOZ cells.

Discussion

Surgical resection is the best therapeutic strategy to GBC, and it is also the major approach to improve the prognosis in GBC patients[1-4]. Nevertheless, a great number of GBC patients lose the optimal surgical opportunity due to the low detective rate of early stage GBC. Clinical symptoms of GBC in the early phase are atypical and GBC is highly invasive, resulting in the high rate of middle and advanced GBC[4-6]. More seriously, GBC relapse *in situ* after surgery is up to 50%[6,7]. It is necessary to illustrate the pathogenesis and etiology of GBC[7-9].

MiRNAs are extensively involved in tumor development[10-13]. They are promising candidates of therapeutic targets and prognostic indicators in tumor diseases[12-16]. In this paper, miR-1231 was downregulated in GBC tissues we collected. Its level was negatively correlated to tumor staging and tumor size of GBC, suggesting that miR-1231 may be a tumor suppressor. Subsequently, our findings demonstrated that overexpression of miR-1231 attenuated proliferative potential, and induced apoptosis in GBC cells. In addition, viability in GBC-SD and NOZ cells dose-dependently decreased following DTX treatment, and overexpression of miR-1231 enhanced DTX sensitivity in GBC cells. DTX intervention may inhibit the activity of GBC-SD and NOZ cells by upregulating miR-1231. Clinical trials have shown that the average survival time of GBC patients treated with DTX is prolonged[19,20]. Hence, we believed that miR-1231 may contribute to improve clinical efficacy of DTX chemotherapy in GBC patients.

Through bioinformatics analysis and Luciferase assay, FOXC2 was the target gene binding miR-1231. FOXC2 level was negatively regulated

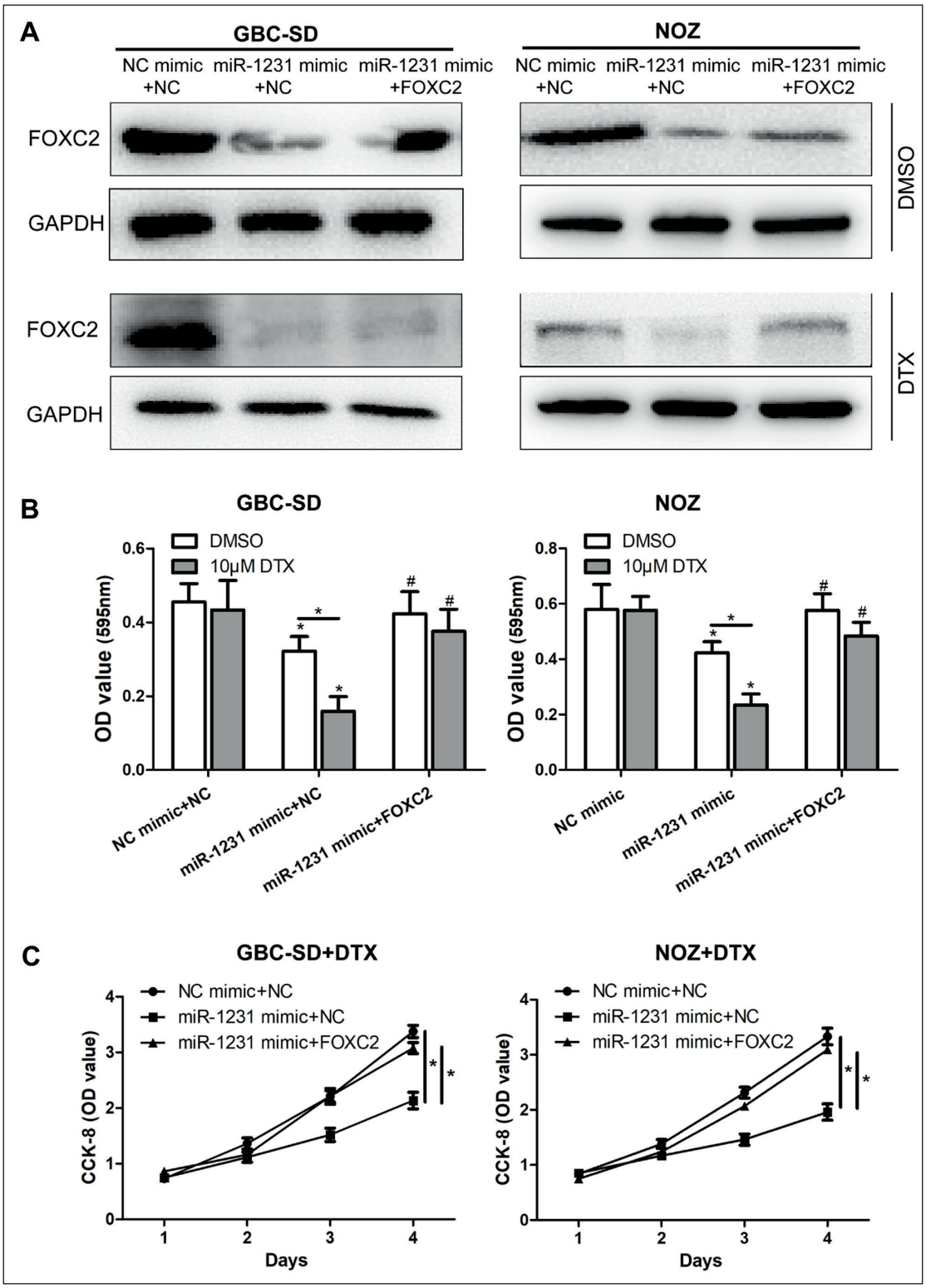

Figure 5. FOXC2 abolished the role of miR-1231 in cell phenotypes and DTX sensitivity in GBC. **A,** Protein level of FOXC2 in GBC-SD and NOZ cells transfected with NC mimic+NC, miR-1231 mimic+NC or miR-1231 mimic+pcDNA-FOXC2. **B,** Cytotoxicity in GBC-SD and NOZ cells induced with 10 μM DTX or DMSO and transfected with NC mimic+NC, miR-1231 mimic+NC or miR-1231 mimic+pcDNA-FOXC2. **C,** CCK-8 assay showed viability in GBC-SD and NOZ cells induced with 10 μM DTX and transfected with NC mimic+NC, miR-1231 mimic+NC or miR-1231 mimic+pcDNA-FOXC2. Data were expressed as mean±SD, *#p<0.05.

by miR-1231 in GBC cells. An interaction between miRNAs and the target genes can influence pathological progression[19,21]. Here, rescue experiments uncovered that FOXC2 was responsible for phenotype changes and DTX sensitivity in GBC regulated by miR-1231. The novelty of this study was that we first explored the role of miR-1231 in the docetaxel sensitivity to gallbladder carcinoma which provides a new therapy for treating gallbladder carcinoma.

Conclusions

MiR-1231 is downregulated in GBC species. Its level is closely linked to tumor staging and tumor size in GBC patients. By downregulating FOXC2, miR-1231 enhances DTX sensitivity to GBC cells and thus alleviates the malignant development of GBC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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