Am J Cancer Res 2022;12(10):4602-4621
www.ajcr.us /ISSN:2156-6976/ajcr0145885

Original Article

HOXC10 promotes carboplatin resistance of ovarian cancer by regulating ABCC3

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Received August 11, 2022; Accepted September 14, 2022; Epub October 15, 2022; Published October 30, 2022

Abstract: HOXC10 has been reported to be upregulated in ovarian cancer (OC) tissues, attributing to the metastasis of OC. However, the specific functions of HOXC10 in OC, especially its role in chemoresistance, remain to be determined. Therefore, in this study, we explored the function and the underlying mechanisms of HOXC10 in carboplatin resistance of OC. A variety of approaches were utilized to analyze the expression of HOXC10 and its related genes. The effect of HOXC10 in cell growth and chemoresistance was investigated in carboplatin-resistant OC subline TOV21G-R and the parental TOV21G-P cells. ROC curve and survival analysis were conducted to determine the predictive value of HOXC10 and ABCC3 combination in carboplatin resistance and the prognosis of OC. Luciferase reporter assay and Chromatin immunoprecipitation (ChIP) assay were used to explore the direct regulation of β-catenin by HOXC10. Our results demonstrated that the expression of HOXC10 was upregulated both in the carboplatin-resistant OC tissues and TOV21G-R cells. Furthermore, the upregulation of HOXC10 could promote the expression of ABCC3 by transcriptionally upregulating β-catenin. Moreover, overexpression of HOXC10 could decrease the sensitivity of cells to carboplatin, while knocking down HOXC10 had the opposite effect both *in vitro* and *in vivo*. Therefore, the expression of HOXC10/ABCC3 could be a novel biomarker for predicting the carboplatin resistance and the prognosis of OC patients.

Keywords: HOXC10, ABCC3, β-catenin, carboplatin resistance, ovarian cancer

Introduction

Ovarian cancer (OC) is one of the most common malignant tumors in the female reproductive system and ranks the fourth among all the tumor-related deaths in women. The current standard therapy is ovarian tumor reduction + platinum-based combination chemotherapy; however, most patients relapse within 12-24 months and die from resistance to chemotherapy [1], reflecting the clinical challenge of treating drug-resistant OC. Therefore, exploring the molecular mechanisms underlying the platinum resistance in OC is of great significance to improve the survival of OC patients.

The human HOX gene family can be divided into four clusters: A, B, C, and D [2]. HOXC10, one of HOX gene family members, is located on the human chromosome 12 of 2,017 bps [3]. Abnormal expression of HOXC10 is reported to be closely associated with the initiation and development of tumors [3]. Our previous studies have shown that HOXC10 is upregulated in OC, as a result of the dysfunction of the upstream regulatory miR-222-3p [4]. Subsequently, the upregulation of HOXC10 promotes the metastasis of OC by transcriptionally regulating the EMT-related gene Slug. We further revealed that Slug could repress miR-222-3p transcription [5], thereby forming a regulatory loop among HOXC10, Slug and miR-222-3p. However, other functions of HOXC10 in OC, especially its involvement in chemoresistance are unclear.

ABCC3 is a member of the ABC transporter family, which pumps small-molecule drugs outside

the cells dependent of ATP hydrolysis [6]. ABCC3 has been shown to be upregulated in many malignant tumors [7, 8] and is associated with poor prognosis; however, the involvement of ABCC3 in the carboplatin resistance of OC patients is largely unknown. In this study, we found that the expression of HOXC10 and ABCC3 was both upregulated in the carboplatin-resistant OC tissues, which was associated with the poor prognosis of OC patients. Hence, our study was the first to indicate that the expression of HOXC10/ABCC3 might be a novel predictive biomarker for the diagnosis, the chemoresistance, and the prognosis of OC. Mechanistically, we revealed that HOXC10 could directly upregulate the transcription of β-catenin and that the HOXC10/β-catenin/ABCC3 axis might serve as a potential therapeutic target for overcoming the carboplatin resistance of OC in clinical treatment.

Materials and methods

Patient samples

A total of 60 OC patients were enrolled from the Xiangya hospital from year 2015 to 2018 for this study. The platinum free interval (PFI) was defined as the duration of the last date of carboplatin dose until the progression of the patients documented. The OC patients were clinically diagnosed as carboplatin-sensitive and carboplatin-resistant based on the PFIs of the patients (> 12 months and < 12 months, respectively). The survival curve was depicted by the clinical follow-up of 59 patients as one patient was lost to follow-up.

Cell culture

The 293T and human normal ovarian epithelial IOSE-29 cells were maintained by the laboratory of Professor Gang Yin (Changsha, China). The OC cell lines TOV21G-P (parental cells) and TOV21G-R (carboplatin-resistant cells) were kind gifts from Professor Jing Tan (Guangzhou, China). The cell lines (293T, IOSE-29 and TOV21G-P) were cultured in RPMI-1640 (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10% FBS (Biological Industries, Kibbutz Beit Haemek, Israel), while TOV21G-R cells were cultured in RPMI-1640 containing 10% FBS and 500 nM carboplatin (S121512, Selleck, Houston, Texas, USA). All cells were maintained in a 37°C humidified incubator with 5% CO_2.

CCK-8 assay

A total of 5,000 cells were seeded into 96-well plates and cultured overnight before being treated for 48 h with different concentrations of carboplatin: 0, 10, 20, 50, 100, 150, 200, 250, 300 μM. Cell viability was determined by CCK-8 assay following the manufacturer's instruction (7sea biotech, Shanghai, China). The optical density (OD) absorbance at 450 nm were measured by a microplate reader.

Colony formation assay

Briefly, a total of 300 cells were seeded into the 12-well plates and cultured overnight before treated with 5 μM carboplatin or vehicle (DNase/RNase-free ddH_2O). The cells were continuously cultured for 14 days and then fixed with 4% paraformaldehyde for 20 min followed by staining with crystal violet for 20 min. Individual colonies were counted and photographed.

Flow cytometry apoptosis assay

Cell apoptosis assay was performed by using the Annexin V-FITC/PI apoptosis kit (70-AP101-100, Multi sciences, Hangzhou, China). Briefly, the cells were seeded into the 6-well plates at about 60-70% and were treated with different concentrations of carboplatin for 48 h. The cells were then harvested to analyze the ratio of apoptotic cells according to the manufacturer's instructions.

IHC staining

Briefly, all tissue specimens were embedded in paraffin and sectioned onto glass microscope slides (0.5 μm). For IHC staining, sections were dewaxed, rehydrated, and pre-treated with the universal two-step kit (pv-9000, ZSGB-bio, Beijing, China) before incubated with the indicated antibodies, as shown in Supplementary Table 1. The staining density score was calculated as previously described [9].

qRT-PCR analysis

Total RNA was isolated with TRizol (Vazyme, Nanjing, China). qRT-PCR was performed following the protocol previously described [4].

GAPDH was used as the internal reference, and $2^{-\Delta\Delta Ct}$ method was used for data analysis. The primers used in this study were listed in Supplementary Table 2.

Western blot analysis

Western blot analysis was performed following the protocol previously described [4]. Primary antibodies used in this study were summarized in Supplementary Table 1. The images from western blot analysis were detected using a chemiluminescence imaging system (Bio-rad).

Plasmids, inhibitors, and agonists

TpcDNA3.1-HOXC10 and pcDNA6-CTNNB1 mammalian expression plasmids were constructed by PCR amplification. For specific gene knockout, the sgRNA sequences with minimal off-target scores were chosen (http://guides.sanjanalab.org/#/) and subcloned into the lentiCRISPR V2 retroviral vector. For luciferase reporter assay, the human β-catenin gene promoter region containing the HOXC10-binding sites was generated by PCR amplification from HO-8910 cells and cloned into pGL3-basic luciferase reporter plasmid (Promega, Madison, WI, USA). For specific gene knockdown, siRNAs were designed, synthesized by RiboBio (Guangzhou, China), and used at a final concentration of 5 nM. Cell transfection was conducted by using jetPRIME kit (Polyplus Transfection, Illkirch, France) according to the manufacturer's instruction. The primer sequences were listed in Supplementary Table 2.

Wnt pathway inhibitor IWR-1-endo (1127442-82-3) and Wnt pathway agonists SKL2001 (909089-13-0) were purchased from Selleck, Houston, Texas, USA. Two concentrations (10 μM and 20 μM) of IWR-1-endo or SKL2001 were used to treat the cells for 24 h before the cell lysates were collected for further analysis.

Dual-luciferase reporter assay and TOP/FOP activity

The 293T cells were seeded into 24-well plates and cultured overnight to reach 50-60% confluence for transfection. Briefly, the cells were transfected with either HOXC10-expressing OE-HOXC10 (or its negative control plasmid, named VECTOR) or si-HOXC10 (or its negative control plasmid, named si-NC) and TOP flash/FOP flash plasmids. At 48 h after transfection, the cell lysates were collected, and the Dual-Luciferase® reporter assay system (Promega, Madison, WI, USA) was used to detect the luciferase activities of TOP/FOP plasmid.

ChIP-qPCR assay

The public database JASPAR was used to identify the HOXC10-binding sites in β-catenin promoter. Chromatin was immunoprecipitated with an anti-HOXC10 antibody (ab153904; Abcam) or an IgG control antibody (ab2410; Abcam), and DNA was extracted and analyzed following the manufacturer's instructions. The primers used in this study were shown in the Supplementary Table 2.

Xenograft tumor model

To investigate the role of HOXC10 on the Carboplatin resistance of OC cells *in vivo*, we generated a xenograft tumor model using 4 weeks old, female, immunodeficient BALB/c nude mice. TOV21G-R cells stably transfected with sg-HOXC10 (for HOXC10 depletion) or with the sg-NC-expressing lentivirus (negative control) were used in the mouse experiments. Briefly, a total of 3×10^6 cells were suspended in 0.1 ml of serum-free culture medium and subcutaneously injected into the anterior armpits of mice. A week later, mice were subcutaneously injected with either PBS or carboplatin (30 mg/kg/w) with or without SKL2001 (Wnt signaling agonist, 6 mg/kg/w) for a period of 4 weeks. The animals were grouped as follows: (1) sgNC + PBS; (2) sgNC + carboplatin; (3) sgHOXC10 + PBS; (4) sgHOXC10 + carboplatin; (5) sgHOXC10 + carboplatin + SKL2001. The tumor size (*L*: length; *W*: width) was measured every 3 days. Tumor volume was calculated by using the following formula: $1/2 \times L \times W^2$. All the treatment of animals was in strict accordance with the guidelines of the Animal Center of Central South University, and all the animal experimental procedures were approved by the Experimental Animal Ethical Committee of Central South University.

Statistics analysis

The experimental results were analyzed by SPSS 20.0 and GraphPad Prism 8.0 software. Comparison between two groups was analyzed by students' test, while comparison among

more than two samples was analyzed by ANOVA. The least significant difference (LSD) test was used to estimate multiple comparisons. A p-value < 0.05 was considered statistically significant.

Results

HOXC10 was upregulated in the carboplatin-resistant cells and OC tissues

To study the function of HOXC10 in carboplatin resistance, we first generated carboplatin resistant TOV21G-R cells by growing the parental TOV21G-P cells in carboplatin-containing medium. After continuous culture in a concentration gradient every 48 h, carboplatin resistant TOV21G-R cells were selected. TOV21G-R cells exhibited an IC50 value of 134.4 μM, while the IC50 value of TOV21G-P cells was 35.28 μM (**Figure 1A**), indicating the carboplatin-resistant index of 3.81. Interestingly, the colony formation assay showed that the proliferation capacity of TOV21G-R cells was higher than that of the parental TOV21G-P cells with or without carboplatin treatment (P < 0.05, **Figure 1B**).

Furthermore, when treated with carboplatin at the concentration of 30 μM, TOV21G-P cells exhibited an increased apoptosis rate when compared to TOV21G-R cells (P < 0.001, **Figure 1C**). Consistently, cleaved Caspase-3 level, the major mediator of apoptosis, was higher in TOV21G-P cells than in TOV21G-R cells. In contrast, the expression of BCL-2, a major anti-apoptosis gene, was significantly higher in TOV21G-R cells than in TOV21G-P cells (**Figure 1D**).

Moreover, IHC staining assay was performed to analyze the expression level of HOXC10 in OC tissues. Both the percentage of positive cells and the staining intensity were increased in the carboplatin-resistant tissues (P < 0.001, **Figure 1E**). In line with this observation, compared with TOV21G-P cells, both the mRNA and protein levels of HOXC10 were increased in TOV21G-R cells (P < 0.001, **Figure 1F**).

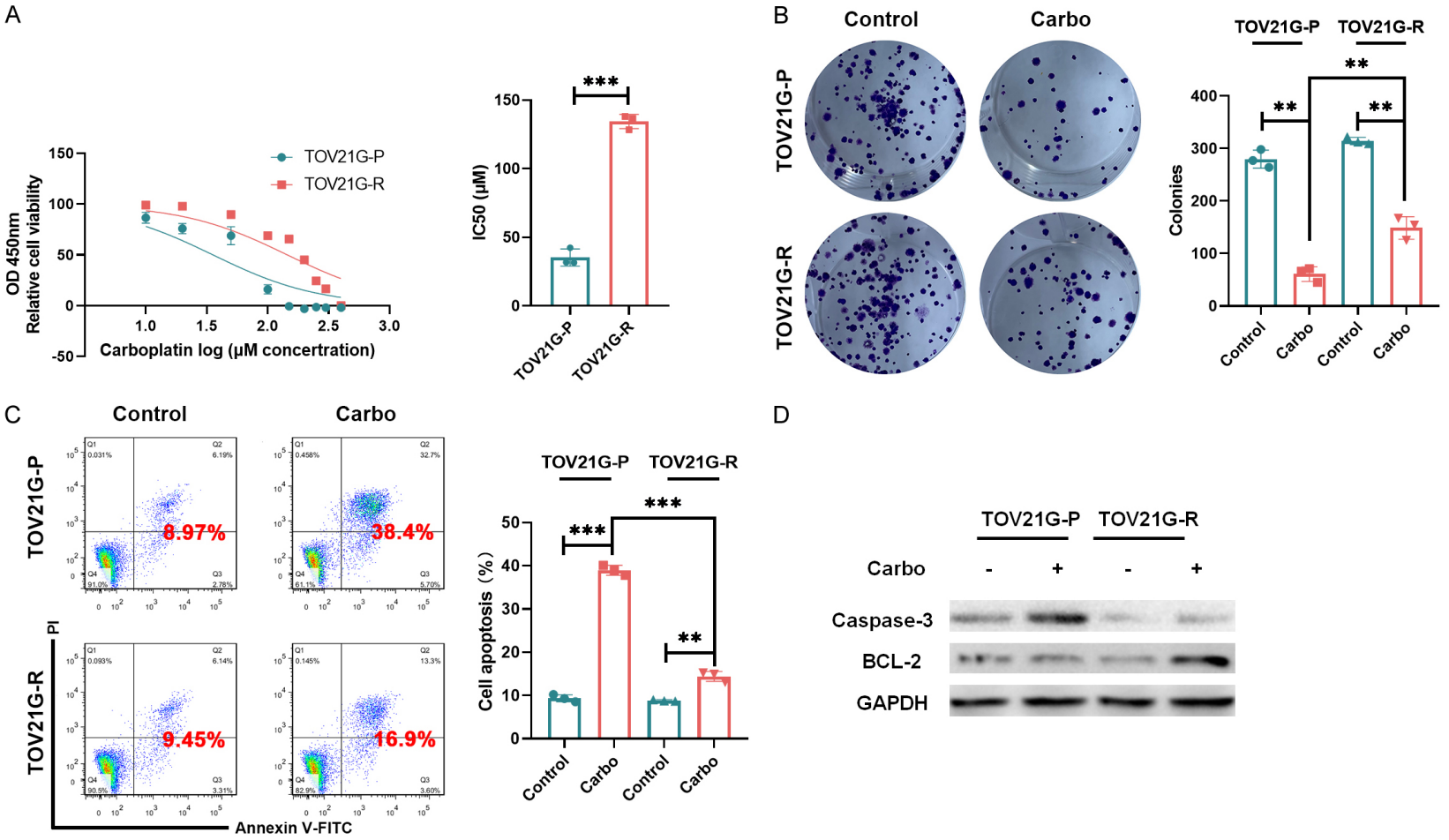
Taken together, we found that the expression of HOXC10 was upregulated both in the carboplatin-resistant cells and tissues when compared with the carboplatin-sensitive cells and tissues.

HOXC10 controlled the sensitivity of the OC cells to carboplatin

To directly determine the role of HOXC10 in carboplatin resistance, we altered HOXC10 expression by either overexpressing or knocking down HOXC10 in cells. The transfection efficiency of the overexpression (OE) plasmids and siRNAs was confirmed by qRT-PCR and western blot analyses (**Figure 2A**). The colony formation assay showed that the over-expression of HOXC10 in TOV21G-P cells reduced the sensitivity of parental cells to carboplatin, whereas knocking down HOXC10 in TOV21G-R increased the sensitivity of carboplatin-resistant cells to carboplatin, leading to decreased colony formation (P < 0.05, **Figure 2B**). The similar results were obtained from flow cytometry analysis (**Figure 2C**). Furthermore, the overexpression of HOXC10 in TOV21G-P decreased the expression of cleaved Caspase-3 and enhanced the expression of BCL-2, compared with control VECTOR expression (**Figure 2D**). Similarly, the downregulation of HOXC10 by siHOXC10 significantly reversed the resistance of TOV21G-R cells to carboplatin by increasing the expression of cleaved caspase-3 and decreasing the expression of BCL-2 (**Figure 2E**). Collectively, these data suggested that the sensitivity of OC cells to carboplatin was regulated by the expression level of HOXC10.

The expression of HOXC10 was positively correlated with ABCC3 and β-catenin

To understand how HOXC10 regulates carboplatin resistance, we performed gene expression correlation analysis by using information from public database. The GEO dataset GSE4122, containing OC and normal control tissues, was downloaded from https://www.ncbi.nlm.nih.gov/gds. The GSEA pathway enrichment analysis showed that the expression of HOXC10 was significantly positively correlated with the expression of ABC transporters and WNT pathway (**Figure 3A**). Importantly, the positive correlation between HOXC10 and ABCC3 expression was experimentally validated by qRT-PCR analysis (**Figure 3B**). Furthermore, we examined the transcriptional activity of WNT pathway in 293T cells either overexpressing HOXC10 or with HOXC10 knockdown by using TOP/FOP luciferase reporter assay. As predicted, the TOP/FOP activity had a positive correlation with


A
OD 450nm
Relative cell viability
TOV21G-P
TOV21G-R
Carboplatin log (μM concertration)
IC50 (μM)
***
B
Control
Carbo
TOV21G-P
TOV21G-R
Colonies
**
C
Control
Carbo
8.97%
38.4%
9.45%
16.9%
PI
Annexin V-FITC
Cell apoptosis (%)
D
Carbo
Caspase-3
BCL-2
GAPDH

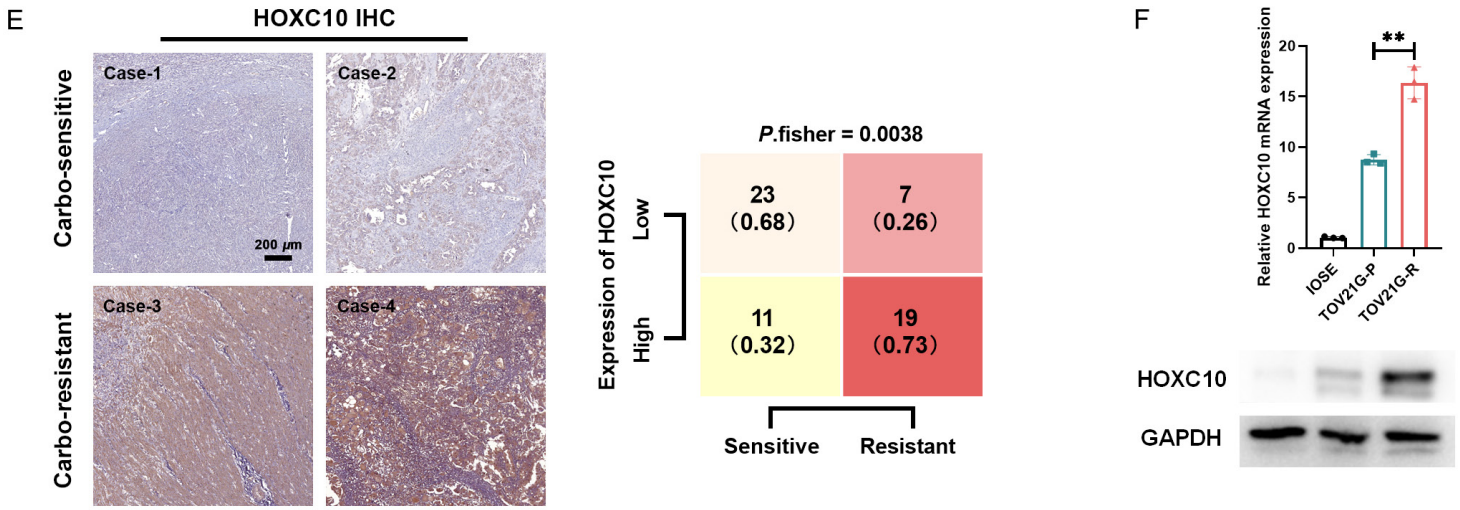
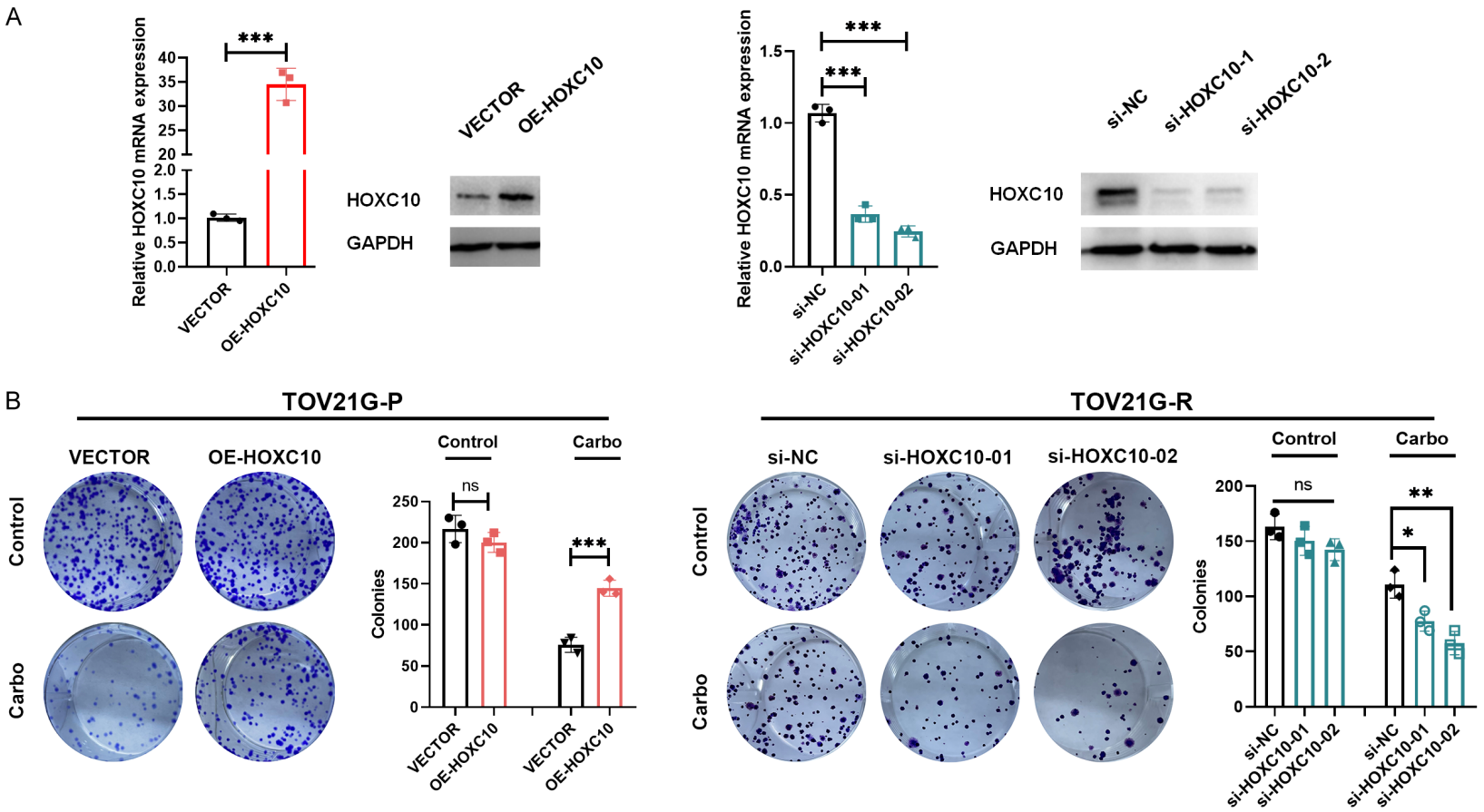


Figure 1. HOXC10 was upregulated in the carboplatin-resistant cells and OC tissues. A. CCK-8 assay to measure the carboplatin IC50 value of the TOV21G-P and TOV21G-R. B. Colonies formation assay to analyze the proliferation ability of the cell lines with or without the treatment of carboplatin. C. Flow cytometry assay to detect the apoptosis rate of the cell lines with or without the treatment of carboplatin. D. Western blot to analyze the expression of cleaved-caspase 3 and BCL-2 with or without the treatment of carboplatin. E. IHC staining to analyze the expression of HOXC10 in the OC tissues. F. qRT-PCR (top) and western blot (bottom) to analyze the expression of HOXC10 in OC cell lines. Carbo is short for carboplatin, ns $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.


A
Relative HOXC10 mRNA expression
***
VECTOR
OE-HOXC10
HOXC10
GAPDH
Relative HOXC10 mRNA expression
***
***
si-NC
si-HOXC10-01
si-HOXC10-02
si-HOXC10-1
si-HOXC10-2
B
TOV21G-P
VECTOR
OE-HOXC10
Control
Carbo
Colonies
ns
***
TOV21G-R
si-NC
si-HOXC10-01
si-HOXC10-02
ns
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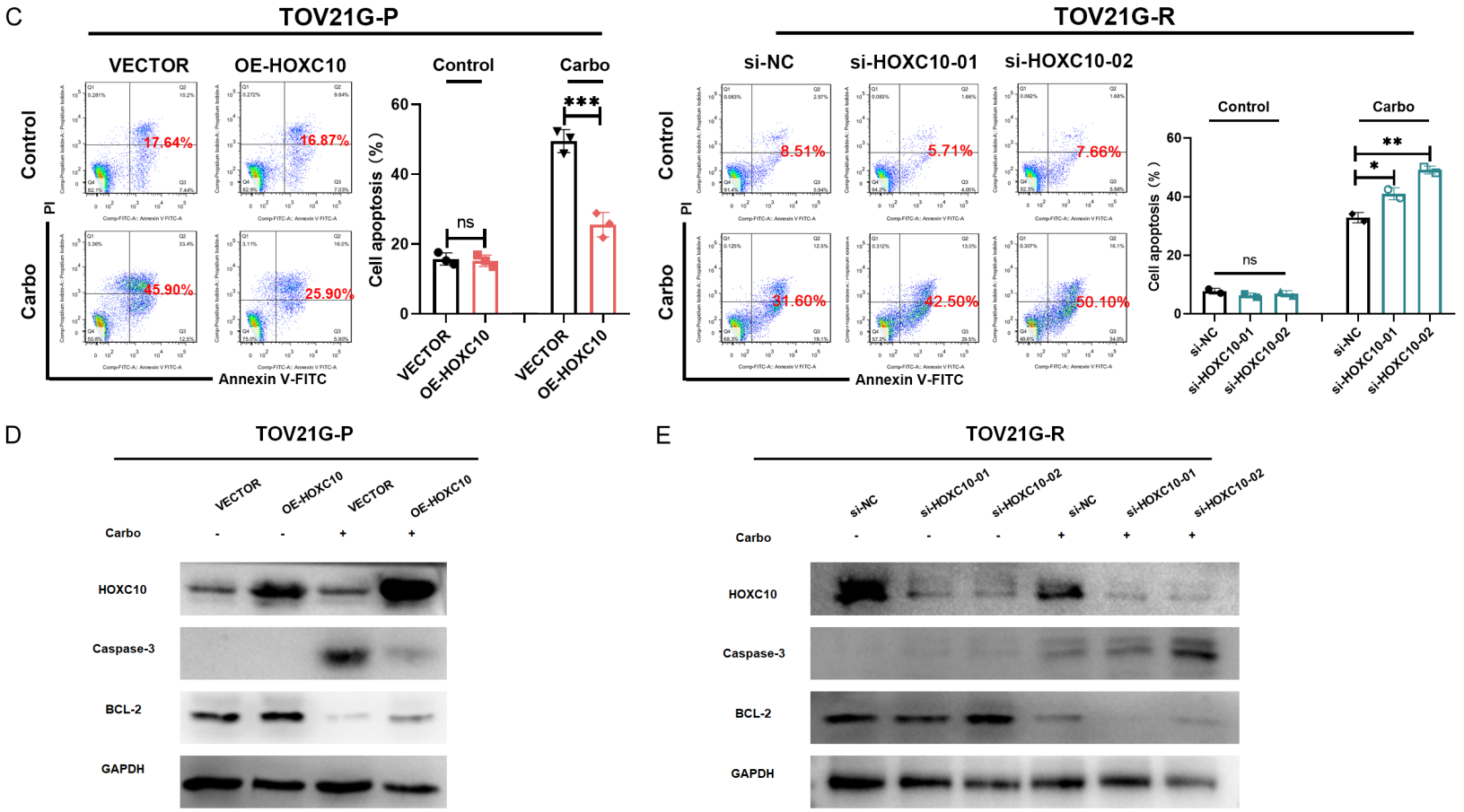
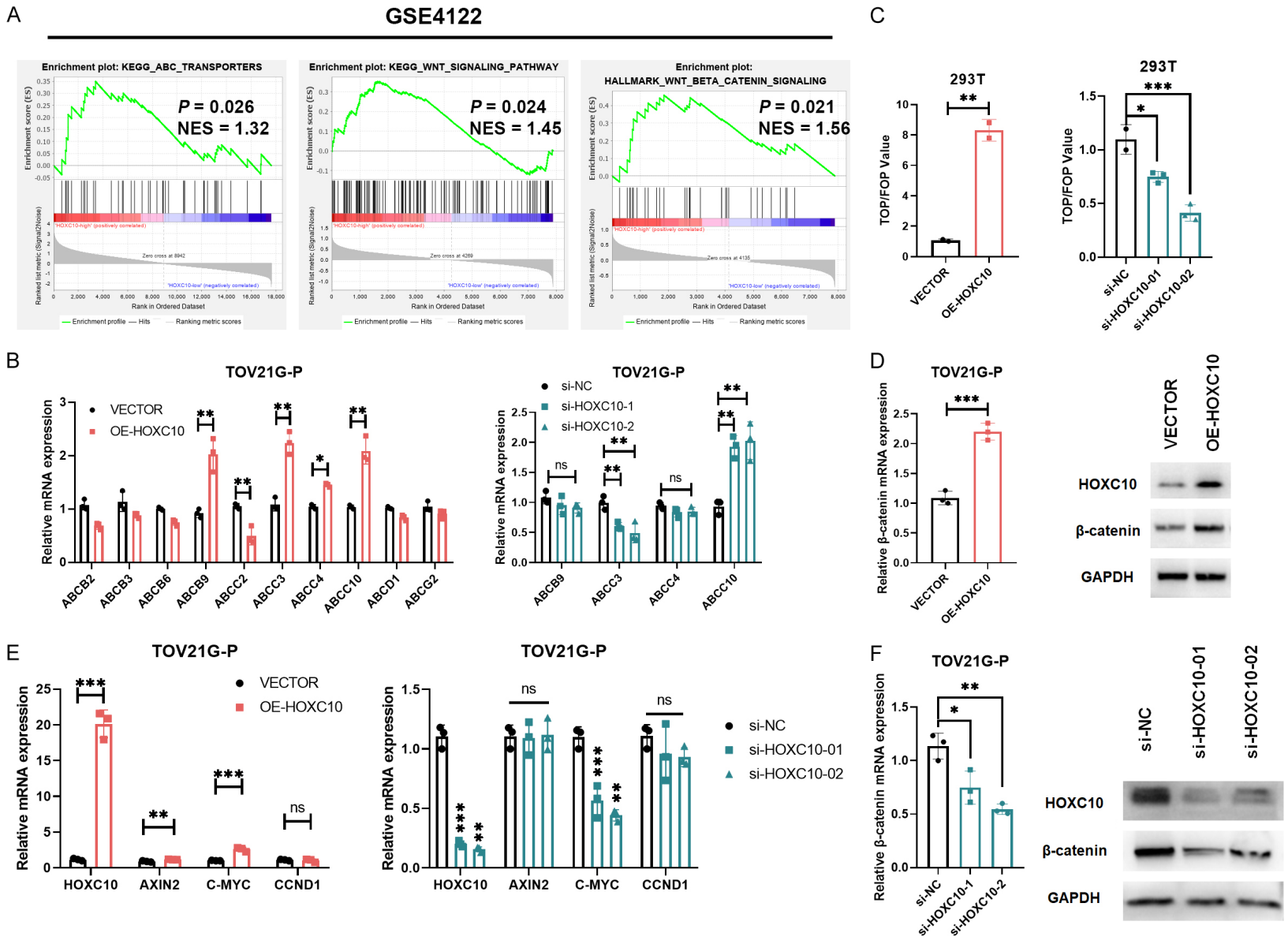


Figure 2. HOXC10 controlled the sensitivity of the OC cells to carboplatin. (A) qRT-PCR and Western blot to check the transfection efficiency of OE-HOXC10 plasmids and siRNAs. (B) Colonies formation assay to detect the proliferation ability of the cells treated with or without carboplatin. (C) Flow cytometry to identify the apoptosis rate of the cells treated with or without carboplatin. Western blot to analyze the cleaved Caspase-3 (D) and BCL-2 (E) expression level in the cells. Carbo is short for carboplatin, ns $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.


A
GSE4122
Enrichment plot: KEGG_ABC_TRANSPORTERS
P = 0.026
NES = 1.32
Enrichment plot: KEGG_WNT_SIGNALING_PATHWAY
P = 0.024
NES = 1.45
Enrichment plot: HALLMARK_WNT_BETA_CATENIN_SIGNALING
P = 0.021
NES = 1.56
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
C
293T
TOP/FOP Value
VECTOR
OE-HOXC10
si-NC
si-HOXC10-01
si-HOXC10-02
B
TOV21G-P
Relative mRNA expression
VECTOR
OE-HOXC10
ABCB2
ABCB3
ABCB6
ABCB9
ABCC2
ABCC3
ABCC4
ABCC10
ABCD1
ABCG2
si-NC
si-HOXC10-1
si-HOXC10-2
D
Relative β-catenin mRNA expression
HOXC10
β-catenin
GAPDH
E
HOXC10
AXIN2
C-MYC
CCND1
si-HOXC10-01
si-HOXC10-02
F

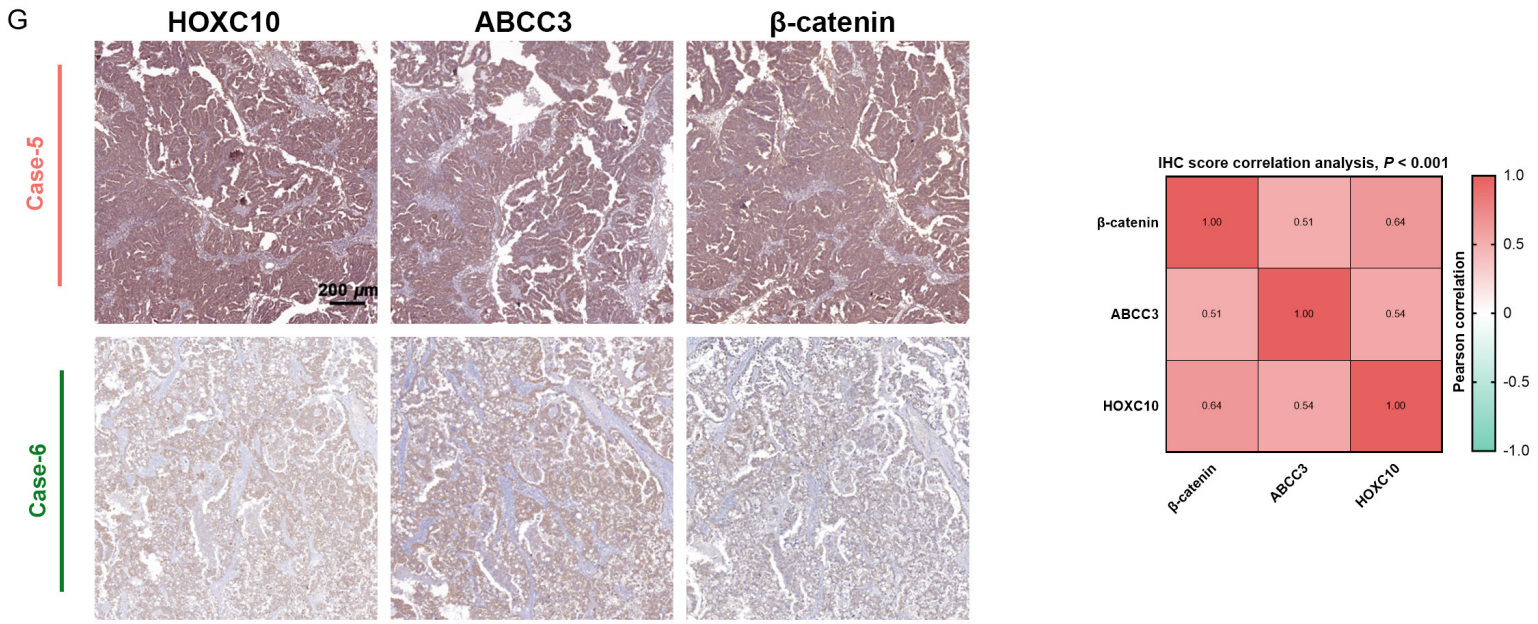


Figure 3. The expression of HOXC10 was positively correlated with ABCC3 and β-catenin. A. Pathway enrichment analysis using GEO dataset and GSEA software. B. qRT-PCR assay was performed to analyze the expression of ABC transporters after up- and down-regulating HOXC10. C. TOP/FOP activity of 293T cells after regulating HOXC10 expression. D, F. qRT-PCR and western blot assay were performed to analyze the β-catenin expression after regulating HOXC10. E. qRT-PCR assay was to analyze the expression of the target genes of WNT signaling pathway after the regulation of HOXC10. G. IHC staining to analyze the expression of HOXC10, ABCC3 and β-catenin in the OC tissues. ns $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the expression of HOXC10 (**Figure 3C**). Since β-catenin is one of the most important regulators of WNT pathway, we explored whether HOXC10 could regulate the expression of β-catenin. Our results showed that the mRNA and protein levels of β-catenin had a positive correlation with HOXC10 ($P < 0.05$, **Figure 3D**, **3F**). Moreover, the effect of manipulating HOXC10 expression on the expression of the downstream target genes of WNT pathway was also analyzed by qRT-PCR, and a positive correlation between them was observed (**Figure 3E**). Lastly, the IHC staining of the OC tissues also demonstrated a positive correlation between HOXC10 and ABCC3/β-catenin (**Figure 3G**), consistent with the results from dataset GSE49577 analysis (Supplementary Figure 1). Hence, we concluded that the expression of HOXC10 was positively related with the expression of ABCC3 and β-catenin.

HOXC10/ABCC3 in the diagnosis, prediction of carboplatin resistance, and prognosis of OC patients

At present, the expression and function of ABCC3 in OC development and chemoresistance remain unclear. Therefore, we used IHC staining to examine the expression of ABCC3 in carboplatin-resistant tissues and found that the protein expression of ABCC3 was significantly upregulated (**Figure 4A**). In addition, the survival analysis showed that high expression of ABCC3 predicted poor prognosis of OC patients (**Figure 4B**, **4C**). The association between the ABCC3 expression and the clinicopathological features of the patients was displayed in **Table 1**. Our results showed that the expression of ABCC3 had a strong negative correlation with the carboplatin sensitivity and the survival state. Since our previously published data indicated that the abnormal upregulation of HOXC10 in OC was associated with the poor prognosis of the patients [4], we further performed the ROC curve analysis to determine the predictive value of using HOXC10 and ABCC3 combination for the diagnosis of OC patients. As shown in the **Figure 4D**, HOXC10 combined with ABCC3 was more effective than them alone in predicting the prognosis of OC patients ($P < 0.001$). Furthermore, our data also showed that the combination of HOXC10 and ABCC3 performed better in predicting the carboplatin resistance in OC than the single indicator did (**Figure 4E**). Moreover, the survival analysis result of the combination of HOXC10 and ABCC3 was consistent with the ROC curve analysis (**Figure 4F**). The OC patients with high expression of both HOXC10 and ABCC3 tended to have the worst prognosis, whereas the prognosis was significantly better if the expression of both HOXC10 and ABCC3 was low. Together, our data suggested that both HOXC10 and ABCC3 played important roles in oncogenesis and carboplatin resistance, and their expression was associated with the poor prognosis of OC patients.

HOXC10 induced the carboplatin resistance by transcriptionally upregulating β-catenin

To further reveal the molecular mechanisms underlying the association of HOXC10 with β-catenin, we explored if HOXC10 as transcription factor could directly regulate the expression of β-catenin. The potential HOXC10 binding sites on the promoter of β-catenin were predicted using the online dataset JASPAR (https://jaspar.genereg.net/, **Figure 5A** top). The dual-luciferase reporter assay was employed to verify the transcriptional activation of β-catenin by HOXC10 ($P < 0.05$, **Figure 5A** bottom). Furthermore, by using ChIP assay, we confirmed the biding sites of HOXC10 on the promoter of β-catenin. The results showed that HOXC10 was mainly bound to the β-catenin promoter at the #2 (position -1385~-1376) and #3 (position -932~-923) regions (**Figure 5B**). The ChIP assays also demonstrated that an enhanced enrichment of HOXC10 was found in the different regions of the β-catenin promoter when HOXC10 was overexpressed, while the enrichment of HOXC10 was attenuated when HOXC10 was knocked down ($P < 0.05$, **Figure 5C**). Moreover, point mutations in the binding sites were generated to further prove the specific binding of HOXC10 to the β-catenin promoter (**Figure 5D**, mutations in the region #2 and region #3 were named as “site 2 MUT” and “site 3 MUT”, respectively; and the double mutation of both region #2 and #3 was named as “site 2&3 MUT”). In sum, HOXC10 could regulate the transcription of β-catenin by directly binding to the promoter of β-catenin.

Importantly, since WNT pathway has been proven to be involved in the drug resistance in cancer [10], we carried out a rescue experiment to verify whether HOXC10 promoted the carboplatin resistance of OC cells through regulating the

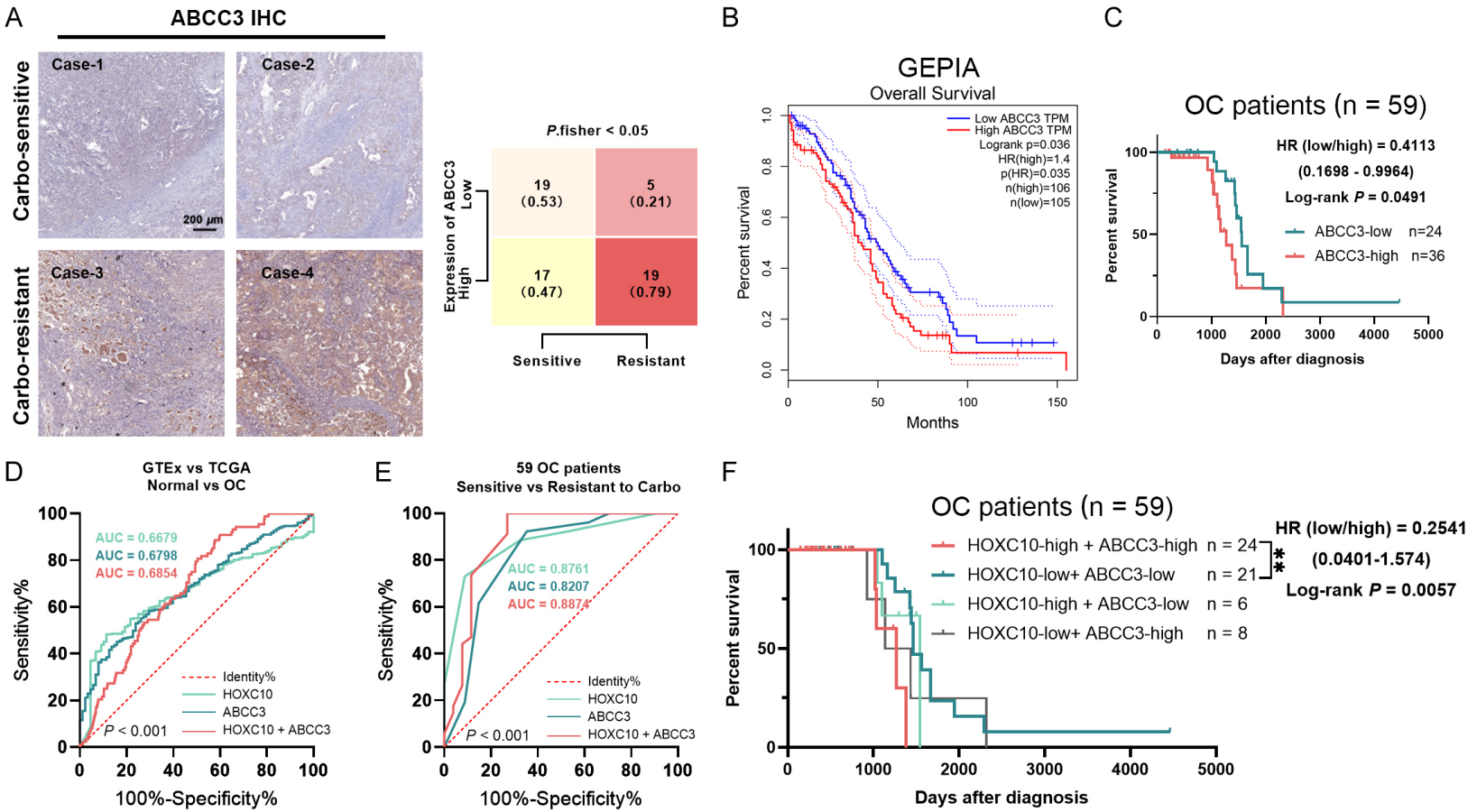


Figure 4. HOXC10/ABCC3 in the diagnosis, prediction of carboplatin resistance, and prognosis of OC patients. (A) IHC staing shows the protein expression of ABCC3 in carboplatin-sensitive and -resistant tissues. The surivial analysis was performed to identify the expression of ABCC3 in the prognosis of OC patients by using online dataset (B) and the collected data in this study (C). The ROC curve analysis was performed to identify the HOXC10 and (or) ABCC3 in the diagnosis of OC by using online dataset (D) and the collected data in this study (E). (F) The survival analysis was performed to identify the HOXC10 combined with ABCC3 in the prognosis of OC. Carbo is short for carboplatin, $^{**}P < 0.01$.

Table 1. The relationship between ABCC3 protein expression and the clinic pathological features in OC

Variable	ABCC3 expression		*P* value
	Low (n = 24)	High (n = 36)	
Age at diagnosis, *N* (%)			0.55
< 50	5 (20.83)	11 (30.56)	
≥ 50	19 (79.17)	25 (69.44)	
Histologic type, *N* (%)			0.76
Serous Cancer	19 (79.17)	26 (72.22)	
Nonserous	5 (20.83)	10 (27.78)	
Distant metastasis, *N* (%)			0.29
Absent	2 (8.33)	7 (19.44)	
Present	22 (91.67)	29 (80.56)	
Sensitivity to carboplatin, *N* (%)			0.0168
Sensitive	19 (79.17)	17 (47.22)	
Resistant	5 (20.83)	19 (52.78)	
Survival state, *N* (%)			0.0489
Dead	12 (50.00)	28 (77.78)	
Alive	12 (50.00)	8 (22.22)	

WNT pathway by overexpressing or knocking down β-catenin. The overexpression of β-catenin by OE-β-catenin transfection and the knockdown of β-catenin by siRNA transfection were confirmed by qRT-PCR and western blot (**Figure 5E**). The results showed that the increased sensitivity of OC to carboplatin caused by HOXC10 knockdown could be partially attenuated by β-catenin overexpression (**Figure 5F**). Taken together, we determined that HOXC10 could promote carboplatin resistance of OC cells by directly upregulating β-catenin expression, and the downregulation of HOXC10 or β-catenin could sensitize the carboplatin-resistant TOV21G-R cell to carboplatin treatment.

HOXC10 regulated the expression of ABCC3 through β-catenin

It has been known that the β-catenin/WNT pathway is functionally associated with the activity of ATP binding cassette (ABC) transporters [11, 12]. In this study, we also found that HOXC10 promoted the expression of ABCC3, and that β-catenin was involved in this regulation, as determined by using the inhibitor or agonist of β-catenin signaling pathway (**Figure 6**). When the cells were treated with different concentrations of the inhibitor or agonist, the expression of β-catenin and ABCC3 changed simultaneously; however, the expression of HOXC10 was not affected, indicating that β-catenin signaling mediated the regulation of ABCC3 by HOXC10 (**Figure 6C**).

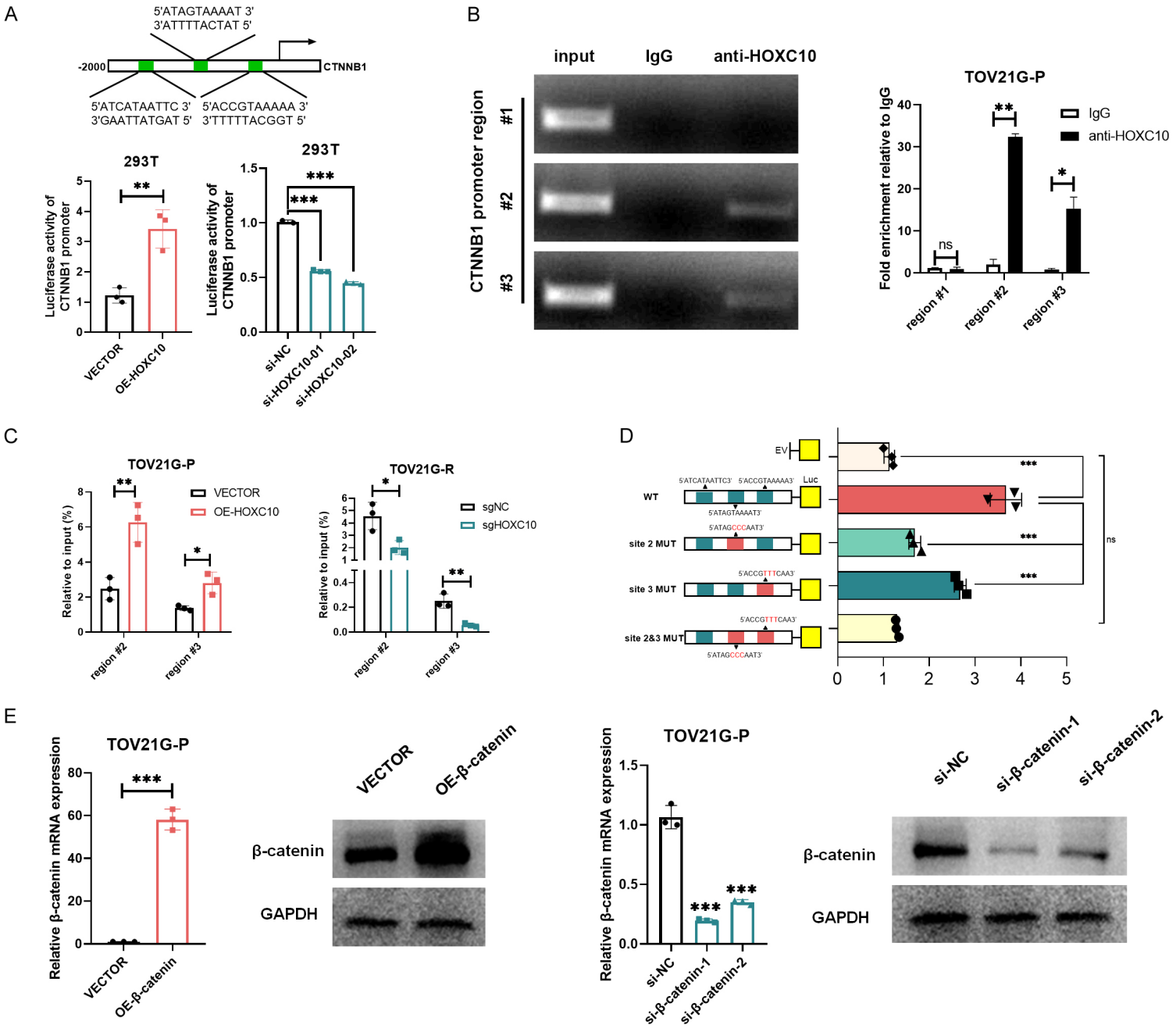
HOXC10 knockout sensitized TOV21G-R cells to carboplatin in vivo

We also used specific sgRNAs to knockout the expression of HOXC10. The stable knockout of HOXC10 in TOV21G-R cells led to a similar phenotype as the TOV21G-R cells with HOXC10 knockdown by siRNA. When HOXC10 was stably knocked out by sgRNAs in TOV21G-R cells, as indicated by the significantly lower expression of HOXC10 (**Figure 7A**), the decreased colony formation (**Figure 7B**) and survival (**Figure 7C**) of TOV21G-R cells caused by carboplatin treatment were reversed. In vivo xenograft tumor model further verified these results. **Figure 7D**, **7E** showed that carboplatin decreased the tumor size and weight, and the depletion of HOXC10 could enhance the effect of carboplatin. Furthermore, β-catenin agonist SKL2001 could partially reverse the increased sensitivity to carboplatin caused by HOXC10 knockdown. The IHC staining of tumor samples in **Figure 7F** were consistent with the previous results.

Based on the results above, we proposed a working model in which HOXC10 was upregulated in the carboplatin-resistant OC tissues and cells and could increase the expression of ABCC3 by directly transcriptionally upregulating β-catenin (**Figure 7G**).

Discussion

Tumor size reduction and paclitaxel platinum-based combined chemotherapy are the standard treatment regimen for patients with advanced OC [13]. Although Platinum and paclitaxel have been widely used in treating OC in clinical application [14], most patients with advanced OC still die of chemo-resistance, tumor recurrence, and metastasis [15]. After the initial chemotherapy, the sensitivity of OC to single drug or combined drugs is reduced by 20-30% [16], which significantly reduces the


A
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3'ATTTTACTAT 5'
-2000
CTNNB1
5'ATCATAATTC 3'
3'GAATTATGAT 5'
5'ACCGTAAAAA 3'
3'TTTTTACGGT 5'
293T
Luciferase activity of CTNNB1 promoter
VECTOR
OE-HOXC10
293T
Luciferase activity of CTNNB1 promoter
si-NC
si-HOXC10-01
si-HOXC10-02
B
input
IgG
anti-HOXC10
CTNNB1 promoter region
#1
#2
#3
TOV21G-P
Fold enrichment relative to IgG
IgG
anti-HOXC10
ns
region #1
region #2
region #3
C
TOV21G-P
Relative to input (%)
VECTOR
OE-HOXC10
region #2
region #3
TOV21G-R
Relative to input (%)
sgNC
sgHOXC10
region #2
region #3
D
EV
WT
site 2 MUT
site 3 MUT
site 2&3 MUT
Luc
ns
E
TOV21G-P
Relative β-catenin mRNA expression
VECTOR
OE-β-catenin
β-catenin
GAPDH
TOV21G-P
Relative β-catenin mRNA expression
si-NC
si-β-catenin-1
si-β-catenin-2
β-catenin
GAPDH

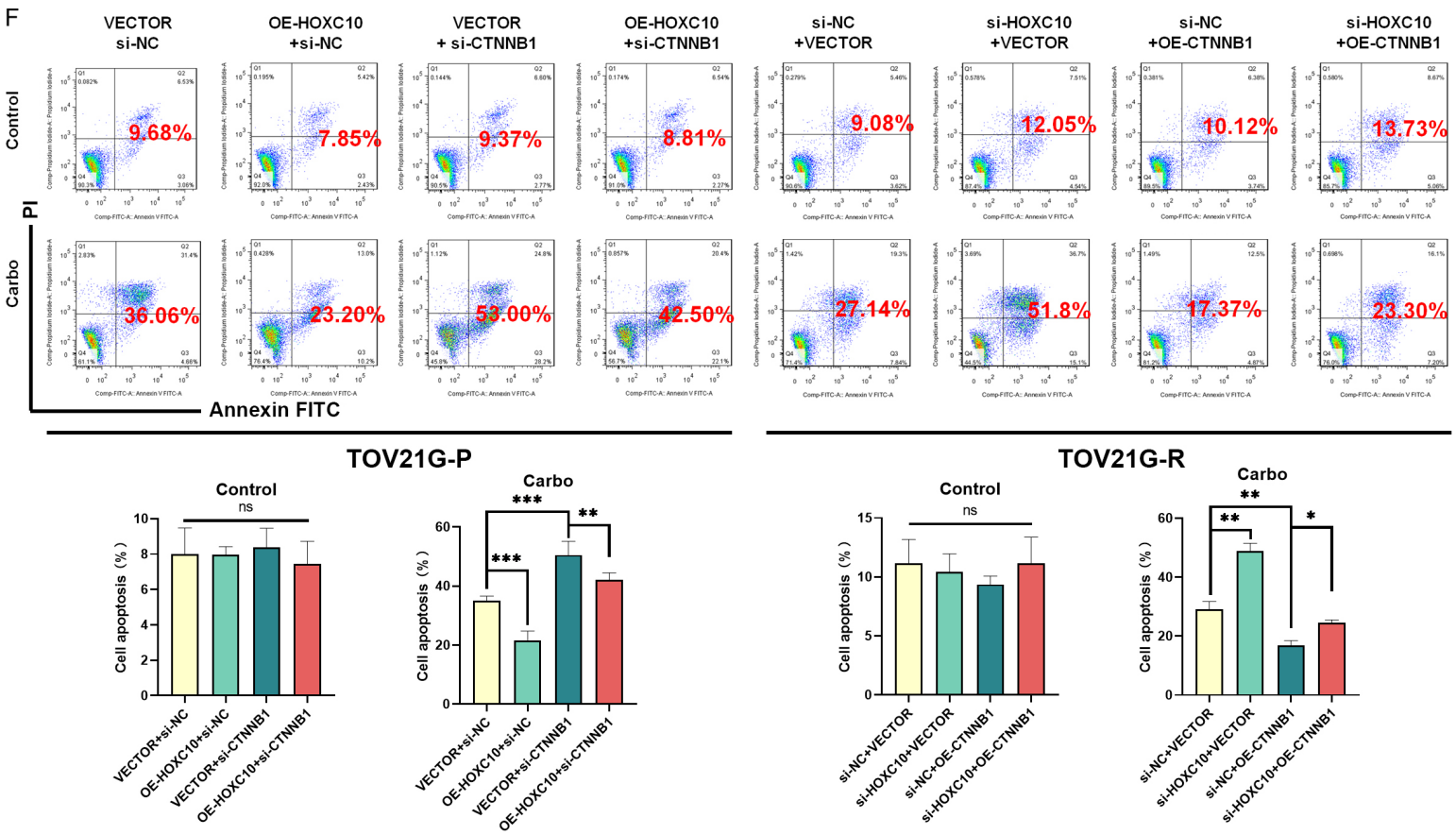


Figure 5. HOXC10 induced the carboplatin resistance by transcriptionally upregulating β-catenin. A. The JASPAR online dataset was used to predict the potential biding regions of HOXC10 with the β-catenin promoter (top), and Dual-luciferase assay was to test the luciferase activity of β-catenin promoter (bottom). B, C. ChIP assay was to verify the specific biding regions of HOXC10 with β-catenin promoter. D. Dual-luciferase assay was to test the luciferase activity of both the WT and mutations of β-catenin promoter. E. qRT-PCR and Western blot assay were to analyze the transfection efficiency of OE-β-catenin plasmids and siRNAs. F. A rescue flow cytometry was to analyze the apoptosis rate of the cells transfected with different plasmids. Carbo is short for carboplatin, ns $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

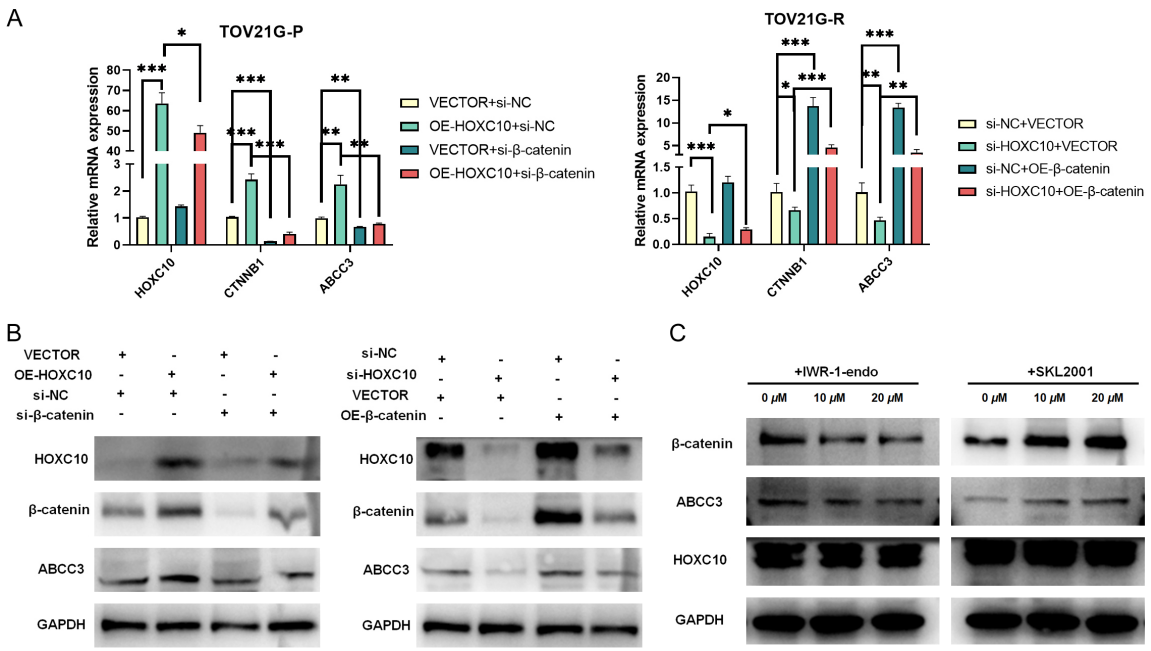


Figure 6. HOXC10 regulated the expression of ABCC3 through β-catenin. qRT-PCR (A) and Western blot (B) were to verify the regulation effect of HOXC10 on β-catenin and ABCC3. (C) Western blot was to detect the expression of β-catenin, ABCC3 and HOXC10 after the treatment with the inhibitor (IWR-1-endo) or agonist (SKL2001) of WNT pathway. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

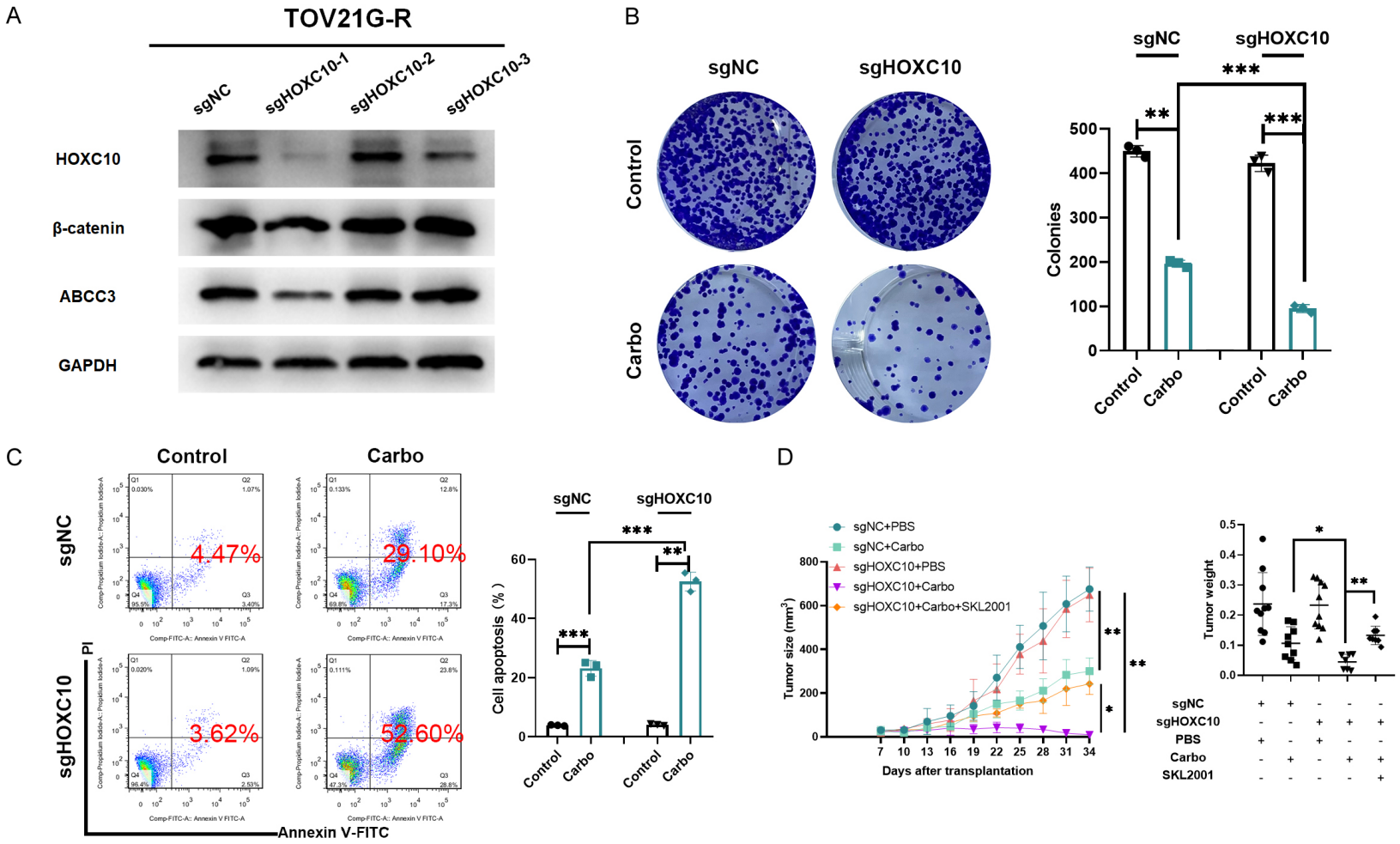
efficacy of chemotherapy. Therefore, overcoming drug resistance is an urgent clinical need.

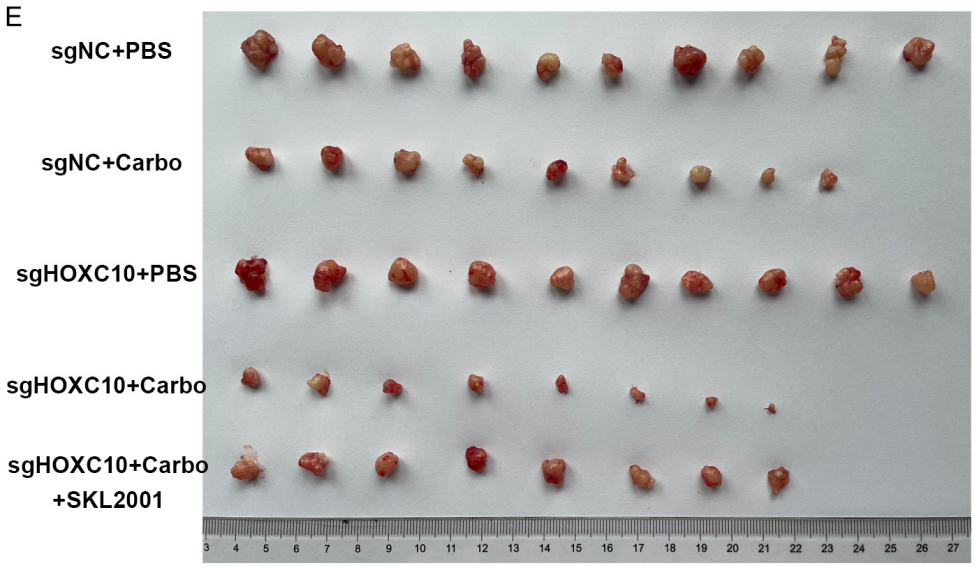
Our previous study has revealed that the expression of HOXC10 is significantly upregulated in OC tissues and has a positive correlation with the tumor metastasis [4]. During the process of analyzing the previous IHC data, we found that the IHC score of HOXC10 seemed to increase in the platinum-resistant ovarian tissues. This prompted us to collect another 60 OC tissues including carboplatin-sensitive and -resistant OC tissues, together with the carboplatin-resistant subline of OC TOV21G cell line named TOV21G-R and the parental line named TOV21G-P. We confirmed that HOXC10 was upregulated in both carboplatin-resistant tissues and cell line, and the sensitivity of the OC cell lines to carboplatin was related to the expression level of HOXC10.

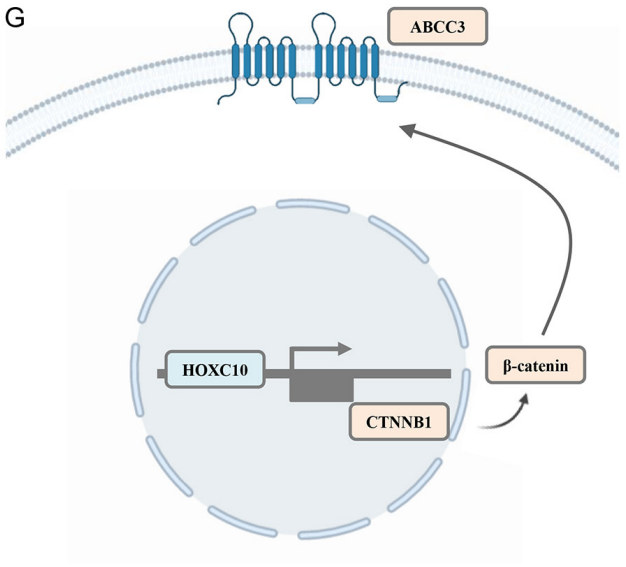
The effectiveness of chemotherapeutic drugs is affected by the ABC transmembrane transporter superfamily which is associated with multidrug resistance (MDR) [17]. According to their sequence homology, 48 ABC transporters are divided into seven subfamilies (A to G) in humans [18, 19]. This study was the first to demonstrate that ABCC3 was upregulated in the carboplatin-resistant OC tissues and that the high expression of ABCC3 predicted the poor outcome of OC patients. Furthermore, our data also indicated that the combination of HOXC10 and ABCC3 played important role in the prediction of carboplatin resistance and the prognosis of OC.

Wnt/β-catenin pathway is a canonical Wnt-signaling pathway that regulates a variety of cellular processes involved in cell growth and differentiation. The abnormal activation of Wnt/β-catenin signaling pathway occurs in many malignant tumors including OC and has an important role in drug resistance [20, 21]. Inhibition of Wnt/β-catenin signaling pathway can effectively inhibit cancer cell growth and chemotherapeutic drug resistance [22], which is considered a potential therapeutic target for malignant tumors. In this study, we found that HOXC10 could promote carboplatin resistance of OC cells by directly upregulating β-catenin, and the downregulation of HOXC10 or β-catenin could partly restore the drug sensitivity of the cells.

Several studies have demonstrated that HOXC10 plays an important role in chemotherapy resistance through different mechanisms.


A
TOV21G-R
sgNC
sgHOXC10-1
sgHOXC10-2
sgHOXC10-3
HOXC10
β-catenin
ABCC3
GAPDH
B
sgNC
sgHOXC10
Control
Carbo
Colonies
C
Control
Carbo
sgNC
sgHOXC10
PI
Annexin V-FITC
4.47%
29.10%
3.62%
52.60%
Cell apoptosis (%)
D
sgNC+PBS
sgNC+Carbo
sgHOXC10+PBS
sgHOXC10+Carbo
sgHOXC10+Carbo+SKL2001
Tumor size (mm3)
Days after transplantation
Tumor weight
PBS
Carbo
SKL2001





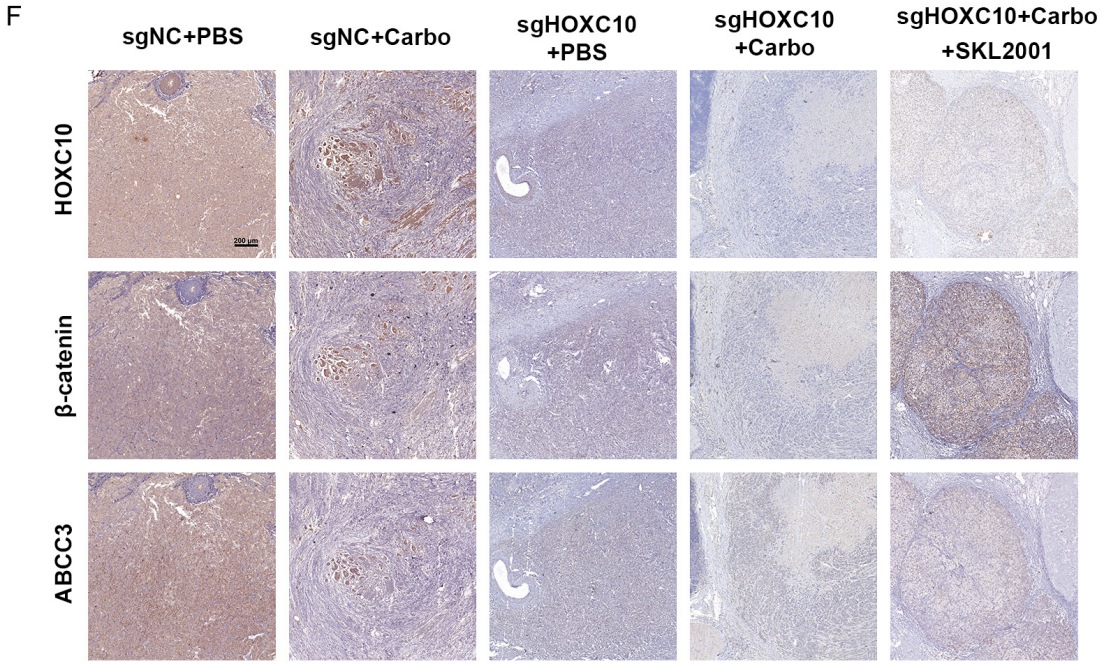


Figure 7. HOXC10 knockout sensitized TOV21G-R cells to carboplatin *in vivo*. A. Western blot assay was to analyze the transfection efficiency of sgRNAs. B. Colonies formation assay was to test the proliferation ability of the cells with or without the treatment of carboplatin. C. Flow cytometry was to analyze the apoptosis rate of cells with or without the treatment of carboplatin. D. The tumor size was measured by using the formula (mm^3): $V = 1/2 \times L \times W^2$. E. Tumor weight of different groups. F. IHC staining of the tissues from different groups. G. Working model of HOXC10 functions. ns $P > 0.05$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

For example, HOXC10 can accelerate the DNA repair mechanism by regulating both the homologous recombination (HR) and checkpoint recovery in breast cancer [23], while HOXC10 can regulate the nonhomologous end-joining (NHEJ) pathway in esophageal squamous cell carcinoma (ESCC) [24]. In this study, we revealed a novel mechanism of HOXC10 in regulating drug resistance. We found that HOXC10 could regulate the expression of ABCC3 through transcriptionally upregulating β-catenin. Furthermore, ABCC3 could be a potential prognostic predictor of OC patients. Therefore, HOXC10/β-catenin/ABCC3 axis might serve as a potential therapy target to overcome the carboplatin resistance of OC.

Acknowledgements

Thanks are due to Prof. Jing Tan for his kindness in giving us the carboplatin-resistant cell line TOV21G-R and its parental cell line TOV21G-P.

Disclosure of conflict of interest

None.

Abbreviations

HOXC10, homeobox C10; OC, ovarian cancer; IHC, Immunohistochemistry; ABCC3, ATP binding cassette subfamily C member 3; Carbo, carboplatin; FIGO, International Federation of Obstetricians and Gynecologists.

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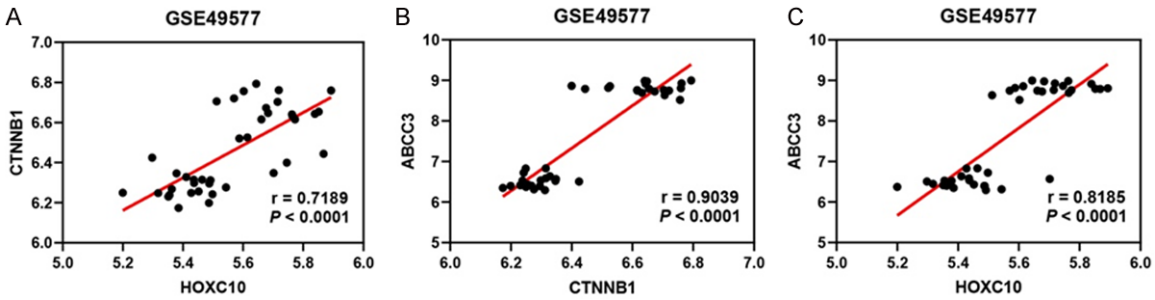
Supplementary Table 1. The IHC and western blot antibodies

Antibody	Brand	Dilution ratio
HOXC10	Abcam, ab153904	1:100 (IHC) 1:1000 (western blot)
β-catenin	Proteintech, Cat# 51067-2-AP	1:1000 (IHC) 1:5000 (western blot)
ABCC3	Cell Signaling Technology [CST], Cat# 39909	1:100 (IHC) 1:1000 (western blot)
GAPDH	Utibody, Cat# UM4002	1:5000 (western blot)
γ-H2AX	CST, Cat# 9718s	1:1000 (western blot)
caspase-3	CST, Cat# 9662s	1:1000 (western blot)
HRP-conjugated Affinipure Goat Anti-Rabbit/Mouse IgG (H+L)	Proteintech Cat# SA00001-1, Cat# SA00001-2	1:5000 (western blot)

Supplementary Table 2. The sequences

Gene	Type	sequences
HOXC10	qRT-PCR	F: AAGCGAAAGAGGAGATAAAGGC
		R: GTCTTGCTAATCTCCAGGCGG
	Overexpression plasmids	F: CTAGCTAGCatgacatgccctcgcaatgt
		R: CCGGAATTCccgcgctctcaggtgaaatt
	siRNA#1	CGCTGGAATTGGAGAAAGA
	siRNA#1	CATTAACCTTACAGACAGA
	sgRNA#1	TGGTCTTGCTAATCTCCAGG
	sgRNA#2	ATAAAGGCAGAAAACACCAC
	sgRNA#3	TTTGACGCGAGAGCGCCGCC
GAPDH	qRT-PCR	F: GTCTCCTCTGACTTCAACAGCG
		R: ACCACCCTGTTGCTGTAGCCAA
CTNNB1	qRT-PCR	F: CACAAGCAGAGTGCTGAAGGTG
		R: GATTCCTGAGAGTCCAAAGACAG
CTNNB1 promoter (ChIP)	-1954-1945	ATCATAATTC
	-1385-1376	ATAGTAAAAT
	-932-923	ACCGTAAAAA
ABCC3	qRT-PCR	F: GAGGAGAAAGCAGCCATTGGCA
		R: TCCAATGGCAGCCGCACTTTGA
c-MYC	qRT-PCR	F: CCTGGTGCTCCATGAGGAGAC
		R: CAGACTCTGACCTTTTGCCAGG



Supplementary Figure 1. A-C. Correlation analysis between HOXC10, β-catenin, and ABCC3. The dataset GSE49577 [37] was downloaded from the GEO database.