Long Non-Coding RNA (lncRNA) BMP/OP-Responsive Gene (BORG) Promotes Development of Chemoresistance of Colorectal Cancer Cells to Carboplatin

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background:	The function of long non-coding RNA (lncRNA) BMP/OP-responsive gene (BORG) has only been studied in breast cancer. We analyzed the role of BORG in colorectal cancer (CRC).
Material/Methods:	BORG in CRC tissues and non-cancer tissues from 66 CRC patients was detected by performing quantitative reverse-transcription PCR (RT-qPCR). BORG in plasma of CRC patients was detected at 3 times-points: before treatment and at 3 and 6 months after treatment. p53 expression in tumor tissues was also detected by RT-qPCR. QPCR was performed to confirm the overexpression of p53 in cells of both CRC cell lines.
Results:	We found that BORG expression was upregulated in CRC tissues and was inversely correlated with p53. With application of carboplatin-based treatment, the expression level of BORG was further upregulated. In CRC cells, carboplatin upregulated the expression of BORG and BORG negatively regulated p53. Under carboplatin treatment, BORG positively regulated the viability of CRC cells. In addition, p53 overexpression attenuated the effects of BORG overexpression.
Conclusions:	BORG promotes the development of chemoresistance of CRC cells to carboplatin.
MeSH Keywords:	**Carboplatin • Colorectal Neoplasms • RNA, Long Noncoding**
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Background

The incidence rate of colorectal cancer (CRC) ranks third and its mortality rate of CRC ranks fourth among all malignancies [1,2]. CRC causes about 700 000 deaths annually and there are 1.3 million new cases every year [3]. With the development of multidisciplinary treatment, survival of CRC patients has been significantly improved during the past several decades. However, early diagnosis of CRC is difficult and most CRC patients cannot be cured by currently available treatment approaches [4]. Most CRC cases diagnosed at advanced stages are treated with systemic chemotherapies, such as carboplatin-based treatment [5,6]. However, chemoresistance will inevitably develop, leading to poor treatment outcomes [7,8].

P53 is a well-studied tumor-suppressor in cancer biology [9]. In addition to the roles of p53 in regulation of cancer cell behaviors, p53 signaling also participates in the regulation of chemosensitivity of cancer cells, such as the sensitivity of CRC cells to chemotherapies [10]. In some cases, p53 participates in the response of cancer cells to chemicals by interacting with long (>200 nt) non-coding RNAs (lncRNAs) [11], which regulate gene expression but do not participate in protein-synthesis [12]. In a recent study, Gooding et al. reported that an lncRNA named BMP/OP-responsive gene (BORG) is an oncogenic lncRNA in breast cancer [13], enhancing the survival of breast cancer cells under chemotherapies [13]. We performed whole-genome transcriptome analysis and observed the inverse correlation between p53 and BORG across CRC specimens. The present study was therefore performed to investigate the possible interaction between BORG and p53 in CRC.

Material and Methods

Study patients and specimens

We selected 66 CRC patients (28 females and 38 males; age range 33–66 years, median age 46.9 years, mean age 45.3±5.6 years) from among the 155 CRC patients who were admitted at Gansu Provincial Hospital from December 2016 to December 2018. Inclusion criteria were: 1) newly diagnosed CRC patients, 2) confirmed by histopathological biopsy, and 3) received carboplatin-based treatment at Gansu Provincial Hospital. Exclusion criteria were: 1) diagnosed at another hospital and transferred to our hospital, 2) recurrent CRC, and 3) diagnosed with any other clinical disorders. All patients were diagnosed by histopathological biopsy, during which non-cancer tissues (within the area 3 cm around tumors) and CRC tissues were collected from each patient. Blood was extracted from each patient before treatment and at 3 and 6 months after the initiation of carboplatin-based treatment. Blood was centrifuged in EDTA tubes for 10 min at 1200 g to prepare plasma. Weights of

tissues ranged from 0.022 to 0.037 g. Based on clinical findings, there were 13, 14, 20, and 19 cases at AJCC stage I-IV, respectively. All patients signed informed consent. The Ethics Committee of our institute approved this study.

CRC cell lines and transient transfections

HS 722.T and RKO 2 human CRC cell lines (ATCC, USA) were used. HS 722.T and RKO cells were cultivated in EMEM (10% FBS) medium under conditions of 5% CO_2 and 37°C.

BORG and p53 expression vector (pcDNA3), empty pcDNA3 vector, negative control siRNA, and BORG siRNA were all from GenePharma (Shanghai, China). HS 722.T and RKO cells were collected at 75% to 85% confluence, followed by transfection of 10 nM BORG and p53 vector, or empty pcDNA3 vector (negative control, NC), 45 nM negative control siRNA (NC), or 45 nM BORG siRNA into 10^5 cells using Lipofectamine 2000 reagent (Thermo Fisher Scientific). Subsequent experiments were performed at 24 h after transfection. Cells without transfections served as a control (C) group.

RT-qPCR

RNAzol reagent (Sigma-Aldrich, USA) was used for RNA extraction from HS 722.T and RKO cells (10^5 cells per 1 ml RNAzol), tissues (0.02 g per 1 ml RNAzol), and plasma (0.2 ml per 1 ml RNAzol). In cases of carboplatin treatment, HS 722.T and RKO cells were treated with carboplatin at dosages of 0, 100, and 300 μM for 24 h before use. Following DNase I digestion, we used the Quantitect Reverse Transcription Kit (Qiagen, Shanghai, China) and QuantiFast SYBR Green PCR Kit Qiagen, Shanghai, China) to synthesize cDNA through reverse transcriptions and preparing PCR reaction mixtures, respectively. Expression of p53 mRNA and BORG was detected with GAPDH and 18S rRNA as endogenous control, respectively.

MTT assay

HS 722.T and RKO cells were harvested at 24 h after transfections, and $4×10^4$ cells were mixed with 1 ml of the cell culture medium. Cells were transferred to a 96-well plate (0.1 ml per well), followed by the addition of carboplatin into each well at dosages of 300 μM. Three replicates were set for each dosage. Under the conditions of 37°C and 5% CO_2, cells were cultivated for 24 h. After that, 10 uL MTT was added into each well and OD values at 570 nm were measured 4 h later.

Western blot analysis

HS 722.T and RKO cell pellets were resuspended in RIPA solution (Sangon, Shanghai, China) at a concentration of 1 ml RIPA solution per 10^5 cells. Protein samples were denatured
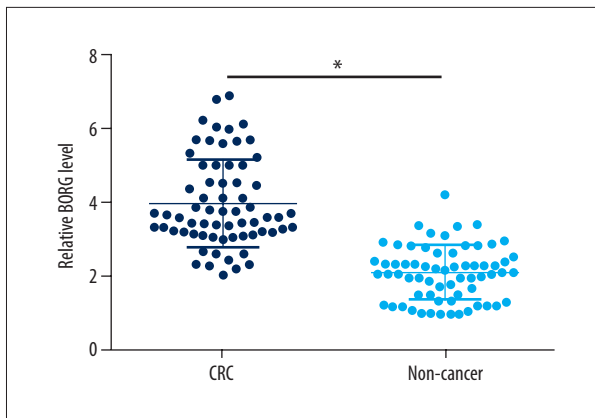
Figure 1. BORG was upregulated in CRC. BORG expression was detected by performing RT-qPCR. Data analysis by paired *t* test showed that expression levels of BORG were significantly higher in CRC tissues than in non-cancer tissues (* p<0.05).

Figure 2. Carboplatin-based treatment upregulated BORG in plasma of CRC patients. BORG expression in plasma of CRC patients was detected before treatment and at 3 and 6 months after transfections. BORG expression data were analyzed by repeated-measures ANOVA, showing that expression levels of BORG were significantly increased with the prolonged carboplatin-based treatment (* p<0.05).

and subjected to 12% SDS-PAGE gel electrophoresis. Proteins were transferred to PVDF membranes. After blocking (5% non-fat milk for 2 h at 25ºC), rabbit polyclonal primary antibodies of GAPDH (1: 1300, ab181602, Abcam) and p53 (1: 1300, ab131442, Abcam) were used to incubate membranes at 4°C for at least 12 h. After that, goat anti-rabbit lgG-HRP secondary antibody (1: 1000, MBS435036, MyBioSource) was used to further incubate with membranes for 2 h at 25°C. Signals were developed by incubating with ECL solution (Sigma-Aldrich, USA) for 15 min at room temperature, and data were processed using Image J 1.48 software.
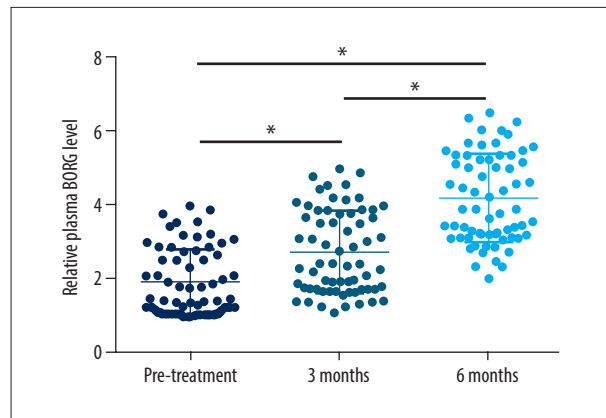
Statistical analysis

Mean values data from 3 biological replicates were calculated. Differences between 2 types of tissues (CRC *vs.* non-cancer) were explored using the paired *t* test. Differences among different time-points were explored using repeated-measures ANOVA. Differences among different cell groups were analyzed by ANOVA (one-way) and Tukey test. Linear regression was used for correlation analysis. p<0.05 was statistically significant.
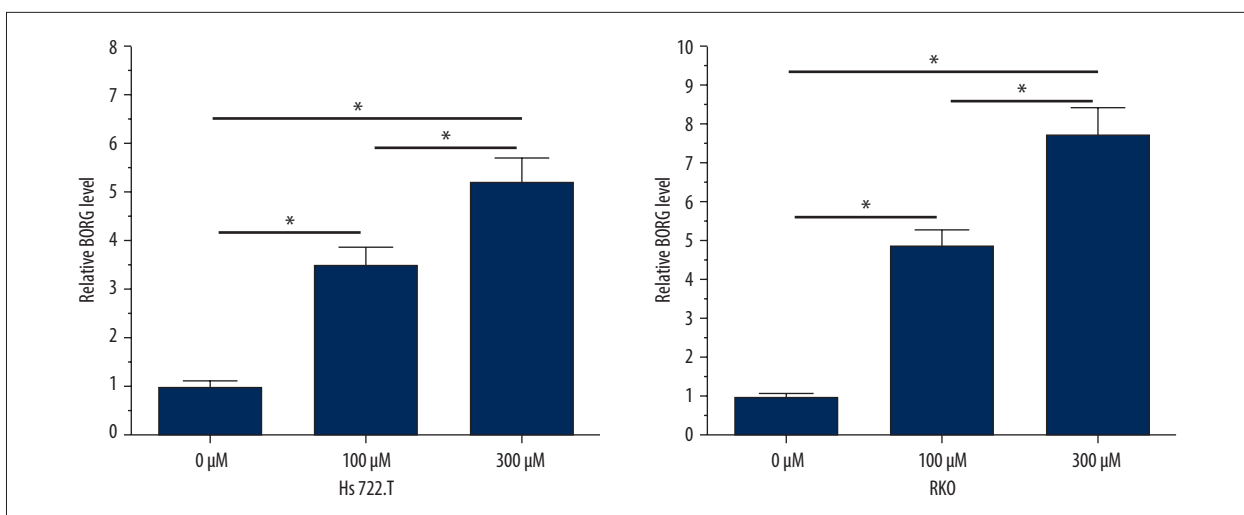

Figure 3. Carboplatin upregulated BORG expression in CRC cells. HS 722.T and RKO cells were treated with carboplatin at dosages of 0, 100, and 300 μM for 24 h. Expression of BORG in HS 722.T and RKO cells was detected by RT-qPCR. Data analysis by ANOVA (one-way) and Tukey test showed that carboplatin treatment upregulated BORG expression in CRC cells in a dose-dependent manner (* p<0.05).
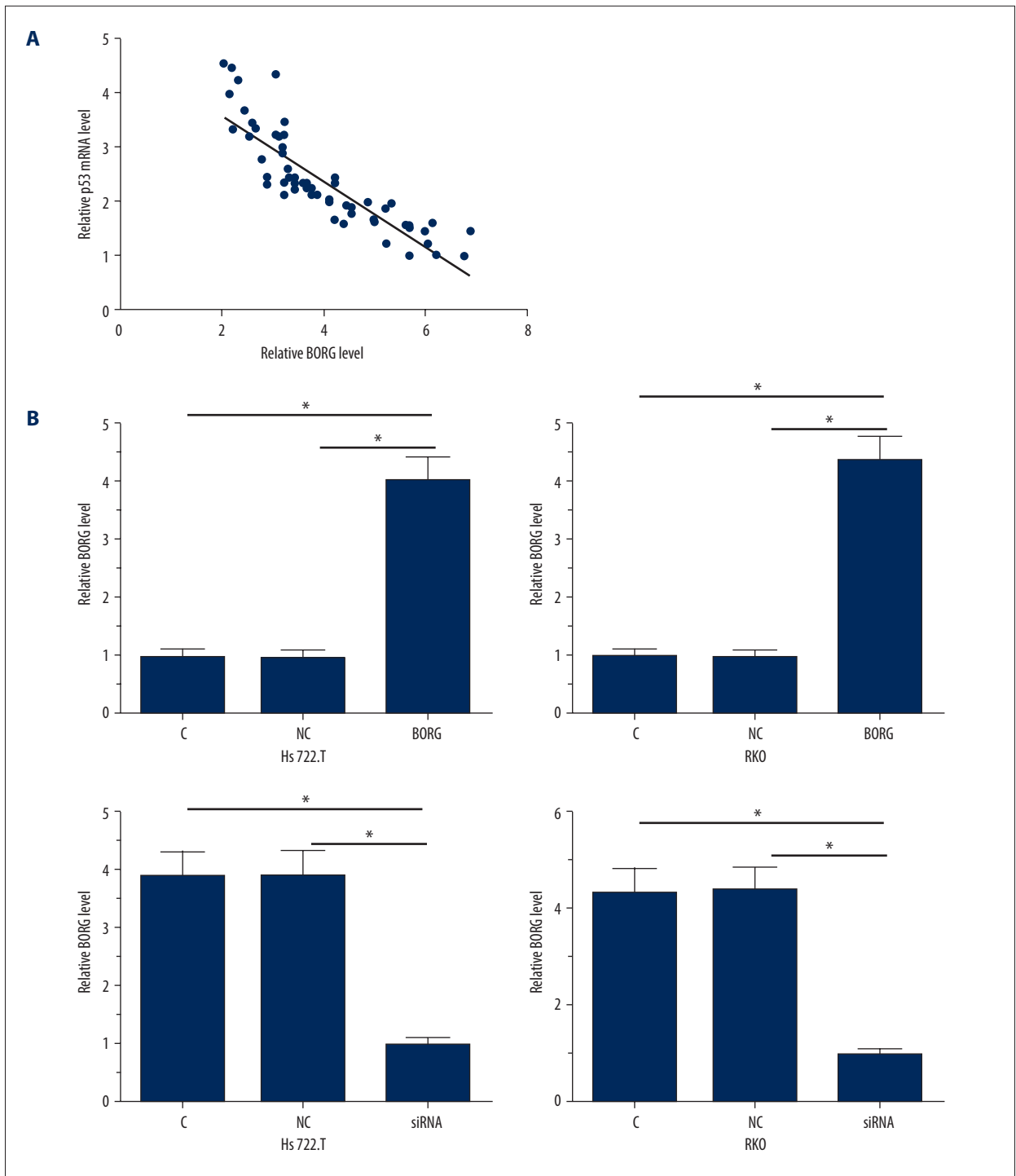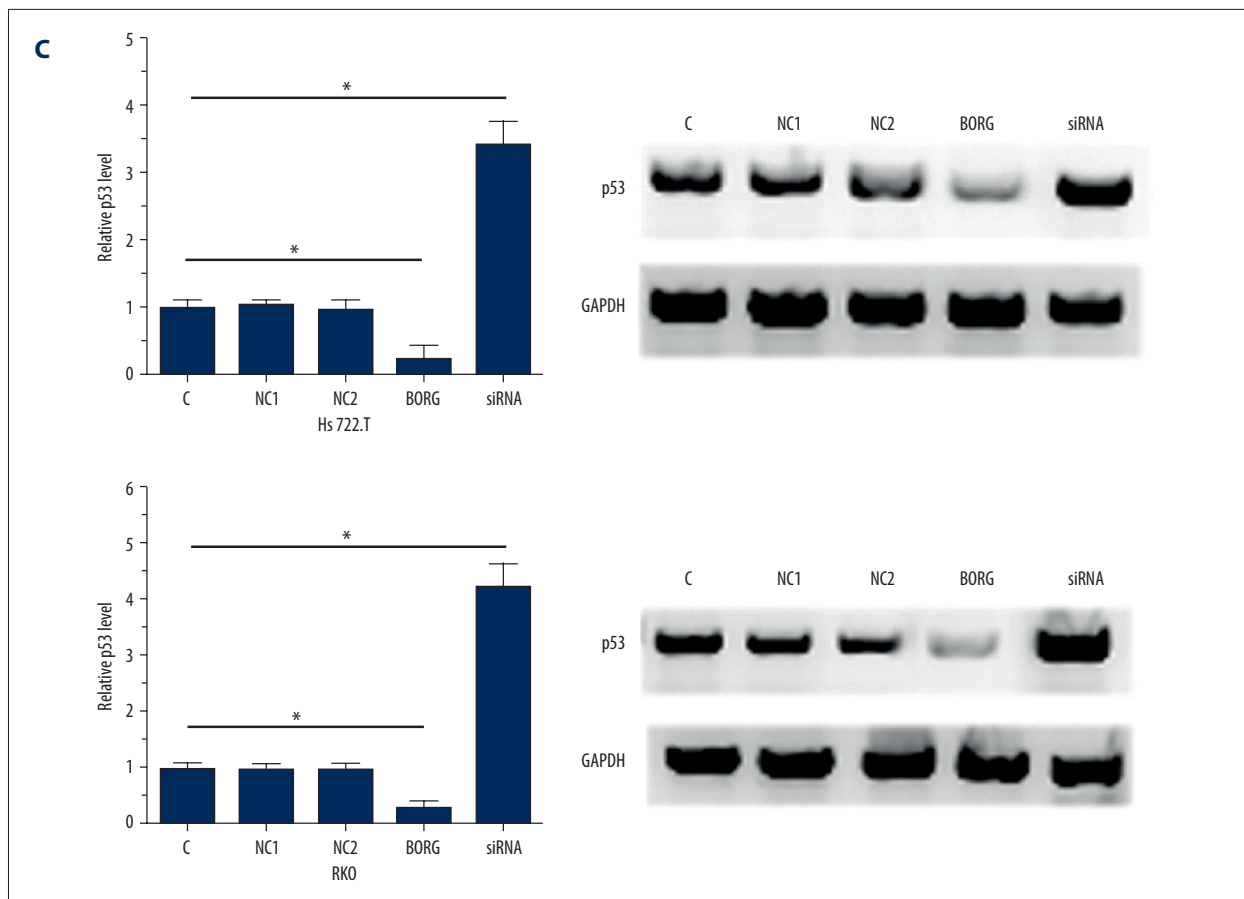
Figure 4. BORG downregulated p53 in CRC cells. Linear regression showed that p53 and BORG were inversely and significantly correlated (**A**). Compared to the C and NC groups, expression levels of BORG were significantly altered at 24 h after transfections, indicating the transfections were successful (**B**). Moreover, overexpression of BORG mediated the downregulation of p53, while BORG siRNA silencing had an opposite effect (**C**), (* $p < 0.05$).

Results

BORG was upregulated in CRC

BORG in CRC tissues and non-cancer tissues from the 66 CRC patients was detected by RT-qPCR. The paired *t* test showed that expression levels of BORG were significantly higher in CRC tissues compared to non-cancer tissues (Figure 1, $p < 0.05$), with a 2.12-fold difference observed.

Carboplatin-based treatment upregulated BORG expression in plasma of CRC patients

BORG in plasma of CRC patients was detected at 3 times-points: before treatment and at 3 and 6 months after treatment. Expression data of BORG were compared among different time-points, and the results showed that expression levels of BORG were significantly increased with the prolonged carboplatin-based treatment (Figure 2, $p < 0.05$), with a 1.32/2.22-fold difference found at 3/6 months after treatment, respectively.

Carboplatin upregulated BORG expression in CRC cells

HS 722.T and RKO cells were treated with carboplatin at dosages of 0, 100, and 300 µM for 24 h. The expression of BORG in HS 722.T and RKO cells was detected by performing RT-qPCR, and expression data were compared by ANOVA (one-way) and Tukey test. It was observed that carboplatin treatment upregulated BORG in HS 722.T cells and RKO cells in a dosage-dependent manner (Figure 3, $p < 0.05$).

BORG downregulated p53 in CRC cells

P53 expression in tumor tissues was also detected by RT-qPCR. Correlation analysis showed that p53 and BORG were inversely and significantly correlated (Figure 4A). BORG expression vectors and siRNAs were transfected into HS 722.T and RKO cells. Compared to the C and NC groups, expression levels of BORG were significantly altered at 24 h after transfections (Figure 4B, $p < 0.05$). Moreover, compared to the 2 control groups, overexpression of BORG mediated the downregulation
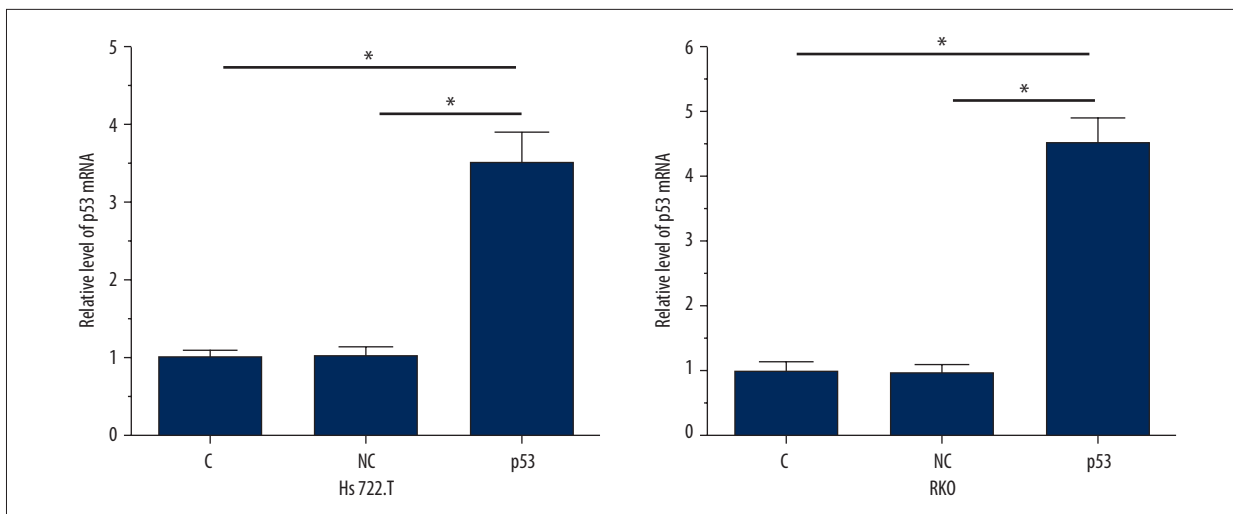
Figure 5. Confirmation of p53 in cells of 2 CRC cell lines. QPCR was performed to confirm the overexpression of p53 in cells of both CRC cells lines. Compared to the C and NC groups, expression levels of p53 mRNA were significantly increased at 24 h after transfections (* p<0.05).
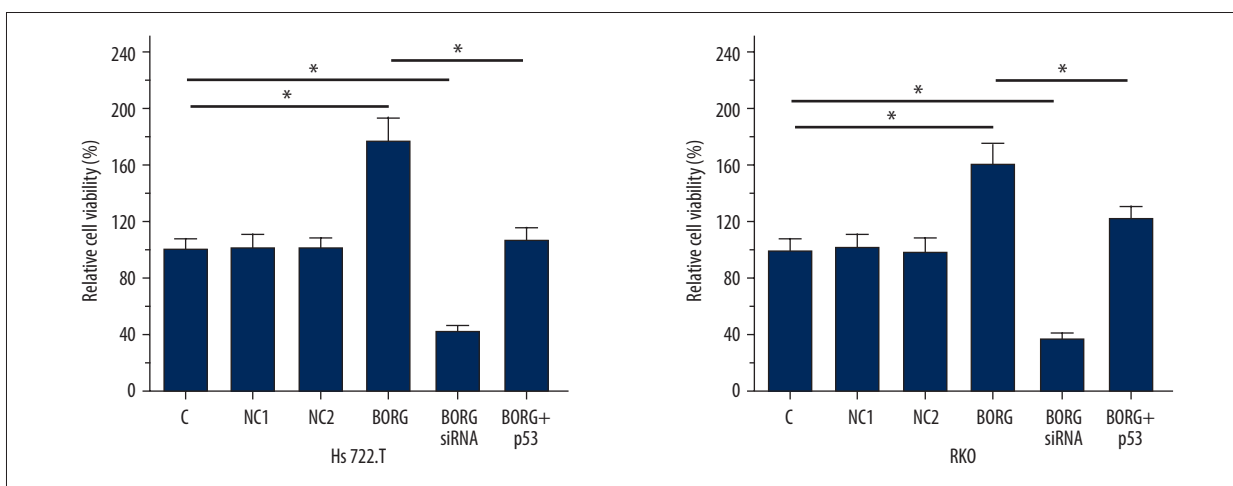

Figure 6. BORG enhanced the viability of CRC cells under carboplatin treatment. MTT assay was performed after cells were treated with carboplatin at dosages of 0, 100, and 300 µM for 24 h. Compared to the C and NC groups, BORG overexpression resulted in increased viability of CRC cells, while BORG siRNA silencing resulted in decreased viability. p53 overexpression attenuated the effects of BORG overexpression (* p<0.05).

of p53, while BORG siRNA silencing had the opposite effect (Figure 4C, p<0.05).

BORG enhanced the viability of CRC cells under carboplatin treatment

QPCR was performed to confirm the overexpression of p53 in cells of both CRC cells lines. Compared to C and NC groups, expression levels of p53 mRNA were significantly increased at 24 h after transfections (Figure 5, p<0.05). Cell viability was detected by performing MTT assay after cells were treated with carboplatin at a dosage of 300 µM for 24 h. Compared to C and NC groups, BORG overexpression resulted in increased

viability of CRC cells, while BORG siRNA silencing resulted in decreased viability. In addition, p53 overexpression attenuated the effects of BORG overexpression (Figure 6, p<0.05).

Discussion

The involvement of BORG and the interaction between BORG and p53 were investigated in the present study. We found BORG was upregulated in CRC and played an oncogenic role in this disease by mediating the development of chemoresistance of CRC cells to carboplatin-based treatment. The actions of BORG are likely mediated by the downregulation of p53.

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Carboplatin-based chemotherapies are widely applied in the treatment of various types of cancers [14,15]. In effect, the use of carboplatin in combination of other chemical drugs, such as Ipilimumab and iniparib, can significantly improve the treatment outcomes compared to the use of single drugs [14,15]. During carboplatin-based treatment, altered expression of many lncRNAs was observed [16,17]. Certain lncRNAs have been proven to be involved in the development of resistance of cancer cells to carboplatin [16,17]. For instance, the upregulation of lncRNA PVT1 in ovarian cancer affects the anticancer action of carboplatin-docetaxel [16]. Moreover, the development of carboplatin resistance in ovarian cancer is affected by the expression pattern of lncRNA HOTAIR [17]. Therefore, regulation of the expression of those lncRNAs can increase the sensitivity of cancer cells to carboplatin. In the present study, we observed upregulated expression of BORG in CRC patients, and carboplatin induced the upregulation of BORG in plasma of CRC and in cells of CRC cell lines. In addition, BORG positively regulated CRC cell viability under carboplatin treatment. Therefore, downregulation of BORG may be a potential therapeutic target to enhance the sensitivity of CRC cells to chemotherapy.

In breast cancer, BORG activates NF-κB signaling to improve the survival of cancer cells [13]. In the present study, we observed an inverse correlation between BORG and p53 in CRC. Further cell line-based in vitro experiments showed that BORG can negatively regulate p53 in CRC cells. It is well known that p53 mutations are closely correlated with the development of chemoresistance in different types of cancer cells, and activation

of p53 increases the sensitivity of CRC cells to chemotherapy [18,19]. Our study also proved that BORG can downregulate p53 in CRC cells to increase the viability of CRC cells under carboplatin treatment. However, the mechanism mediating the interaction between BORG and p53 is unclear and further studies are needed.

Our data suggest that BORG siRNA silencing is a potential therapeutic target to improve the sensitivity of CRC cell to chemotherapies. However, more clinical studies are needed to confirm this hypothesis. In addition, whole-transcriptome analysis is needed to explore the effects of BORG siRNA silencing on the expression of other genes to explore the safety of the BORG siRNA in clinical treatment.

Our data suggest that detecting the expression of BORG can be used to monitor the outcomes of treatment of CRC by carboplatin-based therapies. In addition, regulation of BORG expression might be useful in assisting carboplatin-based therapies.

Our study is limited by its small sample size, and it did not include in vivo animal model experiments. Our future studies will try to include these experiments.

Conclusions

BORG is upregulated in CRC and can negatively regulate p53 to confer resistance of CRC cells to carboplatin-based therapies.

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