Human Helicase RECQL4 Drives Cisplatin Resistance in Gastric Cancer by Activating an AKT–YB1–MDR1 Signaling Pathway

Dongliang Mo1,2, Hongbo Fang1, Kaifeng Niu1,2, Jing Liu1,2, Meng Wu5, Shiyou Li1, Tienian Zhu4, Mohammed A. Aleskandarany6, Aryvind Arora5, Dileep N. Lobo6, Srinivasan Madhusudan5, Adayabalam S. Balajee7, Zhenfen Chi1, and Yongliang Zhao1

Abstract

Elevation of the DNA-unwinding helicase RECQL4, which participates in various DNA repair pathways, has been suggested to contribute to the pathogenicity of various human cancers, including gastric cancer. In this study, we addressed the prognostic and chemotherapeutic significance of RECQL4 in human gastric cancer, which has yet to be determined. We observed significant increases in RECQL4 mRNA or protein in >70% of three independent sets of human gastric cancer specimens examined, relative to normal gastric tissues. Strikingly, high RECQL4 expression in primary tumors correlated well with poor survival and gastric cancer lines with high RECQL4 expression displayed increased resistance to cisplatin treatment. Mechanistic investigations revealed a novel role for RECQL4 in transcriptional regulation of the multidrug resistance gene MDR1, through a physical interaction with the transcription factor YB1. Notably, ectopic expression of RECQL4 in cisplatin-sensitive gastric cancer cells with low endogenous RECQL4 was sufficient to render them resistant to cisplatin, in a manner associated with YB1 elevation and MDR1 activation. Conversely, RECQL4 silencing in cisplatin-resistant gastric cancer cells with high endogenous RECQL4 suppressed YB1 phosphorylation, reduced MDR1 expression, and sensitized cells to cisplatin. In establishing RECQL4 as a critical mediator of cisplatin resistance in gastric cancer cells, our findings provide a therapeutic rationale to target RECQL4 or the downstream AKT–YB1–MDR1 axis to improve gastric cancer treatment.

Introduction

Systemic chemotherapy is the main treatment option for gastric cancer patients as gastric cancer is often diagnosed at an advanced stage and surgical removal is not feasible. However, prognosis and clinical response are highly variable even for the same grade and stage of the tumors and chemoresistance chiefly contributes to treatment failure and recurrence in gastric cancer patients (1). Therefore, understanding the molecular/mechanistic basis for the chemoresistance in gastric cancer is essential not only for devising a better treatment modality but also for developing new chemosensitization strategies to improve gastric cancer patient survival in the future.

Cisplatin (cis-diaminedichloroplatinum) is one of the most frequently used chemotherapeutic drugs in clinical gastric cancer treatment settings. The most prominent mode of its action is to initiate an intrinsic apoptotic pathway through DNA damage response activation (2). Many gastric cancer patients display cisplatin resistance through multiple mechanisms including a decreased uptake and/or an increased efflux of cisplatin mediated by specific transporters, such as MDRs and ATP7B (3–5). Also, constitutive activation of DNA damage response (DDR) pathways owing to elevated expression of DNA repair genes also contributes to chemoresistance by efficient reversal of cisplatin–DNA adducts (6).

RECQL4, a member of the RECQ helicase family with DNA- unwinding activity (7), plays an important role in maintaining the stability of nuclear (8) and mitochondrial genomes (9–11). This is well supported by the existence of three human autosomal recessive disorders Rothmund–Thomson syndrome (RTS; ref. 12), RAPADILLINO (13), and Baller–Gerold syndrome (BGS), all of them owing to mutations in RECQL4 (14). Mutational loss of RECQL4 is associated with increased risk for osteosarcoma development in RTS patients (15). In addition, primary fibroblasts from RTS patients have an increased

*Key Laboratory of Genomic and Precision Medicine, China Gastrointestinal Cancer Research Center, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, China. 2University of Chinese Academy of Sciences, Beijing, China. 3Biological Institute, Hebei Academy of Sciences, Shijiazhuang, China. 4Department of Medical Oncology, Bethune International Peace Hospital, Shijiazhuang, China. 5Division of Cancer and Stem Cells, School of Medicine, University of Nottingham, Nottingham University Hospitals, City Hospital Campus, Nottingham, United Kingdom. 6Gastrointestinal Surgery, National Institute for Health Research Nottingham Digestive Diseases Centre, Biomedical Research Unit, Nottingham University Hospitals and University of Nottingham, Queen’s Medical Centre, Nottingham, United Kingdom. 7REAC/TS, Oak Ridge Associated Universities, Oak Ridge Institute for Science and Education, Oak Ridge, Tennessee.

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D. Mo and H. Fang contributed equally to this article.

Current address for J. Liu: Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China.

Corresponding Authors: Yongliang Zhao, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100101, China. Phone: 8610-8409-7648; Fax: 8610-8409-7648; E-mail: zhaoyongliang@big.ac.cn, and Zhenfen Chi, chizf@big.ac.cn.

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sensitivity to genotoxic agents (16). RECQL4 had recently been found to be significantly associated with clinical outcome and RECQL4 suppression sensitized the breast cancer cells to DNA-damaging agents (17, 18).

Although aberrant expression of RECQL4 has been reported in sporadic osteoblastoma (19), breast and prostate cancer cells and tissues (20, 21), its prognostic and chemotherapeutic significance in gastric cancer are not known. In this study, we demonstrated RECQL4 expression is aberrantly elevated frequently in both clinical gastric cancer samples and tumor cell lines, and a higher RECQL4 expression renders the gastric cancer cells more resistance to cisplatin treatment. We further established a molecular link between RECQL4 expression and cisplatin resistance, in which RECQL4 potentiates YB1 phosphorylation through enhancing AKT and YB1 interaction, suggesting that RECQL4 is a critical determinant of cisplatin resistance in gastric cancer cells through regulation of AKT–YB1–MDR1 pathway.

Materials and Methods

Cell lines and antibodies

The human embryonic kidney HEK293 cell line was ordered in 2012, gastric cancer cell lines AGS and NCI-N87 were ordered in 2013, and SNU-1 and SNU-16 were ordered in 2015 from ATCC. MKN45 and HGC-27 were ordered in 2013 from the Japanese Collection of Research Bioresources and the European Collection of Cell Culture, respectively. MGC–803 was ordered in 2013 from Cell Resource Center, Chinese Academy of Medical Sciences, and authenticated using short tandem repeat (STR) profiling analysis by this Center on December 30, 2015. MKN45, SNU-1, and SNU-16 cell lines were STR-authenticated on November 24, 2015, by Cobioler Biosciences Co. Ltd. AGS, NCI-N87, HGC-27, and HEK293 were STR-authenticated on December 8, 2015, by Shanghai Bioway Applied Biotechnology Co. Ltd.

The antibodies that were used are as follows: RECQL4 from Novus Biologicals (#25470002) and Cell Signaling Technology (#2814); AKT (#9272), p-AKT (#9271), YB1 (#4202), and p-YB1 (#2900) from Cell Signaling Technology; MVP (ab175239) and MDR1 (ab129450) from Abcam; GAPDH (MAB374) from Millipore; and β-actin (A1978) from Sigma.

Gastric cancer tissue array and clinical samples

TissueScan human gastrointestinal cancer tissue qPCR array was ordered from Origene (#HGT101). Eleven pairs of human gastric adenocarcinomas and matched normal gastric specimens were collected from the Department of Medical Oncology, Bethune International Peace Hospital (Shijiazhuang, China). The study on human gastric cancer samples has been approved by the Ethics Committee of Beijing Institute of Genomics.

Immunohistochemistry on gastric cancer patient samples

Patient demographics of about 142 gastric adenocarcinoma cases treated at Nottingham University Hospitals (NUH) between 2001 and 2006 are summarized in Supplementary Table S1. This cohort, comprised of well-characterized patient samples, was used for a wide range of biomarker studies (22–24). Approval from the Ethics Committee of Nottingham University Hospital was obtained for this study.

RECQL4 expression was carried out on tissue microarray (TMA) slides with the primary anti-RECQL4 antibody (#25470002; Novus Biologicals) at a dilution of 1:175. Whole-field inspection of the core was scored and intensities of nuclear or cytoplasmic staining were grouped as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. The percentage of each category was estimated (0–100%). H-score (range 0–300) was calculated by multiplying intensity of staining and percentage staining. Low/negative RECQL4 cytoplasmic expression was defined by mean of H-score of <122. Low/negative RECQL4 nuclear expression was defined by mean of H-score of <179.

Statistical analysis

Statistical analysis of data was performed using SPSS version 20.0 for Windows (SPSS Inc.). Survival rates were calculated from the time of diagnosis until the end of the follow-up period and Kaplan–Meier curves were plotted. The statistical significance of differences between survival rates was determined using the log-rank test. P values ≤ 0.05 was considered as statistically significant.

MTT assay and cisplatin treatment

Cell survival was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 5,000 cells per well in 96-well plates and incubated at 37°C in humidified 5% CO2 for 12 hours. Cells were then exposed to different concentrations of cisplatin for 72 hours, and cellular viability was quantified by the MTT assay following the manufacturer’s instructions (Molecular Probes). Absorbance values at 540 nm were read on a Spectra Max 250 spectrophotometer (Molecular Devices). All MTT assays include 10 duplicated wells for each time-point of each cell line. The data represent mean ± SD from three independent experiments.

Dual luciferase assay

A 256-bp length of MDR1 gene promoter region (–188 to +68) containing Y-box consensus sequence used for luciferase reporter assay was amplified by PCR using the following primer pair: forward: 5’-ggggtaccCACAACGGAAAGGCCAGACAAT-3’; reverse: 5’-cccaagctTGCGAAGACCTAAAGGAAACG-3’. The PCR fragment was then cloned into the KpnI/HindIII sites of pGL3–Basic vector, and verified by sequencing. The pGL3–MDR1pro plasmid was transiently transfected into YB1-silenced MGC803/Tet-on/Flag–RECQL4 cells with YB1 reconstitution and/or doxycycline-induced expression of RECQL4. Luciferase activity was measured following the Dual-Luciferase Assay protocol (Promega).

Adenovirus–mediated knockdown of RECQL4 and YB1 expression

To generate shRECQL4 and shYB1 adenovirus constructs, a 21-mer RECQL4 shRNA (GGTCAAGGCCAATCTGACAGG, 366–386, accession no. NM_004260) and two 21-mer YB1 shRNAs (GGTCAATCGCAACCGAAAGGCCAGACATT-3’; reverse: 5’-cccaagctTGCGAAGACCTAAAGGAAACG-3’) were cloned into U6 promoter pshuttle vector (Agilent Technologies), respectively. The scrambled 21-mer control shRNA sequence is GAGAGAGACCCGACCACTATACGCTACGATTG. Adenovirus particles were produced in HEK293 packaging cells, and used for infecting the cells using the AdEasy Adenoviral Vector System following the manufacturer’s protocol (Cell Biolabs Inc.). Two YB1 shRNA adenoviruses were used concomitantly for knocking down YB1 expression.

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Results

**RECQL4 expression is upregulated in human gastric cancer samples**

To verify whether **RECQL4** expression is aberrantly elevated in human gastric cancer cells, **RECQL4** mRNA level was first determined by real-time quantitative PCR using TissueScan Human gastroesophageal cancer tissue qPCR array (Origene). This array contains cDNA samples of 3 normal gastric tissues (marked), and 20 gastric cancer samples (marked) with different pathologic grades. Other specimens include 3 normal esophageal and 20 esophageal tumor tissues (unmarked). As shown in Fig. 1A, 75% (15 of 20) of gastric cancer tissues displayed a 2.5-fold higher level of **RECQL4** mRNA than normal tissues. **RECQL4** protein level was next examined by Western blotting in 11 pairs of human gastric cancer samples and matched normal controls. In corroboration with elevated **RECQL4** mRNA expression in the clinical gastric cancer samples, **RECQL4** protein elevation was also observed in 8 of 11 gastric cancer samples (Fig. 1B).

**High cytoplasmic **RECQL4** expression correlates with poor survival of gastric cancer patients**

In this study, 92 of 148 tumor cores were found suitable for analysis as some cores within the TMA were missing or lacked tumor and these were eliminated from analysis. Low levels of nuclear and cytoplasmic **RECQL4** expression were found in 18 of 92 (19.6%) samples, whereas 29 of 92 (31.5%) samples showed high nuclear and low cytoplasmic expression. Furthermore, low nuclear/high cytoplasmic **RECQL4** expression was observed in 22 of 92 (23.9%) samples and high nuclear/high cytoplasmic **RECQL4** was found in 23 of 92 (25%) samples (Fig. 1C). As shown in Supplementary Table S2, compared to tumors with low **RECQL4** protein expression, **RECQL4** high nuclear or high nuclear/cytoplasmic coexpression correlated well with grade III tumors ($P = 0.048$), tumor vascular invasion ($P = 0.001$), and circumferential resection margin ($P = 0.041$), suggesting that **RECQL4** high expression has a potential role during gastric cancer tumor progression. Interestingly, tumors with high cytoplasmic **RECQL4** expression had poor disease-specific survival ($P = 0.05$). Although **RECQL4** nuclear expression alone did not influence survival ($P = 0.547$), tumors with both high nuclear/cytoplasmic **RECQL4** appeared to have poor survival compared to tumors with low nuclear/cytoplasmic **RECQL4** expression ($P = 0.05$; Fig. 1D). It is to be mentioned that these patients received only surgery but not any adjuvant/neoadjuvant chemotherapy.

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Figure 1.
**RECQL4** expression in clinical gastric cancer specimens. A, **RECQL4** mRNA level determined by quantitative real-time PCR in normal gastric tissues and gastric cancer specimens with different pathologic grades (TissueScan cDNA array; Origene). Normal gastric and cancer specimens are marked with asterisks (*). Other unmarked samples were esophageal tissue specimens. The values represent mean ± SD from three independent experiments. B, **RECQL4** protein level in 11 pairs of human gastric cancer (T) and matched normal gastric controls (N) determined by Western blotting using **RECQL4** antibody from Novus Biologicals. C, microphotographs of **RECQL4** protein expression in gastric adenocarcinomas. **RECQL4** antibody from Novus Biologicals was used for immunohistochemical staining. D, Kaplan-Meier curves showing disease-specific survival based on **RECQL4** protein expression in different cellular compartments.
*RECQL4* gene is wild type in both gastric cancer cells and clinical gastric cancer specimens with high *RECQL4* expression.

To clarify whether the oncogenic property is driven by gain-of-function owing to mutations in *RECQL4*, the status of *RECQL4* gene was analyzed in 7 gastric cancer cell lines and 11 human surgical gastric cancer specimens by PCR-based single-strand conformation polymorphism (PCR-SSCP) analysis. The primer sets for 21 exons of *RECQL4* coding region and sizes of PCR products were summarized in Supplementary Table S3. As shown in Supplementary Figs. S1–S3, abnormal SSCP band migration in comparison with normal control was observed only in exons 2 to 3 of *RECQL4* gene in AGS and HGC-27 tumor cells. Further sequencing results demonstrated a synonymous GAG to GAA mutation (Glu-Glu at aa 44) in both cell types. In support, genetic polymorphism at this locus has been reported previously by others (12, 25). These results clearly demonstrated that *RECQL4* is wild type in both gastric cancer cells and surgical gastric cancer specimens.

Elevated *RECQL4* expression renders the gastric cancer cells more resistant to cisplatin treatment.

We wished to verify whether elevated *RECQL4* expression confers cisplatin resistance in gastric cancer cells. For this purpose, we first examined the *RECQL4* protein level in 7 gastric cancer cell lines in relative to normal gastric epithelium. In corroboration with elevated *RECQL4* mRNA expression in the clinical gastric cancer samples, *RECQL4* protein level was also elevated in all the gastric cancer cell lines with a much higher level in 4 of the 7 cell lines including AGS, NCI-N87, HGC-27, and SNU-16 when compared with normal control (Fig. 2A). Then the sensitivity to cisplatin treatment was tested by seeding the gastric cancer cells at a density of 5,000 cells and treated with graded concentrations of cisplatin (0, 0.5, 1, 1.5, 2, and 2.5 μg/mL). Cell viability was quantified by the MTT assay. As shown in Fig. 2B, NCI-N87, AGS, and SNU-16 cells with high *RECQL4* expression displayed a significantly higher resistance to cisplatin treatment. In contrast, a much lower cisplatin resistance was observed in MGC-803 cells with a relatively lower *RECQL4* expression. Three cell lines with high (HGC-27) and low (MKN45, SNU-1) *RECQL4* expression showed an intermediate response to cisplatin. Taken together, our results showed a reasonably good correlation between *RECQL4* expression and cisplatin resistance for most of the gastric cancer cell lines examined.

*RECQL4* physically interacts with YB1.

Correlation observed between *RECQL4* expression and cisplatin resistance prompted us to examine the downstream signaling pathway(s) that may be regulated by *RECQL4*. Immunoprecipitation coupled with mass spectrometry was performed to identify potential interactors of *RECQL4*. As shown in Figure 3, *RECQL4* physically interacted with YB1, AKT, and other proteins. These results suggest that *RECQL4* is a key mediator in the regulation of gastric cancer cell growth and survival.
using the lysates of Flag-RECQL4-transfected HEK293 cells. Among the list of candidates identified to interact with RECQL4, YB1 was chosen because YB1 had the highest score in mass spectrometry analysis. Furthermore, YB1 was demonstrated to be not only a transcription factor but also involved in multidrug resistance through regulation of downstream genes such as MDR1 and major vault protein (MVP) genes (26, 27). Interaction between RECQL4 and YB1 was then tested in Flag-RECQL4 overexpressed U2OS cells by coimmunoprecipitation (co-IP) assay. Endogenous YB1 was detected in anti-Flag pull-down lysate with anti-YB1 antibody by Western blotting (Fig. 3A). Likewise, endogenous RECQL4 was observed in anti-YB1 pull-down complex (Fig. 3B), illustrating the interaction between RECQL4 and YB1. YB1 has been shown to be downstream substrate for AKT (28). In support, AKT was detected in the immunoprecipitated complex using either anti-Flag to Flag-RECQL4 or anti-YB1 antibodies (Fig. 3A and B), suggesting that RECQL4 physically interacts with YB1 and AKT.

To further confirm the nature of interaction, purified GST-AKT, His-YB1, and His-RECQL4-Flag proteins expressed in Escherichia coli BL21 and detected by Coomassie brilliant blue staining (Supplementary Fig. S4) were used for the in vitro pull-down assays. Purified His-RECQL4-Flag protein was immobilized on Flag beads and then incubated with His-YB1. Purified GST-AKT was immobilized on glutathione Sepharose beads, and incubated with His-YB1 and/or His-RECQL4-Flag protein. Consistent with the IP results, His-YB1 was pulled down with His-RECQL4-Flag (Fig. 3C), and also with GST-AKT not by GST alone (Fig. 3D), suggesting a direct interaction between RECQL4 and YB1 as well as AKT and YB1. His-RECQL4-Flag can only be pulled down by GST-AKT in the presence of YB1 (Fig. 3D), suggesting an YB1-dependent interaction between RECQL4 and AKT. Through mapping the domain of YB1 specifically interacting with RECQL4 using various GFP-YB1 mutants, the fragment 130–205 aa of YB1 was demonstrated to be responsible for the interaction with RECQL4 (Supplementary Fig. S5).

AKT and YB1 association was enhanced in the presence of RECQL4

Observation of physical interaction between RECQL4 and YB1 prompted us to examine whether RECQL4 promotes the association of YB1 with AKT. *In vitro* pull-down assay (Fig. 3E) revealed that AKT and YB1 interaction was markedly increased by 2.3- to 5.7-fold when RECQL4 protein input was increased from 0 to 0.2 μg (+) and 1 μg (++) respectively. In addition, YB1 and AKT interaction was detected mainly in the cytoplasmic fraction, and their interaction in the cytoplasmic fraction was substantially enhanced when RECQL4 was induced in MGC-803/Tet-on/Flag-RECQL4 cells (Supplementary Fig. S6).

**RECQL4 promotes AKT-dependent YB1 phosphorylation**

It has been reported previously that YB1, being one of the substrates for AKT, enters into nuclear compartment and binds to the promoter region of targeted genes when phosphorylated by AKT (29). Our findings of enhanced association of YB1 and AKT by RECQL4 led us to investigate whether RECQL4 regulates the level of YB1 phosphorylation in gastric cancer cells or not. AGS cells with high RECQL4 expression and RECQL4-inducible MGC-803 cells with low RECQL4 expression were used for evaluating the role of RECQL4 in AKT-mediated YB1 phosphorylation. Immunofluorescent staining results showed that p-YB1 in nuclear was substantially enhanced in RECQL4-induced MGC-803/Tet-on cells (Fig. 4A), whereas it was significantly decreased in RECQL4-suppressed AGS cells (Fig. 4B), suggesting that RECQL4 regulates YB1 phosphorylation and its subsequent recruitment into the nuclear compartment. Consistently, Western blotting analysis showed an increased p-YB1 level in RECQL4-induced MGC-803/Tet-on cells (Fig. 4A). Likewise, RECQL4 suppression in AGS cells resulted in a reduction in YB1 phosphorylation (Fig. 4B). However, AKT phosphorylation was unaffected either by RECQL4 forced expression or by suppression, indicating that RECQL4 performs its functions just downstream of AKT. Inhibition of AKT phosphorylation by treating the above cells with 1 μmol/L of MK-2206 (a p-AKT inhibitor MK-2206 (B)) led us to investigate whether RECQL4 regulates the level of YB1 phosphorylation in gastric cancer cells or not. AGS cells with high RECQL4 expression and RECQL4-inducible MGC-803 cells with low RECQL4 expression were used for evaluating the role of RECQL4 in AKT-mediated YB1 phosphorylation. Immunofluorescent staining results showed that p-YB1 in nuclear was substantially enhanced in RECQL4-induced MGC-803/Tet-on cells (Fig. 4A), whereas it was significantly decreased in RECQL4-suppressed AGS cells (Fig. 4B), suggesting that RECQL4 regulates YB1 phosphorylation and its subsequent recruitment into the nuclear compartment. Consistently, Western blotting analysis showed an increased p-YB1 level in RECQL4-induced MGC-803/Tet-on cells (Fig. 4A). Likewise, RECQL4 suppression in AGS cells resulted in a reduction in YB1 phosphorylation (Fig. 4B). However, AKT phosphorylation was unaffected either by RECQL4 forced expression or by suppression, indicating that RECQL4 performs its functions just downstream of AKT. Inhibition of AKT phosphorylation by treating the above cells with 1 μmol/L of MK-2206 (a p-AKT
inhibitor) for 2 hours specifically abolished YB1 phosphorylation (Fig. 4A and B). This finding together with an elevated YB1–AKT interaction by the increased RECQL4 protein input (Fig. 3E) suggests that RECQL4 promotes AKT-mediated YB1 phosphorylation probably through facilitating the recruitment of YB1 to AKT by some unknown mechanism.

RECQL4 status affects the expression of YB1-targeted genes

We next wished to verify whether RECQL4 affects the expression of YB1-targeted genes such as MDR1 and MVP (26, 30) through modulating p-YB1 level. To this aim, we used qRT-PCR to quantify the mRNA levels of a list of drug resistance–related genes, including MDR1, MRPI-MRP4, BCRP, and MVP. The primer sets for a list of drug resistance-related genes were listed in Supplementary Table S4. As shown in Fig. 5A, only two of the YB1-targeted genes, MDR1 and MVP, were markedly upregulated upon RECQL4 induction in MGC-803/Tet-on/Flag-RECQL4 cells. Elevated MDR1 and MVP proteins upon RECQL4 induction was validated by Western blotting (Fig. 5A). We further tested MDR1 and MVP protein levels in gastric cancer cell lines with varying expression levels of RECQL4. Consistently, cell lines with high RECQL4 expression (AGS, NCI-N87, and SNU-16) had a much higher MDR1 at both mRNA and protein levels relative to cell lines with lower RECQL4 expression (MGC-803, MKN45, and SNL-1; Fig. 5B). Three gastric cancer cell lines (AGS, NCI-N87, and SNU-16) with high RECQL4 expression also have a higher MVP expression at both mRNA and protein levels. HGC-27 cells were found to be an exception with low MVP expression despite a high endogenous RECQL4 expression (Fig. 5C). Consistent with the potentiation of YB1 phosphorylation by RECQL4, expression of YB1 target genes was also enhanced as a function of RECQL4 expression.

RECQL4 regulates MDR1 expression in an YB1-dependent manner

First, MDR1 expression was quantified by real-time RT-PCR in RECQL4-suppressed AGS cells with or without YB1 suppression. As shown in Fig. 6A, suppression of either YB1 or RECQL4 led to a decreased MDR1 expression, whereas no synergistic effect was observed with simultaneous silencing of both YB1 and RECQL4. Luciferase reporter assay was then employed in a reconstituted system to determine whether the regulation of MDR1 expression by RECQL4 is dependent on YB1 or not. Native YB1 was first silenced in MGC-803/Tet-on/Flag-RECQL4 cells by adenovirus-
mediated YB1 shRNA. MDR1 promoter reporter plasmid was then cotransfected with YB1-expressing plasmid with or without RECQL4 induction. Luciferase activities were measured at 72 hours after transfection. As shown in Fig. 6B, MDR1 promoter activity was at a very low level in YB1-silenced cells; however, it was markedly increased by about 5-fold after YB1 reconstitution. In addition, only RECQL4 induction in YB1-suppressed cells failed to activate the MDR1 promoter. In contrast, reconstitution of both RECQL4 and YB1 significantly increased the MDR1 promoter activity in relative to YB1 reconstitution or RECQL4 induction only (Fig. 6B). p-AKT inhibition by treating the cells with 1 μmol/L of MK-2206 (a p-AKT inhibitor) for 2 hours specifically abolished the YB1 phosphorylation and subsequent MDR1 activation even in the presence of RECQL4 induction. These results convincingly demonstrate that MDR1 expression is dependent on RECQL4-mediated transactivation potential of YB1. As with MDR1 promoter, MVP promoter activation was also observed by a luciferase reporter assay upon reconstitution of either YB1 alone or YB1 and RECQL4 in YB1-silenced MGC-803/Tet-on/Flag-RECQL4 cells (Supplementary Fig. S7), suggesting that both of MDR1 and MVP are downstream of RECQL4-YB1 signal pathway.

We further carried out in vitro electrophoretic mobility shift assay (EMSA) and demonstrated both a direct binding of YB1 protein and a YB1-dependent binding of RECQL4 protein to MDR1 promoter (Supplementary Fig. S8). To evaluate the binding efficiency of YB1 to MDR1 promoter, a qPCR-based chromatin immunoprecipitation (ChIP) assay was performed in MGC-803/Tet-on/Flag-RECQL4 cells. As shown in Fig. 6C, YB1 binding to MDR1 promoter was increased by more than 7-fold when RECQL4 was induced, further pointing to the critical role of RECQL4 in regulating YB1 and MDR1 activation.

**Discussion**

The most commonly used treatment regimen in gastric cancer is cisplatin-based chemotherapy (28); however, many gastric cancer patients have cancer recurrence after initial therapy and become refractory to chemotherapy following treatment. Thus, acquisition of cisplatin resistance is a major clinical obstacle for successful treatment of gastric cancer. However, the molecular basis for cisplatin resistance in gastric cancer is not clearly understood.
Figure 7. Altered sensitivity to cisplatin by modulating RECQL4 expression. RECQL4 expression was reconstituted in MKN45, SNU-1, and MGC-803 cells (A) or suppressed in NCI-N87, AGS, and HGC-27 cells (B). Levels of RECQL4, MDR1, MVP, and p-AKT proteins were determined by Western blotting. RECQL4 antibody from Novus Biologicals was used. Cellular viability after treatment with graded concentrations of cisplatin was quantified by MTT assay. The data in the curves represent mean ± SD from three independent experiments. Student t test was used for statistical analysis.
In this study, we have presented a number of novel features that suggest prognostic and therapeutic significance of RECQL4 in gastric cancer: (i) increased expression in gastric cancer cell lines and primary clinical samples; (ii) correlation between high cytoplasmic or nuclear/cytoplasmic expression of RECQL4 with poor survival of gastric cancer patients; (iii) correlation between high RECQL4 expression with cisplatin resistance; (iv) identification of physical and functional interaction of RECQL4 with a major transcription factor YB1; (v) promotion of RECQL4-mediated YB1 phosphorylation by AKT; and (vi) transcriptional activation of a multidrug resistance gene MDR1 by YB1 is regulated by RECQL4 expression.

We have utilized 7 different gastric cancer cells lines in this study, whose genetic makeup is expected to be different from one another. Furthermore, mutational spectrum of genes and the number of genes mutated may also considerably vary. These intrinsic features may influence their cellular phenotypes in vitro especially when these cells are challenged by exogenous DNA damage causing agents such as cisplatin. In spite of the intrinsic variations, a reasonably good correlation between RECQL4 expression and cisplatin sensitivity was observed in most of the gastric cancer cell lines used in this study. Although cisplatin resistance was only marginally increased by forced RECQL4 expression in some of the gastric cancer cell lines with low endogenous RECQL4 expression, the acquired resistance was found to be statistically significant. It is likely that other unidentified mechanism(s) independent of RECQL4 may also contribute to chemoresistance phenotype in gastric cancer cells.

Demonstration of a physical interaction, for the first time in this study between RECQL4 and YB1, indicates the functional significance of RECQL4 upregulation in carcinogenic process (31, 32). YB1 is an oncogenic transcription/translation factor that is overexpressed in a number of cancer types, and is frequently associated with poor outcome and chemotherapy resistance (27, 33). Being a substrate of AKT, YB1 can be phosphorylated at serine 102 by AKT, which is required for YB1 nuclear translocation in cancer cells (28, 34). In support, we found that RECQL4 substantially enhances the interaction between AKT and YB1, resulting in an increased level of p-YB1, illustrating a functional link between RECQL4 overexpression and YB1 activation. Moreover, our data suggest that RECQL4 functions just downstream of AKT and modulates AKT-dependent YB1 phosphorylation possibly by forming a functional complex with AKT through YB1.

Overexpression of the MDR1 gene and corresponding P-glycoprotein (Pgp) efflux pumps is one of the best characterized MDR mechanisms, leading to resistance to a wide variety of anticancer agents in many types of human cancers (35–37). MDR1 expression was shown to be regulated by transcription factors such as YB1 (35). Previous studies have illustrated a plausible association between YB1 and drug resistance in both cultured cancer cells and clinical tumor samples (38, 39). However, there are certain gaps in our understanding with regard to how YB1 becomes activated to induce the expression of MDR1 in cancer cells. Here, we provided evidence that induced expression of RECQL4 in MGC-803/Tet-on/Flag-RECQL4 cells led to a markedly increased level of MDR1 expression. In particular, a fair correlation between RECQL4 status and MDR1 level was observed in gastric cancer cell lines tested. Moreover, ectopic expression of RECQL4 in gastric cancer cells was observed to enhance the YB1 phosphorylation and further its nuclear translocation, and vice versa. The results of luciferase reporter and ChIP–qPCR assays showed that RECQL4 has the potential to modulate MDR1 promoter activity in an YB1–dependent manner. This finding attributes an essential role to RECQL4 in regulating the chemoresistance of gastric cancer cells through regulation of YB1–AKT–MDR1 downstream pathway.

On the basis of our findings, we suggest that RECQL4 is a novel therapeutic target for gastric cancer, and efficient RECQL4-mediated targeting of AKT–YB1–MDR1 pathway is an effective treatment strategy for chemosensitization of gastric cancer. Because RECQL4 has demonstrated roles in DNA replication and repair (40, 41), RECQL4 targeting may have additional advantages for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D. Mo, S. Madhusudan, Z. Chi, Y. Zhao

Development of methodology: D. Mo, H. Fang, S. Madhusudan, Z. Chi

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Mo, H. Fang, T. Zhu, M. A. Aleskandarany, A. Arora, S. Madhusudan, Z. Chi, Y. Zhao

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Mo, H. Fang, M. A. Aleskandarany, A. Arora, S. Madhusudan, Z. Chi, Y. Zhao

Writing, review, and/or revision of the manuscript: D. Mo, M. A. Aleskandarany, A. Arora, D.N. Lobro, S. Madhusudan, A.S. Balajee, Z. Chi, Y. Zhao

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Wu, S. Li, T. Zhu, A. Arora, S. Madhusudan

Study supervision: S. Madhusudan, Z. Chi, Y. Zhao

Other (protein purification): K. Niu

Other (cell line maintenance): J. Liu

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References


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