RecQ-like helicase 4 (RECQL4) exacerbates resistance to oxaliplatin in colon adenocarcinoma via activation of the PI3K/AKT signaling pathway

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ABSTRACT

Oxaliplatin (OXA) resistance is a great challenge for colon adenocarcinoma (COAD) chemotherapy. The promoting role of RecQ-Like Helicase 4 (RECQL4) in chemoresistance to platinum-based drugs has been identified, whereas the effect and specific mechanism of RECQL4 in regulating OXA resistance within COAD have not been explicated yet. In this work, RECQL4 mRNA expression was detected by RT-qPCR. RECQL4, phosphorylated PI3K (p-PI3K), PI3K, phosphorylated AKT (p-AKT), and AKT protein expression were measured by western blotting. CCK-8, flow cytometry, wound healing, and transwell assays were utilized to analyze OXA resistance, cell proliferation, apoptosis, cell cycle, migration and invasion. Herein, we found RECQL4 was upregulated in COAD, especially in OXA-resistant COAD tissues and cells. RECQL4 overexpression facilitated proliferation and metastasis of OXA-resistant COAD cells; on the contrary, RECQL4 knockdown attenuated proliferative and metastatic capabilities in OXA-resistant COAD cells. Moreover, RECQL4 promoted OXA resistance in OXA-resistant COAD cells via activating the P13 K/AKT signaling. To sum up, the results suggest that RECQL4 depletion may be a crucial mechanism to reverse OXA resistance in COAD via inhibition of the P13 K/AKT pathway in vitro, thereby providing a novel target for overcoming OXA resistance in COAD.

Introduction

As a common malignant disease derived from the colonic mucosa epithelium, colon adenocarcinoma (COAD) is a major gastrointestinal malignancy in the world, especially in China [1,2]. Its high incidence and mortality pose a growing threat to public health worldwide [3]. Luckily, most patients with early-stage COAD (Stage I–II) can be cured via surgical resection, and almost 70% of COAD patients at the mid-term stage (Stage III) can be cured via a combination of surgical excision and chemotherapy [4]. However, most COAD patients at advanced stage (stage IV) have a short survival time in spite of improved chemotherapy in recent years [4]. Since COAD is often asymptomatic at early stages, a large number of COAD patients were diagnosed at advanced stage, leading to a poorer prognosis [5]. Oxaliplatin (OXA), one of the third-generation platinum-based drugs, is widely applied in first-line treatment for colorectal cancer (CRC), including COAD [6]. However, OXA resistance is still a serious problem in chemotherapy as it may lead to cancer cell survival or quiescence and even cause tumor recurrence [7]. Therefore, it is quite urgent to identify probable predictive biomarkers for OXA resistance in COAD, thus helping select appropriate chemotherapeutic drugs and predict chemotherapy efficacy for COAD patients.

As a family of DNA-unwinding helicases, RecQ helicases are deeply involved in DNA replication, transcription, recombination, and repair [8,9]. RecQ-Like Helicase 4 (RECQL4), a member of RecQ helicase family, has been reported to act as an oncogene in human cancers, such as prostate cancer [10], breast cancer [11], and hepatocellular carcinoma [12]. In addition, Mo et al. found that RECQL4 induced chemoresistance in gastric cancer [13], suggesting its promoting role in the development of chemoresistance to platinum drugs. Moreover, Lao et al. also observed elevated protein and mRNA levels of RECQL4 in CRC [14].

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However, whether or how RECQL4 could regulate OXA resistance in COAD remains largely unknown.

In this study, we focused on the functions and specific mechanism of RECQL4 in regulating OXA resistance within COAD. Through further investigation, it was determined that RECQL4 modulated COAD resistance to OXA via targeting the PI3K/AKT signaling, thus providing novel insights for personalized chemotherapeutic drug selection in clinical treatment for COAD patients.

Materials and Methods

Clinical specimens

This study was permitted by the Ethical Committee of the Affiliated Shuyang Hospital of Xuzhou Medical University. COAD tissues and paired adjacent non-cancer tissues were obtained from 60 COAD patients who had undergone surgery and accepted OXA chemotherapy at The Affiliated Shuyang Hospital of Xuzhou Medical University. According to follow-up results, the COAD patients were divided into the OXA-resistant group (patients who suffered recurrence within half a year after OXA chemotherapy; n = 24) and the OXA-sensitive group (patients with no recurrence within half a year or with recurrence beyond half a year after OXA chemotherapy; n = 36). Written informed consent was received from each patient.

Cell culture

Normal human colon epithelial cell line (NCM460), as well as COAD cell lines (SW-480, HT-29, T-84, LS-174 T, DLD-1, and LoVo) obtained from BeNa Culture Collection (Beijing, China), were cultivated in DMEM containing 10% FBS (Thermo Fisher Scientific) at 37°C in a humid atmosphere with 5% CO₂.

Establishment of OXA-resistant cell models

Briefly, to construct OXA-resistant COAD cell models (T-84/OXA and DLD-1/OXA), parental T-84 and DLD-1 cells were continuously exposed to OXA for about 6 months with OXA concentration increased in a stepwise manner. Then, the established OXA-resistant COAD cells were cultivated in DMEM containing 10% FBS and 5 μmol/L OXA with 5% CO₂ at 37°C.

Cell transfection

Short hairpin RNA (shRNA) against RECQL4 (sh-RECQL4: 5ʹ-CAAGACGCUUACGUACATT-3ʹ), negative control (sh-NC: 5ʹ-UUCUCCAGUGUCACGUTT-3ʹ), as well as RECQL4 overexpression vector (pcDNA3.1/RECQL4) and empty pcDNA3.1 vector (pcDNA3.1) were purchased from GenePharma. These plasmids were transfected into COAD cells via Lipofectamine 3000 (Invitrogen, USA), and cells were collected 24 hours later.

RT-qPCR

Total RNA was extracted using TRIzol (Invitrogen) and digested with DNase I (Invitrogen) to remove genomic DNA. Thereafter, reverse transcription was performed with cDNA Reverse Transcription Kit (Takara, China). Then, qPCR was performed with SYBR Premix Ex Taq” (Takara) on CFX-96 Real-Time PCR Detection System (Bio-Rad, USA) RECQL4 mRNA expression was calculated by 2− ΔΔCT method, with GAPDH as the internal control. The primers are as follows: GAPDH (Forward (F)):5ʹ-AATCCCATCACATCTCTTCC-3ʹ and Reverse (R): 5ʹ-AGGCTGTGTCATCTTC-3ʹ; RECQL4 (F): 5ʹ-TCAACATGACAGCAACACTAC-3ʹ and R: 5ʹ-CTGCTGTTGTCATACTTC-3ʹ). No-template reaction and no-RT reaction were used as negative controls.

Immunohistochemistry (IHC)

The OXA-sensitive and OXA-resistant COAD tissue sections were embedded in paraffin and then cultivated with citrate buffer or antigen retrieval. Thereafter, tumor sections were cultured with primary antibody against RECQL4 overnight at 4°C, followed by cultivation with HRP-conjugated secondary antibody. Finally, tumor sections were stained via DAB and observed under light microscopy.
Western blotting

Cells obtained were lysed in lysis buffer. By BCA protein assay kit (Thermo Scientific), protein concentrations in cell lysates were measured. Then, equal quantities of proteins were separated by SDS-PAGE gels and transferred to PVDF membranes. After blocked with 5% skim milk, the membranes were cultured with primary antibodies (against phosphorylated PI3K (p-PI3K), PI3K, phosphorylated AKT (p-AKT), AKT, and GAPDH) at 4°C overnight. Then, the membranes were cultivated with horseradish peroxidase-conjugated secondary antibodies for 2 h. The target protein bands were visualized with an ECL kit (Beyotence).

CCK-8

For oxaliplatin resistance analysis, cells (1 × 10⁴/well) were cultured in 96-well plates with increasing oxaliplatin concentration (1, 2, 3, 5, 10, 20 μg/ml) for 24 hours. Then, 10 μl CCK-8 reagent (Beyotence, China) was added to each well and cultured for another 2 hours. Finally, the cells were observed at 450 nm for optical density. The half-maximal inhibitory concentration (IC₅₀) value was calculated accordingly.

For cell proliferation assessment, CCK-8 reagent was added after the cells were cultured for 0, 24, 48, and 72 h. The other steps were the same as those of the oxaliplatin resistance analysis.

Wound healing

To assess cell migration, wound healing assay was applied [15]. Cells in logarithmic growth phase were trypsinized and seeded into 6-well plates. After 24 h, when cell convergence reached about 90%, the tip of a 200 μL pipette was used to make even linear scratches. Thereafter, the plates were rinsed with PBS to eliminate floating cells, and then supplemented with fresh medium. After 24 h, the cells were observed under a light microscope (40× magnification) to measure cell migration distance.

Transwell

Transwell assay was performed to detect cell invasion, the upper chamber is equipped with a Matrigel-coated membrane (BD, USA). Briefly, 1 × 10⁵ cells were seeded into the upper chamber and cultured in serum-free medium. Meanwhile, DMEM containing 10% FBS was added to the lower chamber. After 48 hours’ incubation, the invasive cells passing through the membrane were immobilized with paraformaldehyde, stained with crystal violet, and then counted under a light microscope (Olympus BX61, Japan) [15].

Flow cytometry

For cell apoptosis detection, flow cytometry was performed with FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™). Cells were washed with cold PBS three times and cultured in 100 μl binding buffer together with 2 μl FITC Annexin V and 2 μl for 15 min in darkness. Thereafter, apoptotic cells were observed via flow cytometry (BD Biosciences) [16].

For cell cycle analysis, cells were cultured in six-well plates and allowed to grow to 75–80% confluence. Then, the plates were rinsed twice with PBS to remove non-adherent cells. The left cells were trypsinized, rinsed, and fixed by 70% ethanol overnight. Afterward, the cells were collected by centrifugation and then resuspended in 0.2 mg/ml PI reagent containing 0.1% Triton X-100 and 0.1 mg/ml RNase A. The cell suspension was analyzed via FACScan flow cytometer after incubation in darkness at room temperature for 30 min. Finally, cell percentages in different cell-cycle phases were analyzed by ModFit 5.2 [17].

Statistical analysis

Each experiment was performed three times independently. All data acquired were presented as mean±SD. GraphPad Prism 6.0 was employed for statistical analysis. Student’s t-test or one-way ANOVA was utilized to evaluate significance of comparisons between two or more groups. Any difference with P < 0.05 was considered significant in statistics.

Results

In the current study, we aimed to investigate the role of RECQL4 in regulating chemoresistance to OXA
in COAD. Through a series of in vitro assays, it was revealed that RECQL4 promoted OXA resistance in COAD via activating the PI3K/AKT signaling pathway. Therefore, this work explored the functional effects of RECQL4 in regulating OXA resistance in COAD for the first time, showing a novel direction for COAD chemotherapy.

**RECQL4 expression in OXA-resistant COAD**

Data from both GEPIA and UALCAN websites showed that RECQL4 expression was markedly increased in COAD tissues (Figure 1a and b). Moreover, according to GEPIA analysis, RECQL4 expression is also elevated in other human cancer tissues.
types, such as ACC, BLCA, BRCA, CESC, DLBC, and HNSC (Figure 1c), indicating RECQL4 is upregulated in multiple human cancers. Next, the correlation between RECQL4 expression and COAD clinical features was analyzed. According to Table 1, high RECQL4 expression was positively correlated with positive lymph node metastasis and high TNM stage. Moreover, RT-qPCR results indicated that RECQL4 mRNA expression was apparently upregulated in COAD samples, relative to normal tissues; besides, OXA-resistant COAD specimens showed higher RECQL4 mRNA expression than OXA-sensitive COAD specimens (Figure 1d). IHC assay further confirmed that RECQL4 protein expression was higher in OXA-resistant COAD samples than OXA-sensitive COAD samples (Figure 1e). Then, RT-qPCR assay revealed that RECQL4 mRNA expression was distinctly lifted in COAD cell lines (SW-480, HT-29, T-84, LS-174 T, DLD-1, and LoVo) than normal human colon epithelial cell line (NCM460) (Figure 1f). As RECQL4 expression was relatively higher in T-84 and DLD-1 cells, they were applied to establish OXA-resistant COAD cell models. CCK-8 assay disclosed that OXA-resistant COAD cell lines (T-84/OXA and DLD-1/OXA) exhibited higher IC₅₀, relative to OXA-sensitive COAD cell lines (parental T-84 and DLD-1 cells) (Figure 1g). In addition, RT-qPCR and western blotting assays manifested that RECQL4 enrichment was markedly higher in T-84/OXA and DLD-1/OXA cell lines than parental T-84 and DLD-1 cells (Figure 1h and i). To sum up, RECQL4 was upregulated in OXA-resistant COAD.

**Table 1.** Correlations between RECQL4 level and clinicopathological characteristics.

|                | RECQL4 level | P-value |
|----------------|--------------|---------|
| **Gender**     |              |         |
| Female         | Low (n = 31) | 0.744   |
|                | High (n = 29)|         |
| Male           | 7            |         |
|                | 8            |         |
| **Age (years)**|              | 0.071   |
| ≤55            | 17           |         |
|                | 11           |         |
| >55            | 14           |         |
|                | 18           |         |
| **Tumor size (diameter, cm)** |           | 0.238   |
| ≤5.0           | 21           |         |
|                | 17           |         |
| >5.0           | 10           |         |
|                | 12           |         |
| **Lymph node metastasis** |       | 0.027*  |
| Negative       | 17           |         |
|                | 9            |         |
| Positive       | 14           |         |
|                | 20           |         |
| **TNM stage**  |              | 0.019*  |
| I–II           | 19           |         |
|                | 10           |         |
| III–IV         | 10           |         |
|                | 21           |         |

Note: *indicates a statistical significance (P < 0.05).

**Overexpression of RECQL4 promotes proliferation and metastasis of OXA-resistant COAD cells**

To evaluate the biological role of RECQL4 in the OXA resistance of COAD cells, gain-of-function assays were performed. RT-qPCR and western blotting revealed that the mRNA and protein levels of RECQL4 were increased in OXA-resistant COAD cells transfected with RECQL4 overexpression plasmid (Figure 2a and b). As displayed in Figure 2c, the IC₅₀ value of OXA-resistant cells transfected with pcDNA3.1/RECQL4 was significantly increased, compared with the pcDNA3.1 group (Figure 2c). CCK-8 assay and flow cytometry analysis showed the upregulation of RECQL4 promoted the cell viability and cell cycle progression but reduced cell apoptosis in OXA-resistant COAD cells (Figure 2d-f). In addition, wound healing and transwell assays indicated that overexpression of RECQL4 markedly promoted the cell migration and invasion of OXA-resistant cells compare with the control group (Figure 2g and h). The above data suggested that the upregulation of RECQL4 enhanced the chemoresistance of OXA-resistant COAD cells to OXA.

**RECQL4 knockdown inhibits OXA-resistant COAD cell proliferation and metastasis**

Compared with the control group, RECQL4 knockdown decreased RECQL4 level in T-84/OXA and DLD-1/OXA cells (Figure 3a and b). As indicated by CCK-8 assay, RECQL4 depletion distinctly reduced OXA IC₅₀ value in OXA-resistant cells (Figure 3c). Moreover, RECQL4 silencing also alleviated cell proliferative ability and induced cell apoptosis and cell-cycle retardation in T-84/OXA and DLD-1/OXA cells (Figure 3d-f). Additionally, RECQL4 depletion also impaired migrative and invasive capabilities of T-84/OXA and DLD-1/OXA cells (Figure 3g and h). Therefore, these findings disclosed that RECQL4 suppression restrained OXA resistance in OXA-resistant COAD cells.

**RECQL4 affects the PI3K/AKT signaling in OXA-resistant COAD Cells**

The positive correlation observed between RECQL4 expression and OXA resistance inspired us to explore putative downstream signaling
Figure 2. Overexpression of RECQL4 promotes proliferation and metastasis of OXA-resistant COAD cells. (a and b) RECQL4 mRNA and protein levels in T-84/OXA and DLD-1/OXA cells transfected with pcDNA3.1/RECQL4 or pcDNA3.1 were assessed by RT-qPCR and Western blotting. (c) IC50 value of OXA was determined in T-84/OXA and DLD-1/OXA cells treated with pcDNA3.1/RECQL4 or pcDNA3.1 by CCK-8 assay. (d) CCK-8 was used to detect cell viability. (e and f) Flow cytometry was utilized to detect cell apoptosis and cell cycle. (g and h) Wound healing and Transwell assay were utilized to assess cell migration and invasion. The results are presented as the mean ± standard deviation (SD) from at least three independent experiments. *P < 0.05.
**Figure 3.** RECQL4 knockdown inhibits OXA-resistant COAD cell proliferation and metastasis. (a and b) RECQL4 mRNA and protein levels in T-84/OXA and DLD-1/OXA cells transfected with sh-RECQL4 or sh-NC were assessed by RT-qPCR and Western blotting. (c) IC_{50} value of OXA was determined in T-84/OXA and DLD-1/OXA cells treated with sh-NC or sh-RECQL4 by CCK-8 assay. (d) CCK-8 was used to detect cell viability. (e and f) Flow cytometry was utilized to detect cell apoptosis and cell cycle. (g and h) Wound healing and Transwell assay were utilized to assess cell migration and invasion. The results are presented as the mean ± standard deviation (SD) from at least three independent experiments. *P < 0.05.
regulated by RECQL4 in COAD resistance to OXA. As a key pathway frequently activated in human cancers, the PI3K/AKT signaling is often implicated in cellular transformation, tumorigenesis, cancer progression, and drug resistance \cite{18,19}. Besides, PI3K/AKT signaling pathway has also been identified as a vital regulator in COAD carcinogenesis and a potential therapeutic target of COAD \cite{20,21}. To further explore whether RECQL4 regulates the PI3K/AKT pathway in COAD cells, western blotting was employed to detect the activity of the PI3K/AKT pathway. As shown in Figure 4a, RECQL4 knockdown had no significant influence on the total PI3K, and AKT expressions, while the p-PI3K and p-AKT levels were apparently reduced by RECQL4 deficiency in COAD cells. Therefore, the activation of the PI3K/AKT pathway was impaired in COAD cells by RECQL4 depletion.

**RECQL4 regulates the OXA resistance of COAD cells through the PI3K/AKT signaling**

To further evaluate whether RECQL4 exerted a regulatory effect on OXA resistance of COAD cells through PI3K/AKT signaling pathway. OXA-resistant COAD cells were treated with PI3K activator IGF-1. Western blot showed that IGF-1 effectively abolished the inhibition of PI3K/AKT signaling by RECQL4 blocking (Figure 5a). As illustrated in Figure 5b, the declined IC\textsubscript{50} value induced by RECQL4 knockdown was reversed after IGF-1. Moreover, the enhanced cell viability was observed in sh-RECQL4+ IGF-1 group of OXA-resistant COAD cells, comparable to sh-RECQL4 group (Figure 5c). Besides, G0/G1 phase arrest and cell apoptosis of OXA-resistant COAD cells caused by RECQL4 knockdown were abrogated by IGF-1 treatment (Figure 5d and e). In addition, the cell migration and invasion abilities were declined in sh-RECQL4 group, but markedly increased in sh-RECQL4+ IGF-1 group (figure 5f and g). Conclusively, RECQL4 enhanced the OXA resistance of COAD cells through the PI3K/AKT pathway.

**Discussion**

OXA is widely applied as a first-line drug for chemotherapy of a variety of human cancers, including COAD. However, OXA chemoresistance is a great obstacle for COAD chemotherapy. Further understanding of the molecular mechanisms associated with OXA resistance in COAD may help to improve the efficacy of OXA chemotherapy for COAD patients.

As a human RecQ helicase, RECQL4 was identified as oncogenic in multiple malignancies. For example, Lyu et al. revealed that RECQL4 was highly expressed and promoted tumorigenesis in esophageal cancer \cite{22}. Arora
**Figure 5.** RECQL4 regulates the OXA resistance of COAD cells through the PI3K/AKT signaling. (a) Western blotting was used to assess p-PI3K, PI3K, p-AKT, and AKT in T-84/OXA and DLD-1/OXA cells transfected with sh-NC, sh-RECQL4, or sh-RECQL4+ IGF-1. (b) IC50 value of OXA was determined in T-84/OXA and DLD-1/OXA cells treated with sh-NC, sh-RECQL4 or sh-RECQL4+ IGF-1 by CCK-8 assay. (c) CCK-8 was used to detect cell viability. (d and e) Wound healing and Transwell assay were utilized to assess cell migration and invasion. (f and g) Flow cytometry was utilized to detect cell apoptosis and cell cycle. The results are presented as the mean ± standard deviation (SD) from at least three independent experiments. *P < 0.05.
et al. demonstrated that RECQL4 was upregulated and played a tumor-promoting role in breast cancer [23]. In addition, Król et al. elucidated that RECQL4 was abnormally expressed and promoted cell proliferation as well as chemoresistance in glioma [24]. In consistence with the above results, GEPIA and UALCAN databases showed that RECQL4 was overexpressed in COAD. We also found that RECQL4 expression was largely increased in COAD tissues and cells, especially OXA-resistant COAD tissues and cells, suggesting RECQL4 might promote OXA-resistance in COAD. Moreover, functional assays showed that RECQL4 upregulation remarkably increased OXA resistance, promoted cell proliferation and cell-cycle progression, reduced cell apoptosis, and facilitated cell migration, and invasion of OXA-resistant COAD cells; conversely, RECQL4 deficiency remarkably suppressed OXA resistance, impaired cell viability, induced cell-cycle regression and cell apoptosis, and largely restrained cell metastatic capabilities of OXA-resistant COAD cells. To sum up, our data demonstrated that RECQL4 exacerbated OXA resistance in COAD in vitro.

As PI3K/AKT signaling has been proven to promote tumorigenesis and chemoresistance in several cancers [25–28], it is a promising target for improving therapeutic effects of tumor chemotherapy. In our study, by silencing RECQL4 in OXA-resistant COAD Cells, PI3K/AKT signaling was inhibited, which led to reduced OXA resistance, inhibited cell viability, increased apoptotic rate and cell-cycle retardation, and impaired cell metastatic capabilities. Such effects of RECQL4 knockdown could be partially abolished by the PI3K activator, IGF-1. These data suggest that RECQL4 promotes OXA resistance in COAD cells via targeting the PI3K/AKT signaling.

**Conclusion**

In summary, our findings indicated that RECQL4 enhanced OXA resistance of COAD via activating the PI3K/AKT signaling in vitro, which might provide a promising therapeutic target for patients with OXA-resistant COAD. In the future, in vivo experiments will be performed to further support our findings.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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