RECQL5 is an important determinant for camptothecin tolerance in human colorectal cancer cells

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Synopsis

CPTs (camptothecins) are an important class of effective anticancer agents that target type I topoisomerase in humans. Irinotecan and topotecan are currently used to treat various types of cancers and many CPT derivatives are being developed. However, these drugs are only effective in a small percentage of each type of cancer and the molecular underpinning for this individualized response to the drug has remained elusive. Thus, identification of the main determinants for cell survival in response to this unique class of drug should help to improve their clinical applications. In the present study, we examined whether RECQL5 constitutes an important determinant of CPT resistance in colon cancer cells. Specifically, RECQL5-deficient derivatives of both DDL1 and HCT116 cells, two colorectal cancer cell lines were generated by adenovirus-based somatic gene-targeting experiments and the CPT sensitivity between the RECQL5-proficient parental lines and their corresponding RECQL5-deficient derivatives were examined. We found that deletion of RECQL5 from DDL1 and HCT116 cells both resulted in a significant enhancement in CPT sensitivity under in vitro culture conditions. More importantly, xenograft tumours derived from RECQL5-deficient HCT116 cells, but not those from the parental line, could be cured by a CPT-based therapy in nude mice. Thus, the present study has identified RECQL5 as a major determinant for CPT resistance in colorectal cancer cells and a potential candidate as a biomarker for irinotecan-based treatment for colon cancer.

Key words: camptothecin (CPT), colorectal cancer, drug tolerance, RECQL5, topoisomerase I (Topo I) inhibitor

INTRODUCTION

CPT (camptothecin) and its derivatives are a class of anticancer agents collective known as Topo I (topoisomerase I) inhibitors [1,2]. These agents target type I topoisomerase and kill human cells by interfering with the function of Topo I during DNA replication [3–7]. Two of them, irinotecan and topotecan have been approved by the U.S. Food and Drug Administration for several types of human cancers, including non-small-cell lung cancer and advanced colorectal cancer [6,7]. Importantly, these drugs are currently being tested in many different multi-agent treatments for various types of cancers. For examples, irinotecan are being tested in multi-agents therapies for both colorectal cancers as well as other types of cancer with promising results [8–10]. Thus, these anticancer drugs are among the most promising classes of anticancer agents [1]. In this regard, an improved understanding of the mechanisms of drug tolerance for this unique class of anticancer agents would be empirical in the rational design of multi-agent combinatorial therapies with these agents and/or the development of biomarkers for personalized therapies.

We have reported recently that Recql5, a member of the mammalian RecQ helicase family, functions to promote cell survival after CPT treatment in a homologous recombination-independent manner and that Recql5-deficient cells are uniquely hypersensitive to CPT but not many other DNA-damaging agents, including methyl methanesulfonate, cisplatinum, mitomycin C and etoposide [11–13]. These findings demonstrate that Recql5 plays an important role in a novel pathway of CPT resistance. Importantly, as the mouse Recql5 and human RECQL5 proteins are also highly conserved, RECQL5 may represent an important determinant for resistance to Topo I inhibitors in human cells. The aim of the present work was to investigate whether RECQL5, the human homologue of mouse Recql5, also plays an important role in the cytotoxic response of human colorectal cancer cells to...
irinotecan, a derivative of CPT, which is currently used to treat colorectal cancer patients.

MATERIALS AND METHODS

Gene targeting in HCT116 and DLD1 cells

The HCT116 cell line was generously provided by Dr Bert Vogelstein at Johns Hopkins University, while DLD1 cell was purchased from the A.T.C.C. The procedure for gene targeting using the AVV (adeno-associated virus) system has been described [14]. Briefly, two RECQL5-AAV targeting vectors, \textit{RECQL5TVN} and \textit{RECQL5TVP}, were constructed to delete exon 5 of the human \textit{RECQL5} gene. They differ only by their selection markers (\textit{PGKneo} for \textit{RECQL5TVN} and \textit{PGKpuro} for \textit{RECQL5TVP} respectively) (Figure 1A and results not shown). These vectors were transfected along with pAAV-RC and pHELPER plasmid DNA into AAV-293 cells to obtain the AAV viral stocks to be used to infect HCT116 or DLD1 cells. Stably transfected cells were selected by growth in the presence of G418 or puromycin and then subjected to a PCR-based genotyping screen to identify those with the proper gene-targeting events (see Figure 1). Also, the detailed information regarding both the construction of targeting vectors and the identification of the targeted clones will be provided by G.L. upon request. Briefly, HCT116 or DLD1 cells were
infected with AAV viruses with the targeting vectors. After AAV transfection, transfected cells were selected in either G418- or puromycin-containing containing medium. For RECQL5 knockout in HCT116 cells, RECQL5TVN-AAV virus was first used to obtain clones with one targeted RECQL5 allele. One of these clones was selected and transfected with a Cre-expressing adenovirus and then plated at a density of 200 cells/10 cm plate to obtain single cell-derived clones. These clones were then screened by PCR for those in which the PGKneo cassette had been deleted. One clone was then selected for further manipulations. It was first re-transfected with the same RECQL5TVN-AAV virus to obtain clones in which two RECQL5 alleles had been targeted. One of these clones was then transfected with the RECQL5TVP-AAV virus to obtain clones in which all three copies of the RECQL5 genes have been targeted. Two such clones, HCT116G2 and HCT116E6, were selected for subsequent experiments. For RECQL5 targeting in DLD1 cells, RECQL5TVN-AAV virus was first used to obtain clones with one targeted RECQL5 allele. Then one of these clones was then transfected with the RECQL5TVP-AAV virus vector to obtain clones in which both copies of the RECQL5 gene had been targeted. One such clone, DLD1A1, was selected for further study. Finally, putative RECQL5-deficient clones were analysed by Western blotting to determine their status of RECQL5 protein expression.

**Western blot analysis**

Proteins were isolated using RIPA buffer supplemented with protease inhibitor mixture (Roche Applied Sciences). Western blot analysis was performed using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology). Polyclonal rabbit anti-RECQL5 antibodies were produced against a recombinant polypeptide corresponding to amino acid 661–880 of human RECQL5β [15]. Polyclonal rabbit r-tubulin antibody was obtained from a commercial vendor (Cell Signaling Technology).

**Cell viability test and clonogenic survival assay**

For each experiment, a defined number of cells were plated on to 10-cm plates and allowed to recover for 24 h. Cells were treated with various concentrations of CPT (Sigma–Aldrich) for 16 h. For the flow-sorting experiment, cells were allowed to recover for various times in drug-free medium and then processed for flow sorting as described [13]. For the clonogenic survival experiment, the cells were allowed to grow for 8 days after the CPT treatment. Next, the plates were stained with Methylene Blue for 4 h and the numbers of colonies with more than 50 cells in each plate were determined. A set of untreated plates were included as controls to determine the plating efficiency for individual cell lines. The effective number of cells/plate was determined on the basis of the plating efficiency of each cell line and the number of cells plated per plate. These numbers were then used to calculate the percentage survival. Also, each treatment was performed in triplicates in order to determine the S.D. for each data point.

**Cell cycle analysis and flow cytometry**

A standard propidium iodide staining approach was used to analyse the cell cycle profile. Briefly, cells were seeded and treated with CPT (10 nM for HCT116 and HCT116G2 and 20 nM for DLD1 and DLD1A1 respectively) for 16 h. At different time points after the treatment, cells were harvested and fixed with 80% ethanol at −20 °C, washed with PBS, and resuspended in the staining solution (50 μg/ml PI, Sigma; 200 μg/ml RNase A, Roche, Indianapolis, IN, U.S.A.) for flow analysis. All the flow cytometry data were collected using a Counter EPICS XL-MCL Cytometer (Beckman Coulter, Fullerton, CA, U.S.A.).

**Xenograft tumours in nude mice**

The experimental procedure was approved by the Institutional Animal Care and Use Committee of Case Western Reserve University and the experiment was carried out at the Athymic Core Facility at Case Western Reserve University School of Medicine as described previously [16]. Briefly, RECQL5-proficient or -deficient HCT116 tumour cells (5 × 10³) were injected into the right or left flanks of individual female BALB/c athymic nude mice (6–8 weeks of age). The tumours developed after injection were measured with calipers using the equation: tumour volume (V) = (L × W²)/2, in which L is the largest diameter and W is the diameter that is perpendicular to the largest diameter of the tumour. Those with tumour nodules of approx. 100 mm³ were selected and assigned randomly to control (n = 6) or treatment groups (n = 6). Individual animals in the treatment groups were injected once every other day with CPT at 4 mg/kg body weight in a 200 μl of saline solution; while those of the control groups were injected with 200 μl of saline. Tumour measurements were taken prior to each drug treatment (every other day). In the meantime, the mice were also photographed.

**RESULTS**

We have shown previously that Recql5-knockout mouse ES cells were hypersensitive to CPT [11], raising the possibility that RECQL5, the human homologue of mouse Recql5, may have a similar role in CPT resistance in human cells. As irinotecan, a CPT derivative, is currently used for treating several human cancers, we were particularly interested to investigate whether RECQL5 is an important determinant in their resistance to Topo I inhibitors in human colorectal cancer cells. To address this question, we decided to generate RECQL5 knockout colorectal cancer cells by gene targeting and then compare their CPT sensitivity with their isogenic counterparts. Standard AAV-based somatic cell targeting technology [14] was first used to delete the RECQL5 gene from the genome of HCT116, a colorectal carcinoma cell line with a relatively stable karyotype and hence is a preferable choice for the genetic manipulation experiment. As HCT116 cells possess a total of three copies of RECQL5 genes [17], three consecutive rounds of gene-targeting experiments were performed to mutate all three copies of RECQL5
three experiments. Also, for both (C) and (D), each data point represents the mean from
of the relative frequency of the sub-G1 population in untreated cells or in
in HCT116G2 cells at 72 h after the CPT treatment. (B) Quantification
are shown on the
x-axis. Note a prominent peak of sub-G1 population
in HCT116G2 cells at 72 h after the CPT treatment. (B) Quantification of the relative frequency of the sub-G1 population in untreated cells or in the
treated cells at 72 h after the treatment shown in (A). (C, D) Results
of clonogenic survival experiments with CPT and cisplatin respectively.
The y- and x-axis in (C) indicate percentages of cell survival and drug
concentrations respectively. Note that the y-axis is a logarithmic scale. Also,
also, for both (C) and (D), each data point represents the mean from
three experiments.

genes (Figure 1A). The clones with the desirable targeting events
were identified by PCR-genotyping (Figure 1A–1D). After three
rounds of targeting, RECQL5 null clones, e.g., RECQL5G2, were
obtained (Figures 1D and 1E). A similar targeting procedure was
carried out to generate the RECQL5 null clone DLD1A1 using
another colon cancer cell line DLD1, which has two copies of the
RECQL5 gene (Figure 1F). The fact that we were able to generate
RECQL5 null cells indicates that RECQL5 is not essential for
cell viability in these two cell lines.

Having obtained these RECQL5 null colon cancer cell lines,
we examined whether these cells exhibit any differences from
their parental lines, which are RECQL5 proficient in response to
CPT, the prototype experimental representative for this class of
anticancer agents [1,7]. First, a flow cytometric analysis was con-
ducted to examine the acute cytotoxic response, i.e. the induction
of the sub-G1 population following CPT treatment. The result of
a preliminary study suggested that growth of the RECQL5-
deficient but not RECQL5-proficient HCT116 cells was sig-
nificantly inhibited by 10 nM CPT (results not shown). Thus, the
response to this particular treatment was examined in detail. We
found that a 16-h CPT treatment resulted in an effective arrest of
both wild-type and mutant HCT116 cells at the late S/G2 phase.
Moreover, in neither case was the arrest was accompanied by a

significant increase in the sub-G1 populations (Figure 2A, 0 h),
demonstrating that the treatment did not cause a significant im-
mediate cell death response, which is consistent with previous
reports [13,18]. Yet, at 72 h after removal of CPT, the mutant,
but not the wild-type control, exhibited a significant increase in
the sub-G1 population (Figure 2A, 72 h and Figure 2B), sug-
gesting increased cell death in these CPT-treated mutant cells.

Further independently derived RECQL5-deficient HCT116 cell
line also exhibited a similar phenotype (results not shown). A
clonogenic survival experiment was also used to access the role
of RECQL5 in CPT tolerance. The result of this experiment also
showed that RECQL5-deficient HCT116 cells were significantly
more sensitive to CPT than their RECQL5-proficient counterpart
(Figure 2C). Thus, these data indicate that RECQL5 plays an
important role in CPT resistance in HCT116 cells.

We have previously shown that Recq5 deletion in mouse ES
cells resulted in a unique hypersensitivity to CPT, but not many
other DNA-damaging agents, including cisplatin [13]. Thus, we
next examined the sensitivity of HCT116 and its RECQL5 null
derivative to cisplatin and found that these two types of cells
exhibited a similar sensitivity to this particular type of drug (Figure
2D), suggesting that RECQL5 also has a unique role in CPT
tolerance in human colon cancer cells and possibly in most human
cells.

We then asked whether RECQL5 also played an important
role in CPT tolerance in DLD1, another human colorectal cancer

Figure 2 Effect of RECQL5 deletion on CPT tolerance in HCT116
cells
(A) DNA content profiles of cells without any treatment (NT) or with
treatment of 10 nM CPT for 16 h followed by 0 or 72 h of growth after
drug release. For each plot, the y-axis represents the number of events,
while x-axis indicates the DNA content of individual events. Also, the
relative positions of the 2n (G1) and 4n (G2/M) peaks of DNA content
are shown on the x-axis. Note a prominent peak of sub-G1 population
in HCT116G2 cells at 72 h after the CPT treatment. (B) Quantification
of the relative frequency of the sub-G1 population in untreated cells or in
the treated cells at 72 h after the treatment shown in (A). (C, D) Results
of clonogenic survival experiments with CPT and cisplatin respectively.
The y- and x-axis in (C) indicate percentages of cell survival and drug
concentrations respectively. Note that the y-axis is a logarithmic scale. Also,
also, for both (C) and (D), each data point represents the mean from
three experiments.

Figure 3 Effect of RECQL5 deletion on CPT tolerance in DLD1
cells
(A) DNA content profiles of cells without any treatment (NT) or with
treatment of 20 nM CPT for 16 h followed by 0 or 72 h of growth after
drug release. For each plot, the y-axis represents the number of events,
while x-axis indicates DNA content of individual events. Also, the relative
positions of the 2n (G1) and 4n (G2/M) peaks of DNA content are shown
on the x-axis. (B) Quantification of the relative frequency of the sub-G1
population in untreated cells or in the treated cells at 72 h after the treatment shown in (A).

RECQL5 and camptothecin tolerance

Figure 4 Effect of RECQL5 deletion on the response of HCT116-derived xenograft tumours to a CPT-based treatment

(A, C) Photographs of a representative nude mouse at various time points (indicated on top of each photo) after injection with HCT116 cells (on the right flank) and RECQL5-deficient HCT116G2 cells (on the left flank) either with (C) or without (A) the CPT treatment. Note that day 1 was the first day when the tumour-bearing mice were selected and separated randomly into the control and treatment groups. This was also the day of the first CPT injection. Note in (C) the disappearance of RECQL5-deficient tumour (on the left flank) at 15 days after the treatment had been initiated. (B, D) Growth curves of xenograft tumours derived from HCT116 (RECQL5+/+) or HCT116G2 (RECQL5−/−) either with (D) or without (B) the CPT treatment. The y- and x-axis indicate tumour volume (mm³) and time (days) after the tumour-bearing mice have been selected as in (A, C) respectively. In both cases, each data point of tumour volume represents the mean value from six animals.

sensitivity to CPT suggests that RECQL5 may be an important determinant in the tolerance to CPT and its derivatives and therefore the expression status of this protein may also affect the response to anticancer treatments that use one of these CPT derivatives. To begin to address this important issue, we examined whether RECQL5 deletion also affects the effectiveness of CPT-based anticancer treatment in a xenograft tumour model. Specifically, we implanted subcutaneously RECQL5-deficient and -proficient HCT116 cells on the right and left side of individual nude mice and examined their response to a CPT-based chemotherapy, which involves intraperitoneal injection of 4 mg/kg CPT once every other day for 2 weeks. First, we found that, in the absence of any interventions, implants derived from RECQL5-proficient and -deficient HCT116 cells developed into visible xenograft tumours with similar characteristics (Figures 4A and 4B), indicating that the deficiency of RECQL5 did not significantly affect the tumorigenic potential of HCT116 cells under this specific xenograft condition. Remarkably, however, when a similar set of such mice were treated with CPT, the tumours derived from RECQL5-deficient cells, but not those from their RECQL5-proficient counterpart, became progressively smaller. Surprisingly, all RECQL5-deficient tumours eventually disappeared after the 2-week CPT treatment (Figures 4C and 4D). These data clearly demonstrate that RECQL5 also has an important role in CPT tolerance under a simulated preclinical setting.

Meanwhile, we also noticed that, following the CPT treatment, the tumours that were derived from RECQL5-deficient HCT116 cells in fact exhibited an initial growth before their progressive regression (Figures 4C and 4D). This initial growth likely reflects a delay in the cytotoxic effect on the tumours. Importantly, this initial growth before its subsequent regression in individual tumour has enabled the visual observation of shrinkage of individual tumour following the treatment and suggested that a curative outcome has been achieved. Yet, this important issue could not be addressed by this particular experiment because, in this experimental setting, mice also carried tumours derived from RECQL5-proficient HCT116 cells, which upon the discontinuation of the CPT treatment grew rapidly and these mice had to be killed (results not shown). Thus, a new set of experiments was carried out in which individual mice (n = 6) were implanted with RECQL5-deficient HCT116 cells only and the tumour-bearing mice were subjected to the same treatment as before. We found that, once again, the RECQL5-deficient tumours also disappeared after the 2-week CPT treatment (results not shown). More importantly, these mice were able to recover. They were then allowed to live for another 6 months without any additional interventions. No tumours were ever observed again in these mice. Thus, it appears that under this simulating preclinical condition, the progressions of the RECQL5-proficient tumours were inhibited by the treatment. But the same treatment led to the complete elimination of all RECQL5-deficient tumours.

DISCUSSION

The modest response rates of irinotecan and topotecan as single agents have severely limited their clinical use. Meanwhile, these agents have demonstrated great potential in various combinat-
oral therapies. Thus, understanding the molecular basis of drug tolerance for this unique class of anticancer drugs could enable the identification of biomarkers that can be used to guide patients selection and/or the rational design of multi-agent therapy.

Mechanisms that are involved in mitigating the challenge imposed by CPT, i.e. CPT-induced DNA damage, are the natural candidates for conferring tolerance to this drug. Historically, it has been well established that CPT induces DNA DSBs (double-stranded breaks). Thus, HR (homologous recombination)-mediated DSB repair should provide an important mechanism of CPT tolerance. Indeed, cells that are defective in HR, for example, Brca2-deficient cells are hypersensitive to CPT [19]. Thus, defective HR in some human cancers could be a contributing factor to their favourable response to treatments with CPT derivatives. Yet, to date, there has not been any conclusive evidence that a defective HR in any given cancer types is correlated with more favourable responses to treatments involving either irinotecan or topotecan.

In the meantime, CPT is also well known for its unique S-phase-specific cytotoxicity. We have shown recently that, in CPT-treated mouse cells, a novel Recql5-dependent mechanism acts prior to the formation of DSBs, i.e. the collapse of replication forks, to prevent formation of DSBs and promote cell survival. We have shown that this Recql5-dependent mechanism functioned in parallel with HR to provide optimal protection from CPT-induced toxicity [13]. This novel mechanism can potentially explain the CPT tolerance in human cells. Here, we provide experimental evidence to demonstrate that a similar RECQL5-dependent mechanism of CPT tolerance does exist in human colorectal cancer cells. Conceivably, defects in this mechanism, as well as factors thereof, in some human cancers may constitute the molecular basis for their favourable response to CPT derivatives such as irinotecan or topotecan.

We show that deletion of RECQL5 in two different colorectal cancer cell lines both resulted in a dramatic increase in CPT sensitivity. Importantly, our study demonstrated that RECQL5 has a significant role in cell survival in response to low doses of CPT that are not cytotoxic to RECQL5-proficient cells. Therefore, this mechanism is expected to be relevant under the conditions of clinical treatments in which the effective drug concentrations are expected to have little or no adverse effect on the non-cancer compartments of the patients. Remarkably, we show that a treatment with a low dose of CPT that had only a modest growth inhibition effect on HCT116-derived tumours resulted in a curative outcome on the corresponding RECQL5-deficient tumours. This finding has underscored the potential significance of the RECQL5-dependent mechanism in the response to treatments that are based on CPT and its derivatives. Importantly, irinotecan-based therapies are currently used to treat advanced colorectal cancer. Thus, the demonstration that RECQL5 deficiency could greatly enhance the CPT sensitivity of two independent colorectal cancer cell lines suggests that colorectal cancers that are deficient in this protein, if there exists any, could also be more responsive to irinotecan-based treatments. Therefore, RECQL5 and/or other factors involved in this novel RECQL5-dependent pathway, represent potential candidates for useful classifier for treatments that are based on irinotecan, or perhaps other CPT derivatives.

The underlining molecular basis for this unique feature of RECQL5 is not yet clear. However, the RECQL5-deficient cells created in this study could serve as important reagents to interrogate this novel RECQL5-dependent mechanism as well as its relationship to other drug tolerance mechanisms in the genetic hierarchy of DNA damage response and repair network. Such information could prove invaluable in the rational design of multi-agent anticancer therapies involving CPT derivatives, a strategy that has already shown promising results in a number of studies that use irinotecan-based multi-agent therapies for treating colorectal cancers [8–10].

Alternatively, inhibition of RECQL5 function may offer a means for enhancing the cytotoxic effect of CPT and its derivatives. That is, agents that inhibit RECQL5 may function as sensitizer for CPT and its derivatives. Interestingly, deletion of Recql5 in mice did not have any major adverse effect on the embryonic development or postnatal life of the animals [12]. Since mouse Recql5 and human RECQL5 are functionally conserved, it is reasonable to predict that inhibition of RECQL5 in humans may not have any major adverse effects either. The cell lines derived from the present study could be very useful in the identification of such RECQL5-inhibiting agents.

AUTHOR CONTRIBUTION
Xiaqi Wang generated most of the experimental data for this paper. Xincheng Lu initiated this study and generated important preliminary data. Guangjin Zhou provided anti-RECQL5 antibodies for this work. Hua Lou played an important role in the overall design of the study, the analysis of the data and the preparation of the paper. Guangbin Luo oversaw the overall design and execution of this project and wrote the paper.

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