Circadian Clock Gene CRY2 Degradation Is Involved in Chemoresistance of Colorectal Cancer

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Abstract

Biomarkers for predicting chemotherapy response are important to the treatment of colorectal cancer patients. Cryptochrome 2 (CRY2) is a circadian clock protein involved in cell cycle, but the biologic consequences of this activity in cancer are poorly understood. We set up biochemical and cell biology analyses to analyze CRY2 expression and chemoresistance. Here, we report that CRY2 is overexpressed in chemoresistant colorectal cancer samples, and CRY2 overexpression is correlated with poor patient survival. Knockdown of CRY2 increased colorectal cancer sensitivity to oxaliplatin in colorectal cancer cells. We also identify FBXW7 as a novel E3 ubiquitin ligase for targeting CRY2 through proteasomal degradation. Mechanistic studies show that CRY2 is regulated by FBXW7, in which FBXW7 binds directly to phosphorylated Thr300 of CRY2. Furthermore, FBXW7 expression leads to degradation of CRY2 through enhancing CRY2 ubiquitination and accelerating the CRY2’s turnover rate. High FBXW7 expression downregulates CRY2 and increases colorectal cancer cells’ sensitivity to chemotherapy. Low FBXW7 expression is correlated with high CRY2 expression in colorectal cancer patient samples. Also, low FBXW7 expression is correlated with poor patient survival. Taken together, our findings indicate that the upregulation of CRY2 caused by downregulation of FBXW7 may be a novel prognostic biomarker and may represent a new therapeutic target in colorectal cancer. Mol Cancer Ther; 14(6); 1–12. ©2015 AACR.

Introduction

Colorectal cancer is one of the leading causes of cancer mortality. 5-Fluorouracil (5-FU)–based chemotherapy is routinely used to treat those patients who are at high risk of developing recurrence or those with advanced or metastatic disease. As a consequence of early diagnosis, prevention, and adjuvant chemotherapy, death rate of colorectal cancer has decreased by 40% in the past four decades (1). However, a significant proportion of patients receiving chemotherapy become chemoresistant (2). Therefore, understanding the mechanisms underlying chemoresistance can help us to identify a subgroup of patients who may benefit from chemotherapy and avoid overtreatment. Studies have shown that multiple cellular processes, including DNA repair, cell apoptosis, and proliferation, may play important role in chemoresistance (3–6). Several clinical studies have been performed in an attempt to find biomarkers that predict the benefit from chemotherapy. For example, BRAFV600E mutations confer poor prognosis, but limited data suggest lack of antitumor activity from anti-EGFR monoclonal antibodies in the presence of a BRAF mutation status (7–9). However, except KRAS/NRAS-activating mutations on exon2 and other RAS mutations (10–12), only few of the studied markers have an impact on the clinical management of colorectal cancer so far. There is an urgent need for discovering better markers that can enhance the prognostic strength.

Circadian clock is an endogenous biochemical mechanism shared by most organisms, which can influence nearly all aspects of physiology and behavior. Deregression of circadian rhythm has been found to accelerate malignant growth and increase the risk of certain kinds of cancer, such as breast cancer, prostate cancer, and colorectal cancer, implying the involvement of the circadian clock in cancer development and tumor progression (13–19). Circadian genes are also related to the clinical outcome of cancer patients (14, 18). Several studies have shown that anticancer drugs were more efficacious at a certain circadian time, indicating that chronotherapy, a circadian-based chemotherapy, may be considered with patient treatment (20–23). It has been suggested that prevention of chemotherapy-induced circadian disruption might reduce toxicity and improve efficacy in cancer patients (22). Cryptochrome 2 (CRY2), one of the identified circadian clock proteins, has been shown to be involved in DNA-damage checkpoint control and regulating important cell-cycle progression genes (24, 25). In addition, CRY mutation can increase the cell sensitivity to apoptosis induced by genotoxic agents and also protect p53-mutant mice from the early onset of cancer (26). Also, breast cancer cells with reduced CRY2 have accumulated greater mutagen-induced DNA damage (15, 26, 27). These studies indicated that CRY2 might correlate with DNA damage and chemotherapeutic outcome of patients. Understanding these molecular alterations can help improve clinical care.

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F-box and WD repeat domain-containing 7 or FBW7) is a component of conserved SCF (complex of SKP1, CUL1, and F-box protein)-type ubiquitin ligase (28, 29). SCFFBXW7 is known to degrade several proto-oncogenes that function in cellular growth and division pathways, including cyclin E (30), Aurora B (31), Aurora A (32), MYC (33, 34), JUN (35, 36), and Notch (37). Loss-of-function of tumor-suppressor FBXW7 increased prosurvival protein MCL1 levels and conferred resistance to Taxol-induced cell death, which indicated that FBXW7 is also involved in drug resistance (38). Interestingly, the ubiquitin-mediated protein degradation pathway plays an essential role in maintaining normal circadian clock function (39, 40). It is not clear whether FBXW7 and CRY2 are involved in chemoresistance.

In this study, we found that CRY2 was overexpressed in chemoresistant colorectal cancer patient samples. We have shown that FBXW7 negatively regulated CRY2 via the ubiquitination pathway and sensitized colorectal cancer cells to chemotheraphy. Low FBXW7 expression was correlated with CRY2 overexpression in colorectal cancer patient samples. Our studies provide important insight into the signal deregulation of the FBXW7–CRY2 axis in the chemosensitivity of colon cancer and help elucidate CRY2’s role as a therapeutic intervention target in colorectal cancer treatment.

Materials and Methods

Human tissue

First, 16 patients with stage III primary rectal cancer who consecutively underwent neoadjuvant chemotherapy (fluorouracil–leucovorin–oxaliplatin) were randomly selected from the Biobank of the Department of Colorectal Surgery, the Sixth Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China). Tumor specimens were obtained by colonoscopy prior to neoadjuvant therapy. The effect of chemotherapy on the tumor was assessed as the three-dimensional volume reduction rate or tumor response rate. The tumor response was evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST), which is defined as the following: complete response (CR; disappearance of the disease), partial response (PR; reduction of ≥30%), stable disease (SD; reduction <30% or enlargement ≤20%), or progressive disease (PD; enlargement ≥20%). Of these, 8 patients were defined as CR/PR and others were defined as SD/PD. Paraffin-embedded samples of primary colorectal adenocarcinomas were included from 289 patients from the First Affiliated Hospital of Sun Yat-Sen University. Our study protocol was approved by the ethics committee. Overall survival was the endpoint of this study. Survival time was calculated from the date of surgery to the date of death or the last follow-up time. Written informed consent from all patients regarding tissue sampling was obtained.

TMA construction and immunohistochemistry

Tissue microarrays were constructed; in brief, paraffin-embedded tissue blocks were overlaid with corresponding slides of hematoxylin and eosin–stained tissue sections for tissue microarray sampling. Duplicate 0.6-mm-diameter cylinders were punched from representative tumor areas of an individual donor tissue block and reembedded into a recipient paraffin block at predefined positions using a tissue-embedding instrument (Beecher Instruments). Immunohistochemical (IHC) staining was performed on 5-μm tissue sections. The sections were placed on poly-lysine–coated slides, deparaffinized in xylene, and then rehydrated using graded ethanol. The slides were then placed in 3% hydrogen peroxide to quench for endogenous peroxidase and then processed for antigen retrieval by microwave heating for 10 minutes in 10 mmol/L citrate buffer (pH 6.0). The primary antibody against CRY2 (1:1,000; Abcam) or FBXW7 (1:1,000; Invitrogen) was diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and incubated at 4°C overnight. The next day, immunostaining was performed using the Invitrogen SuperPicture 3rd Gen IHC Detection Kit, which resulted in a brown precipitate at the antigen site. Then, the slides were counterstained with hematoxylin (Zymed Laboratories), mounted in nonaqueous mounting medium, and coverslipped. The original IHC slides were scanned with the Advanced CCD Imaging Spectrometer that captured digital images of the immunostained slides. The ACIS calculates a score for regions selected by the pathologist. The receiver operating characteristic (ROC) curve was used to define the cutoff point.

Data mining

The CRY2 gene mRNA expressions in colon tissue were obtained by the Gene Expression Omnibus. Cohort: GSE-14333 consisting of 223 colon cancer patients. DNA constructs and reagents

PCR-amplified human CRY2 was cloned into PCMV5 or PDEST15. CRY2 T300A mutant was made by the Herculase II Fusion Enzyme Kit (Agilent Technologies). siRNA oligos were designed and manufactured by Ruibo, targeting CRY2, sense: 5′-GAACGAAUUGGACAGAUUU-3′. Flag-FBXW7 has been previously described (31). Cycloheximide and MG132 were obtained from Sigma. Ni-NTA Agarose was obtained from Invitrogen (#R901-15). Antibodies used were: Flag (M2 monoclonal antibody; Sigma; F3165), CRY2 (Abcam), and myc (Santa Cruz Biotechnology; sc-40).

Cell culture and transfection

The colon cancer cell lines DLD-1 and SW480 were purchased from the ATCC (authenticated by short-tandem repeat analysis). Cells were maintained in DMEM/F12 (Gibco), supplemented with 10% (v/v) fetal calf serum (FCS). HCT116 FBXW7+/+ and HCT116 FBXW7−/− (a kind gift from Dr. Bert Vogelstein, The Johns Hopkins Medical Institutions, Baltimore, MD; authenticated with Western blot analysis routinely) were cultured in McCoy’s 5A media (Hyclone) supplemented with 10% (v/v) FCS. All cells were incubated in a humidified atmosphere of 5% CO2 at 37°C. Plasmids and CRY2 siRNA were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were harvested 48 hours after transfection to assay for transfection efficiency by qPCR.

RNA isolation and quantitation

RNA samples were isolated from harvested cells using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. A total of 1 μg of RNA from each sample was reversed transcribed using oligo-dT primers and the ReverAid First Strand cDNA Synthesis Kit (Fermentas, ELI). Quantitative real-time PCR was performed using the Thunderbird SYBR qPCR Mix (TOYOBO) in the Applied Biosystems 7500 Real-Time PCR system (ABI). The primers used for CRY2 amplification were: forward: GTCCT-GGATGTTTACGCTT; reverse: CCACACAGGAGGACGAGAT. mRNA quantity was normalized using GAPDH content, and
fold change of expression was calculated according to the ΔΔC_{T} method.

Cell proliferation assay

Cell proliferation was determined using the MIT assay as previously described (41). Briefly, 5 × 10^3 cells per well were plated into 96-well plates, incubated at 37°C, 5% CO_{2} overnight. After transfection, cells were treated with different concentrations of fluorouracil or oxaliplatin (Sigma) for 72 hours. Cells were incubated in medium containing 0.1 mg/mL MIT (Sigma) for an additional 4 hours. The cells were then lysed in 150-μL DMSO (Sigma). Cell viability was determined by measuring the absorbance at 550 nm using a 680 BioRad Microplate Absorbance Reader (Bio-Rad).

Annexin V/PI staining

The cell was seeded on 96-well plates at a density of 10^5 cells per well and cultured overnight. Cells were transfected with 100 mmol/L negative control or CRY2 siRNAs. After 48 hours of treatment with oxaliplatin, cells were collected and washed twice with PBS by spinning at 1,000 rpm for 10 minutes. Cell pellets were resuspended in a FITC-labeled Annexin V and propidium iodide (PI) staining solution (BD Bioscience) and incubated for 15 minutes at room temperature. The samples were then analyzed on a FACSCalibur (FACSCanton II; BD).

TUNEL assay

Apoptosis was determined by the terminal deoxynucleotidyl transferase--mediated dUTP nick end labeling (TUNEL) method using the TMR Red kit (Roche) according to the manufacturer’s protocol. Briefly, cells were rinsed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Cells were then incubated in TUNEL reaction mixture at 37°C for 60 minutes, protected from light. After three rinses with PBS, the samples were analyzed under a fluorescence microscope (Leica) and emission wavelength of 580 nm. The number of cells was determined by DAPI staining. At least 200 cells were counted for each experiment.

Immunoprecipitation and immunoblotting

Total cell lysates were processed as described previously (42–44). Cell lysates for Western blot analysis or immunoprecipitation were collected from tissue culture dishes after two rinses with cold PBS. Cells were centrifuged at low speed for 10 minutes and supernatants were discarded. Pellets were then lysed with 200-μL 1× lysis buffer [0.5 L batch: 7.5-g 1 mol/L Tris (Fisher), 15-mL 5 mol/L NaCl (Fisher), 0.5-mL NP-40 (USB Corp.), 0.5-mL Triton X-100 (Sigma), and 1-mL 0.5 mol/L EDTA (Fisher)] for 20 minutes at 4°C. Lysis buffer also contained a cocktail of protease/phosphatase inhibitors: 5 mmol/L NaV, 1 mmol/L NaF, 1 mmol/L dithiothreitol (DTT), 0.1 mg/mL Pepstatin A, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), and 1,000× Complete Cocktail Protease Inhibitor (Roche). Lysates were immunoblotted with indicated antibodies. For immunoprecipitations, cell lysates were prepared and standardized as before, and 1-mg protein was immunoprecipitated with appropriately diluted antibody in lysis buffer overnight. Antibody was pulled down with 50 μL of either Protein A or G beads (Santa Cruz Biotechnology) for 1 hour. Beads were centrifuged at low speed for 10 minutes, and supernatants were discarded. Dried beads were mixed with 2× loading dye and boiled for 5 minutes. Lysate samples were loaded onto gels, and SDS-PAGE was performed as before.

In vitro binding assay

Flag-FBXW7 and myc-CRY2 were prepared by in vitro transcription and translation using the TNT coupled system (Promega). TNT products were mixed and immunoprecipitated followed by immunoblotting as described in the legend to Figure 3.

Molecular docking of CRY2–FBXW7 complex structure

The information-driven docking program HADDOCK (version 2.0; ref. 45) was used to generate the Cry2–FBXW7 complex model. The starting structures for docking were an X-ray crystal structure of FBXW7 (PDBID: 2ovp; ref. 30) and the homology model of Cry2. The Cry2 model was constructed using the crystal structure of Cryptochrome-2 from Mus musculus (PDBID: 416g) as the template model, and the modeled residues were from residues 22 to 511. On the basis of the Skp1–FBXW7–Cyclin E peptide complex structure (PDB: 2ovp), specific degron motif (TPXXS) binding to narrow face of the WD40 domain of FBXW7 was used to generate this complex model. The WD40 domain of FBXW7 structure contained sets that included the active residues W425, T439, S462, T463, R465, R479, Y519, Y545, and S585, and the neighbors of these active residues were selected as passive residues. The Cry2 model contained sets that included the active residues from R275 to S304, and the neighbors of these active residues were selected as passive residues. The active residues

| Table 1. Correlation between expression of CRY2 and clinicopathologic features in 289 case of colorectal cancer |
|-----------------|-----------------|-----------------|-----------------|
| Gender          | Low expression  | Overexpression  | P               |
| Male            | 147 (50.9)      | 85 (51.5)       | 62 (50.0)       | 0.813*          |
| Female          | 142 (49.1)      | 80 (48.5)       | 62 (50.0)       |                |
| Agea            |                 |                 |                 | 0.073*          |
| <59             | 130 (45.0)      | 82 (49.7)       | 48 (38.7)       |                |
| ≥59             | 159 (55.0)      | 83 (50.3)       | 76 (61.3)       |                |
| Histologic grade |                 |                 |                 | 0.233*          |
| G1              | 20 (6.9)        | 12 (7.3)        | 8 (6.5)         |                |
| G2              | 230 (79.6)      | 126 (76.4)      | 104 (83.9)      |                |
| G3              | 39 (13.5)       | 27 (16.4)       | 12 (9.7)        |                |
| PT status       |                 |                 |                 | 0.111*          |
| T1              | 7 (2.4)         | 7 (4.2)         | 0 (0.0)         |                |
| T2              | 39 (13.5)       | 24 (14.5)       | 15 (12.1)       |                |
| T3              | 237 (82.0)      | 131 (79.4)      | 106 (85.5)      |                |
| T4              | 6 (2.1)         | 3 (1.8)         | 3 (2.4)         |                |
| pN status       |                 |                 |                 | 0.226*          |
| N0              | 173 (59.9)      | 104 (63.0)      | 69 (55.6)       |                |
| N1              | 116 (40.1)      | 61 (37.0)       | 55 (44.4)       |                |
| PM status       |                 |                 |                 | 0.246*          |
| M0              | 259 (89.6)      | 151 (91.5)      | 108 (87.1)      |                |
| M1              | 30 (10.4)       | 14 (8.5)        | 16 (12.9)       |                |
| Clinical stage  |                 |                 |                 | 0.430*          |
| I               | 30 (10.4)       | 20 (12.1)       | 10 (8.1)        |                |
| II              | 124 (42.9)      | 73 (44.2)       | 51 (41.1)       |                |
| III             | 105 (36.3)      | 58 (35.2)       | 47 (37.9)       |                |
| IV              | 30 (10.4)       | 14 (8.5)        | 16 (12.9)       |                |
| Chemotherapy    |                 |                 |                 | 0.221*          |
| No              | 211 (73.0)      | 136 (75.6)      | 75 (68.8)       |                |
| Yes             | 78 (27.0)       | 44 (24.4)       | 34 (31.2)       |                |

*P values were calculated in SPSS16.0 using a chi-square test. P values <0.05 were considered to indicate statistical significance.

aMedian age.
were chosen on the basis of the Skp1–FBXW7–Cyclin E peptide complex structure (PDB: 2ovp). During the rigid body energy minimization, 10,000 structures were calculated, and the 162 best solutions based on the intermolecular energy were used for the minimization, 10,000 structures were calculated, and the 162 best complex structure (PDB: 2ovp). During the rigid body energy

were pulled down by the Ni-NTA. The protein complexes were

m

50

g/mL of MG132 for 6 hours. The ubiquitinated proteins

were analyzed as previously described (46–48). Then, cycloheximide was added into the media at 60

m

50

g/mL of MG132. At 24 hours after transfection, cells were treated with
described (34, 43). DLD-1 cells were cotransfected with indicated plasmids. At 24 hours after transfection, cells were treated with 50 μg/mL of MG132 for 6 hours. The ubiquitinated proteins were pulled down by the Ni-NTA. The protein complexes were then resolved by SDS-PAGE and probed with indicated antibody to observe the ubiquitinated level.

were transfected with indicated plasmids and incubated in the 37°C with 5% (v/v) CO₂ for 24 hours. Turnover rate was analyzed as previously described (46–48). Then, cycloheximide was added into the media at 60 μg/mL of final concentration. The cells were harvested at the indicated time points after cycloheximide treatment. The protein levels were analyzed by immunoblotting.

UBiquitination assay

were chosen on the basis of the Skp1–FBXW7–Cyclin E peptide complex structure (PDB: 2ovp). During the rigid body energy minimization, 10,000 structures were calculated, and the 162 best solutions based on the intermolecular energy were used for the semiflexible, simulated annealing. The best 162 docked models were clustered a cutoff of 3.5Å with a minimum of four structures in each cluster, which yielded seven clusters. In terms of HADDOCK score and the basis of the FBXW7–CycE peptides complex binding motif, the best structure was selected as a final model of CRY2–FBXW7 complex structure.

Turnover assay

The cells were transfected with indicated plasmids and incubated in the 37°C with 5% (v/v) CO₂ for 24 hours. Turnover rate was analyzed as previously described (46–48). Then, cycloheximide was added into the media at 60 μg/mL of final concentration. The cells were harvested at the indicated time points after cycloheximide treatment. The protein levels were analyzed by immunoblotting.

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Statistical analysis

Statistical analyses were performed using SPSS (standard version 16.0; SPSS). The correlation between CRY2 expression and clinicopathologic features of colorectal cancer patients was analyzed by the χ² test or the Fisher exact test. For univariate survival analysis, survival curves were obtained by the Kaplan–Meier method, and differences between survival curves were assessed with the log-rank test. The Cox proportional hazards regression model was used to identify the independent prognostic factors. All in vitro experiments were performed in triplicate. The results are described as mean ± SD. Statistical analysis was performed by the one-way analysis of variance (ANOVA), and comparisons among groups were performed by the independent-sample t test. Results were considered significant at a P value less than 0.05.

Results

CRY2 expression is correlated with chemoresistance and poor outcome in colorectal cancer patients

To investigate whether CRY2 expression relates to chemosensitivity in colorectal cancer patients, we first compared the expression of CRY2 levels in colorectal cancer colonoscopy samples from 16 rectal cancer patients who subsequently underwent neoadjuvant chemotherapy. IHC revealed that the level of CRY2 expression was higher in cancer tissue samples from stable/progressive patients than in samples from complete/partial response patients (Fig. 1A). Statistical analysis revealed that IHC score between two groups was significantly different (P = 0.001; Fig. 1B).
To determine the clinical relevance, we subjected a tissue microarray containing 289 human colorectal cancer specimens to IHC staining for CRY2. The clinicopathologic characteristics of the colorectal cancer patients are summarized in Table 1. Among the colorectal cancer patients, 9 of 30 metastatic colorectal cancer patients (30%) received the oxaliplatin-based chemotherapy, and 69 of 259 patients (26.7%) without metastasis received the chemotherapy. To assess statistical significance, the ROC curve was plotted to determine cutoff scores for CRY2 expression. We divided the colorectal cancer patients into high and low CRY2 expression groups according to cutoff scores. In the cohort, high expression of CRY2 was found in 124 of 289 (42.9%) of colorectal cancer patients. There was no significant association between CRY2 expression and clinicopathologic features, such as patient gender, age, T classification, N classification, distant metastasis, and clinical stage (Table 1). The expressions of CRY2 as well as other clinicopathologic factors were further examined by the multivariate Cox regression analysis. Results indicated that histologic grade, N stage, M stage, and CRY2 overexpression were independent prognostic factors for colorectal cancer patients (Supplementary Table S1). Importantly, the Kaplan-Meier analysis showed that high expression of CRY2 was correlated with poor survival, especially in colorectal cancer patients who received adjuvant chemotherapy (Fig. 1C). Therefore, CRY2 can be identified as a biomarker that can predict outcomes of colorectal cancer patients.

CRY2 knockdown leads to chemosensitivity of colorectal cancer cell lines

To examine further whether CRY2 expression has an impact on chemoresistance of colon cancer cells in vitro, CRY2 was downregulated by specific siRNA in two colorectal cancer cell lines DLD-1 and SW480. We found that DLD-1 and SW480 cells with reduced CRY2 were more sensitive to oxaliplatin compared with control cells (Fig. 2A). Because CRY2 was involved in DNA damage-specific control of cell death, we then examined the contribution of CRY2 to oxaliplatin-induced apoptosis in DLD-1 and SW480 cells. In the presence of 4 μM oxaliplatin, CRY2 depletion significantly increased number of apoptotic cells (P < 0.001; Fig. 2B), as evident by Annexin V staining. Cells were also analyzed for the presence of the cleaved form of poly-(ADP-ribose) polymerase cleavage (PARP), an apoptosis marker. CRY2 knockdown cells showed more PARP cleavage compared with the control cells (Fig. 2C). Conversely, overexpression of CRY2 prevents oxaliplatin-induced PARP cleavage (Fig. 2C). In addition, CRY2 depletion by siRNA resulted in six times more apoptotic cells than the cells transfected with scramble siRNA, as evident by TUNEL staining (Fig. 2D). Taken together, these results indicate that CRY2 expression leads to chemoresistance.

FBXW7 negatively regulates CRY2

Because FBXW7 can regulate the chemosensitivity of cancer cells (38), we explored whether the FBXW7 is involved in CRY2-mediated chemosensitivity. CRY2 levels were decreased when cells were transfected with FBXW7 in a dose-dependent manner (Fig. 3A). Given that FBXW7 is involved in the destabilization of CRY2, it is possible that there is an interaction between FBXW7 and CRY2. To investigate this potential interaction, we analyzed cell lysates cotransfected with FBXW7 and CRY2. Indeed, exogenous expressed FBXW7 was able to associate with endogenous CRY2 as assayed by Co-IP (Fig. 3B). In vitro binding assay confirmed the interaction between FBXW7 and CRY2 (Fig. 3B). FBXW7 preferentially binds to target proteins with the degron TPXXS motifs (36). We found that CRY2 contains a consensus sequence (298NSTPLPLSL305) that is highly similar to the FBXW7-binding sites in c-Myc, cyclin E, Notch1, and c-Jun (Fig. 3C). FBXW7-binding site on CRY2 is conserved in different species (Fig. 3C). To determine whether the consensus sequence is critical in mediating the binding between FBXW7 and CRY2, we constructed T300A site mutant of CRY2 and assessed the binding by immunoprecipitation and immunoblotting. The result showed that CRY2 T300A mutant reduced its binding to FBXW7 (Fig. 3C), suggesting that T300 of CRY2 is important site for mediating FBXW7 binding.

To gain insight into the structural basis of CRY2 recognition by FBXW7, we generated a model of the CRY2–FBXW7 complex using HADDOCK docking protocol (45). Briefly, the crystal structures of WD40 domain of FBXW7 and CRY2 were used for modeling the protein–protein complex. We defined structure-based degron motif (ϕ-X-ϕ-ϕ-ϕT/S-P-X-pS/T, with ϕ representing a hydrophobic residue and X any amino acid) as active bases in CRY2. Residues 298–305 (comprising degron motif) are acid positions shared by degron motif in cyclin E, Notch-1, c-Myc, and c-Jun (Fig. 3C). Structures of the FBXW7–CycE peptide complexes show that the CycE peptide binds to the narrow face of the WD40 domain of FBXW7 extending from blades six and seven at one side of the surface across the central channel. After the HADDOCK docking protocol similar to FBXW7–CycE peptide complexes, we have shown the best structure interactions between CRY2 and WD40 domain of FBXW7 (Fig. 3D). The degron motif of CRY2 residues 298–305 is docked to the narrow face of WD40 domain of FBXW7 (Fig. 3D). We also plotted the intermolecule H-bonds and hydrophobic interactions observed in the
Figure 3.
FBXW7 interacts with CRY2. A, FBXW7 negatively regulates the steady-state expression of CRY2. DLD-1 cells were transfected with indicated plasmids and increasing amounts of FBXW7. Equal amounts of cell lysates were immunoblotted with the indicated antibodies. B, interaction of FBXW7 with CRY2. Equal amounts of DLD-1 cell lysates transfected with Flag-FBXW7 were immunoprecipitated with anti-CRY2 and immunoblotted with anti-Flag. FBXW7 interacts with CRY2 in vitro. Flag-FBXW7 and Myc-CRY2 cDNAs were transcribed and translated in vitro (TNT). FBXW7 and CRY2 proteins were incubated overnight and immunoprecipitated with anti-Myc followed by immunoblotting with anti-Flag. (Continued on the following page.)
binding interface (Fig. 3D). The degron motif of CRY2 binds to the narrow face WD40 domain of FBXW7. Residues T300 and P301 (in the TPXX5 motif) of CRY2 make hydrogen bonds with R465 and R479 of FBXW7 (Fig. 3D). Besides the interactions involving in the TPXX5 motif, some additional interactions are also observed from this CRY2–FBXW7 complex model. Some electrostatic interactions are made between the charged residues R178, K183, K184, and K296 of CRY2 and the charged residues D381, D399, D440, and R689 of FBXW7 (Fig. 3D). This complex structural model reveals the interactions between CRY2 and WD40 domain of FBXW7.

FBXW7 regulates CRY2 via enhancing ubiquitin-mediated degradation

To investigate the impact of FBXW7 on CRY2 stability, we examined the turnover rate of CRY2 in the presence of de novo protein synthesis inhibitor cycloheximide in HCT116 and HCT116 FBXW7−/− cells, and found that HCT116 FBXW7−/− cells had a decelerated turnover rate of endogenous CRY2 (Fig. 4A). On the other hand, we transfected FBXW7 and performed the cycloheximide experiments. The data showed that CRY2 had a faster turnover rate in the presence of FBXW7 (Fig. 4A). FBXW7-mediated CRY2 downregulation was

(Continued.) C, the consensus FBXW7-binding motif is highlighted. Sequences of CRY2 and other known FBXW7 substrates are shown for comparison (left). Sequence alignment of CRY2 containing FBXW7-binding motifs from different species is shown (right). Equal amounts of cell lysates from DLD-1 cotransfected with WT myc-CRY2 or myc-CRY2-T300A and Flag-FBXW7 plasmids were immunoprecipitated with anti-Flag and immunoblotted with indicated antibodies. D, ribbon presentation of the CRY2–FBXW7 complex model. The loop of CRY2 degron motif (residues T300–S304) interacts with the WD40 domain of FBXW7. Close-up view of the interface in the CRY2–FBXW7 complex. The interaction residues involving in the interface of CRY2–FBXW7 molecules are shown in stick.
suppressed by MG132, a proteasome inhibitor, suggesting the involvement of the 26S proteasome (Fig. 4B). Indeed, we found that FBXW7 increased the ubiquitination level of CRY2 (Fig. 4A). We then identified that CRY2 T300A mutant had a slower turnover rate than WT CRY2 even in the presence of FBXW7 (Fig. 4C). Accordingly, CRY2 T300A mutant is resistant to FBXW7-mediated ubiquitination (Fig. 4D). These results suggest that FBXW7 activity negatively regulates CRY2 stability via enhancing the levels of CRY2 ubiquitination, thereby increasing CRY2 turnover rate.

FBXW7 enhances chemosensitivity of colorectal cancer cell line

We examined whether FBXW7 expression had an impact on chemoresistance of colon cancer cells and found that DLD-1 cells with overexpressed FBXW7 were more sensitive to oxaliplatin compared with control cells (Supplementary Fig. S1A). Accordingly, overexpressed FBXW7 resulted in more oxaliplatin-induced PARP cleavage than the control cells (Fig. 5A). As expected, FBXW7 overexpression led to downregulation of CRY2 during this process (Fig. 5A). Another line of evidence showed that in the presence of 4 μmol/L oxaliplatin, overexpressed FBXW7 significantly potentiated the drug-mediated apoptosis, as evident by Annexin V staining (Fig. 5B and Supplementary Fig. S1B). These data suggest that the FBXW7–CRY2 axis is critical for chemosensitivity.

Low FBXW7 expression correlates with high CRY2 expression in colorectal cancer and manifests poor survival

To determine the clinical relevance, we subjected a tissue microarray containing 289 human colorectal cancer specimens to IHC staining for CRY2 and FBXW7. The colorectal cancer samples had high CRY2 expression, which correlated with low FBXW7 expression (representative case 1). Accordingly,
colorectal cancer samples with low CRY2 expression had high FBXW7 expression (representative case 2; Fig. 5C). The CRY2 and FBXW7 expression levels were reversely correlated with each other (Table 2). Importantly, the Kaplan–Meier analysis showed that high expression of CRY2 and low expression of FBXW7 were correlated with poor survival (Supplementary Fig. S1C). These results strongly suggest that the FBXW7–CRY2 axis is deregulated during the development of human colorectal cancer.

Discussion

Adjuvant chemotherapy with 5-FU and oxaliplatin currently remains a standard treatment for patients with advanced colorectal cancer. However, chemotherapy resistance leading to treatment failure and local recurrence is still a critical problem. One of the biggest challenges is to identify the subpopulation of patients who are most likely to respond to a specific therapy. If one or more biomarkers could predict patient response to chemotherapy, one could spare the nonresponders from ineffective treatment and direct them to alternative treatment strategies that could be more effective. There was some evidence that showed that the circadian clock can modulate drug metabolism and efficacy of antitumor therapy. Patient response to chemotherapy was closely correlated to the functional status of the circadian genes. However, chronotherapeutic approach has not become routine in clinical practice, perhaps in part, because of the lack of a clear mechanistic basis (49). Noticeably, we found that circadian clock protein CRY2 is overexpressed in chemoresistant colorectal cancer and that FBXW7 has unprecedented biologic activity in downregulating CRY2 to mediate chemosensitivity; this demonstrates that circadian clock proteins may play an important role in colorectal cancer chemosensitivity.

There has been evidence that the circadian clock could modulate multidrug resistance genes, thereby playing important roles in chemoresistance (50), as mdrla gene can be affected by the circadian clockwork. Also, overexpression of the circadian clock gene Bmal1 can increase sensitivity to oxaliplatin in colorectal cancer (51). These findings suggest that dysregulation of the circadian clock may be associated with drug sensitivity. Paradoxically, we have shown that high expression of CRY2 was significantly associated with poor chemo-therapeutic outcomes in colorectal cancer patients who received standard adjuvant chemotherapy. It is possible that CRY2 expression is involved in DNA-damage response, thereby affecting the chemosensitivity. It was reported that mutation of Cry could protect p53-mutant mice from early onset of cancer and extend their survival time after ionizing radiation (26), suggesting that Cry may be involved in p53-mediated DNA-damage response. However, there was no study regarding the correlation between CRY2 expression and p53 in patients in terms of clinical outcome. Interestingly, our in vitro study did show that repression of CRY2 in DLD-1 and SW480 cells (both are p53 mutant) leads to decreased cell viability and increased apoptosis in the presence of oxaliplatin, thereby resulting in an increased sensitivity to chemotherapy drugs. Thus, previous findings and current study indicate that CRY2 expression level and p53 status might be factors in predicting chemoresistance in colorectal cancer patients.

Ubiquitination-mediated degradation of clock proteins has been highlighted as a key regulatory process in clockwork. The FBXL3 and FBXL21 are critical for generating the CRY expression dynamics and behavior rhythms (39, 40, 52). Both CRY1 and CRY2 repressor proteins are regulated by ubiquitin-mediated degradation for temporally orchestrating the transcription of clock genes. Our studies have shown that FBXW7 can bind to and destabilize CRY2 through enhancing ubiquitin-mediated degradation of CRY2. FBXW7 is a tumor-suppressor (53) protein through recognizing and degrading several oncoproteins such as cyclinE, c-JUN, Aurora B, and MYC (33, 35, 54). Mutant FBXW7 or downregulation of FBXW7 causes tumorigenesis as it fails to decrease target oncoproteins (37, 55). Although FBXW7 has a critical function in cancer regulation, very few circadian clock proteins are the known targets of FBXW7. Giving that three F-Box proteins are all involved in CRY2 ubiquitination, the relationship between FBXW7, FBXL3 and FBXL21 in terms of regulating CRY2 stability certainly warrants further investigation. Nonetheless, it makes sense that a tumor-suppressor protein FBXW7 is involved in degrading CRY2 when CRY2’s overexpression leads to poor clinical outcome. Indeed, we found that CRY2 overexpression is quite common in chemoresistant colorectal cancer. CRY2 overexpression was negatively correlated with FBXW7 protein expression in human colon cancer samples. These findings demonstrate that FBXW7 downregulation can, at least partially, account for CRY2 overexpression in colon cancer and provide important insights into the mechanisms underlying CRY2 overexpression in chemoresistant colorectal cancer. The inverse relationship between FBXW7 and CRY2 is demonstrated for the first time in cancer.

In summary, our mechanistic studies explain the regulatory relationship among CRY2, FBXW7, and chemosensitivity (Fig. 5D). That CRY2 overexpression in chemoresistant colorectal cancer raises the possibility that inhibiting the CRY2 is an efficient therapeutic approach in overcoming chemoresistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: L. Fang, L. Wang, J. Wang, M.-H. Lee
Development of methodology: L. Fang, Z. Yang, M.-H. Lee
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Fang, Z. Yang, J. Zhou, Y. Deng, M.-H. Lee
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Fang, J.-Y. Tung, C.-D. Hsiao, M.-H. Lee
Writing, review, and/or revision of the manuscript: L. Fang, Z. Yang, J.-Y. Tung, C.-D. Hsiao, M.-H. Lee
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Fang, Z. Yang, L. Wang, P. Wang, M.-H. Lee
Study supervision: L. Fang, J. Wang, M.-H. Lee
Other (used computational molecular docking analysis methods to interpret the interactions between Crys and Bw7): J.-Y. Tung

Table 2. Correlation between CRY2 and FBXW7 expression in colorectal cancer patient samples (P < 0.001)

<table>
<thead>
<tr>
<th>CRY2 FBXW7</th>
<th>Low FBXW7</th>
<th>High FBXW7</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low CRY2</td>
<td>48 (17%)</td>
<td>86 (30%)</td>
<td>134 (46%)</td>
</tr>
<tr>
<td>High CRY2</td>
<td>117 (40%)</td>
<td>38 (13%)</td>
<td>155 (54%)</td>
</tr>
<tr>
<td>Total</td>
<td>165 (57%)</td>
<td>124 (43%)</td>
<td>289 (100%)</td>
</tr>
</tbody>
</table>

NOTE: Fisher exact test.
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