Overexpression of the circadian clock gene Bmal1 increases sensitivity to oxaliplatin in colorectal cancer

Zhao-lei Zeng\textsuperscript{1,2,§}, Hui-yan Luo\textsuperscript{1,3,§}, Jing Yang\textsuperscript{1,2,§}, Wen-jing Wu\textsuperscript{1,3}, Dong-liang Chen\textsuperscript{1,3}, Peng Huang\textsuperscript{1,2}, Rui-hua Xu\textsuperscript{1,3,*}

\textsuperscript{1}State Key Laboratory of Oncology in Southern China, \textsuperscript{2}Department of Experimental Research and \textsuperscript{3}Department of Medical Oncology, Cancer Center, Sun Yat-sen University, Guangzhou, Guangdong, China.

Running title: Bmal1 increases oxaliplatin sensitivity in colorectal cancer

Keywords: Bmal1; Oxaliplatin; Colorectal cancer; Circadian clock gene.

§These authors contributed equally to this work.

This work was supported by the Natural Science Foundation of China (No. 81000888); the National High Technology Research and Development Program of China (863 Program) (No.2012AA02A506); the Science and Technology Department of Guangdong Province, China (No. 2012B031800088) and the Medical Scientific Research Foundation of Guangdong Province, China (No. C2011019).

*Corresponding author:
Rui-hua Xu, M.D., Ph.D.
State Key Laboratory of Oncology in Southern China, Departments of Medical Oncology, Cancer Center, Sun Yat-sen University, Guangzhou 510060, China. Phone: +86(20)87343228, Fax: +86(20)87343392. E-mail: xurh@sysucc.org.cn.

The authors declare no conflicts of interest.

The total word count of the text (including the title, abstract and figure legends) is 4783.
Translational relevance

The circadian clock gene Bmal1 controls cellular sensitivity to DNA damage and may play an important role in cancer proliferation. Here, we show that Bmal1 inhibits colorectal cancer cell proliferation and that Bmal1 overexpression increases the sensitivity of colorectal cancer cells to oxaliplatin in vitro and in vivo. Furthermore, Bmal1 protein expression correlates with the outcome of advanced colorectal cancer patients receiving oxaliplatin-based first-line chemotherapy. Moreover, the effect of Bmal1 was mechanistically associated with its ability to regulate cell cycle arrest in G2/M phase by activating the ATM signaling pathway. In summary, these findings suggest that Bmal1 overexpression increases cellular sensitivity to oxaliplatin. Bmal1 may represent a novel prognostic biomarker as well as a new therapeutic target in colorectal cancer.
Abstract

Purpose: The circadian clock gene Bmal1 is involved in cancer cell proliferation and DNA damage sensitivity. The aim of this study was to explore the effect of Bmal1 on oxaliplatin sensitivity and to determine its clinical significance in colorectal cancer (CRC).

Experimental Design: Three CRC cell lines, HCT116, THC8307 and HT29, were used. The Bmal1-mediated control of CRC cell proliferation was tested in vitro and in vivo. MTT and colony formation assays were performed to determine the sensitivity of CRC cells to oxaliplatin. Flow cytometry was used to examine changes in the cell cycle distribution and apoptosis rate. Proteins expressed downstream of Bmal1 upon its overexpression were determined by Western blotting. Immunohistochemistry was used to analyze Bmal1 expression in 82 archived CRC tumors from patients treated with oxaliplatin-based regimens.

Results: Bmal1 overexpression inhibited CRC cell proliferation and increased CRC sensitivity to oxaliplatin in three CRC cell lines and HCT116 cells model in vivo. Furthermore, the overall survival of CRC patients with high Bmal1 levels in their primary tumors was significantly longer than that of patients with low Bmal1 levels (27 vs. 19 months; \( p = 0.043 \)). The progression free survival of patients with high Bmal1 expression was also significantly longer than that of patients with low Bmal1 expression (11 vs. 5 months; \( p = 0.015 \)). Mechanistically, the effect of Bmal1 was associated with its ability to regulate G2/M arrest by activating the ATM pathway.

Conclusion: Bmal1 shows the potential as a novel prognostic biomarker and may represent a new therapeutic target in CRC.
Introduction

The circadian rhythm is a biochemical and biophysical process that has an approximately 24-hour cycle and is driven by the endogenous clock system (1). The hierarchically organized network of the circadian clocks regulate various physiological processes in the human body, including blood pressure, hormone secretion, sleep and immune activity (2). Deregulation of circadian rhythms can result in increased risk of cardiovascular diseases, immune system diseases and cancer (3, 4).

The Bmal1 gene, encodes the Bmal1 protein, is a core component of the circadian clock in mammals (5). Bmal1 belongs to the bHLH-PAS structural domain transcription factor family, which controls the circadian genes Per, Cry and Rev-Erbα and maintains the normal circadian rhythm (6, 7). In addition to its function in controlling biological rhythm, Bmal1 is closely related to aging, angiocardiopathy, immune disorders and cancers (8-12). Recent evidence suggests that Bmal1 is correlated with proliferation and the cell cycle, indicating that Bmal1 may play an important role in tumorigenesis (11, 13-15). For instance, Bmal1 has been shown to affect the cell cycle and cell proliferation by regulating the p53/p21 signaling pathway (14, 15). Epigenetic inactivation of Bmal1 is associated with the development of B-cell lymphoma and certain types of leukemia, whereas the reintroduction of Bmal1 inhibits of cancer cell growth in vitro and in vivo (11).

Colorectal cancer (CRC) is the third most common cancer diagnosed in both men and women, and is the third leading cause of cancer-related deaths in the United States according to the American Cancer Society in 2013. Over 1 million new cases of CRC are clinically diagnosed each year, and more than 500,000 patients die from CRC annually (16). More than 20% of CRC cases are diagnosed at a late stage, and this percentage is higher in developing countries (17). Thus, many patients experience recurrence after colorectal resection.

Oxaliplatin, a platinum-based, antineoplastic agent, is used in combination with
5-fluorouracil as the standard first-line chemotherapy for CRC (18, 19). The timing of oxaliplatin administration affects its toxicity and efficacy. For example, when compared with constant-rate administration, the administration of oxaliplatin, 5-fluorouracil and leucovorin using a programmable infusion pump confers a 5-fold improvement in patient tolerability and a nearly 2-fold increase in antitumor activity in patients with advanced CRC (20). This could be due in part to the fact that the circadian system controls drug metabolism and the expression of drug targets as well as cell cycle progression, DNA repair and apoptosis (21, 22). Disturbance of the circadian rhythms results in accelerated tumor growth in mice and poor survival in CRC patients undergoing oxaliplatin-based chemotherapy (23-25).

Bmal1-deficient fibroblasts show reduced sensitivity to DNA-damaging anticancer drugs, such as etoposide and daunorubicin (8). However, the clinical significance of Bmal1 in CRC remains largely unknown, and the relationship between Bmal1 and oxaliplatin is unclear. Furthermore, the mechanism by which Bmal1 affects the sensitivity of cancer cells to DNA-damaging anticancer drugs requires further study.

In this study, we provide evidence suggesting that Bmal1 might inhibit the proliferation of CRC cells and that Bmal1 upregulation could enhance the sensitivity of CRC cells to oxaliplatin in vitro and in vivo. Furthermore, we show that Bmal1 protein expression correlates with the outcome of oxaliplatin-based first-line chemotherapy in advanced CRC patients. Moreover, the effect of Bmal1 appears to be mechanistically associated with its ability to regulate G2/M phase arrest by activating the ataxia telangiectasia mutated (ATM) signaling pathway.
Materials and Methods

Cell lines and cell culture

The HCT116 and HT29 CRC cell lines were purchased from ATCC (Manassas, VA, USA) in 2009, and cultured in McCoy's 5A Medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA). The THC8307 cell line was a gift from the Institute of Hematology (CAMS & PUMC, Tianjin, China) in 2010, and was cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) containing 10% FBS. All cell lines were tested and authenticated by Beijing Microread Gene Tech. Co., Ltd. (Beijing, China) by short tandem repeat DNA testing in 2012. Cells were seeded in 6-well plates (NEST, Shanghai, China) at an initial density of 5-8×10^5 cells/well. The next day, they were treated with 50% fetal bovine serum for 2 h for synchronization. After synchronization, some of the cells were harvested for RNA and protein extraction, and others were reseeded in 6-well or 96-well for cell growth, colony formation, MTT and flow cytometry analysis.

Xenografted tumor model

Male BALB/c nude mice (4-5 weeks of age, 18-20 g) were purchased from the Guangdong Province Laboratory Animal Center (Guangzhou, China). All mice were synchronized with a 12-h light/dark cycle in an autonomous chronobiological animal facility (Sujing, Suzhou, China), with the lights on from 6 am (Zeitgeber time 0) to 6 pm (Zeitgeber time 12) for 3 weeks, after which they were randomly assigned to groups. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. Two in vivo experiments were designed. In experiment 1, the mice were randomly divided into the HCT116_EV, HCT116_Bmal1, HCT116_EV+L-OHP and HCT116_Bmal1+L-OHP groups (n=6 per group), and equal amounts of HCT116_Bmal1 or control cells (2×10^6) were injected subcutaneously into the flank of each mouse. In experiment 2, the mice were also
randomly divided into the above-mentioned 4 groups (n=5 per group), and different amounts of HCT116_Bmal1 (3×10^6) or control (2×10^6) cells were injected subcutaneously into the flank of each mouse. Oxaliplatin was administered at a dose of 5 mg/kg at 10 am twice a week for 3 weeks. Treatment began on day 7, when the tumors were measurable. The tumors were examined twice weekly; length and width measurements were obtained with calipers, and the tumor volumes were calculated. On day 25, the animals were euthanized, and the tumors were excised and weighed.

**Patients and samples**

Eighty-two patients with histopathologically and clinically diagnosed metastatic CRC were enrolled in our study from 2000 to 2007. All patients received FOLFOX (fluorouracil, leucovorin and oxaliplatin) or XELOX (capecitabine and oxaliplatin) regimens as first-line chemotherapy at the Sun Yat-sen University Cancer Center (Guangzhou, China). Prior to chemotherapy primary tumor samples were obtained during colonoscopy biopsies or palliative operations and embedded in paraffin. The clinical and clinicopathological classification and stage were determined according to the American Joint Committee on Cancer (AJCC) criteria. Prior to using these clinical materials for research purposes, patient consent and approval from the Institutional Research Ethics Committee were obtained.

Overall survival (OS) was defined as the interval between the date that first-line chemotherapy treatment was started and the date of death or the last known follow-up. Progression-free survival (PFS) was defined as the interval between the date that first-line chemotherapy treatment was started and the date of disease progression.

The criteria for inclusion were as follows: age >18 years; histologically confirmed CRC with at least 1 measurable lesion as defined by the Response Evaluation Criteria in Solid Tumors; Eastern Cooperative Oncology Group performance status (ECOG PS) of B2, life expectancy >3 months. Other criteria also included: adequate hematologic (neutrophil count: }
1,500/L, platelet count: 100,000/L), hepatic (bilirubin: 1.5 times the upper limit of normal, SGOT and SGPT: 2.5 times the upper limit of normal) and renal (creatinine clearance: 30 mL/min) functions. No previous chemotherapy for the target lesions was allowed; however, patients who had received adjuvant/neoadjuvant chemotherapy were included if the interval after the end of chemotherapy was > 12 months. Patients were excluded based on the following criteria: unresolved bowel obstruction or malabsorption syndrome, central nervous system metastases, bone metastases as the only disease site, active uncontrolled infection, other malignancies in the past 5 years, uncontrolled diabetes mellitus, a history of neurological or psychiatric disorders or congestive heart failure.

For the XELOX regimen, oxaliplatin (130 mg/m²) was diluted in 500 mL of 5% dextrose and infused intravenously over 2 h on day 1; this treatment was followed by oral administration of capecitabine (1,000 mg/m²) twice a day from days 2-15. This cycle was repeated every 21 days. For the FOLFOX regiment, oxaliplatin (85 mg/m²) was given intravenously with 5-FU/LV (400 mg/m² LV, equivalent to 200 mg/m² L-folinic acid), by infusion on day 1, followed by a bolus of 400 mg/m² 5-FU and a 46-h continuous infusion of 2,400 mg/m² 5-FU. This therapy was repeated every 2 weeks for a minimum of 6 cycles. Oxaliplatin was administered at approximately 10 am and over a period of >2 h. In mild allergic cases, the oxaliplatin infusion time was extended to 6 h. Treatment was continued until 8 cycles of XELOX or 12 cycles of FOLFOX had been completed, disease progression, unacceptable toxicity, patient withdrawal, or physician’s decision. The target lesions were measured by computed tomography or magnetic resonance imaging within 1 month prior to study entry. Tumor assessments were repeated every six weeks during therapy and at the end.
of treatment.

Vectors and siRNA transfection

A Bmal1 expression construct was generated by sub-cloning PCR-amplified full-length human Bmal1 cDNA into the pMSCV plasmid. Recombinant retroviral vectors were generated and viral infection was performed as previously described (26). Stable cell lines expressing Bmal1 were selected over 10 days with 2 μg/mL puromycin. To verify the stable high expression of Bmal1 in these cell lines, western blotting was performed after 30 generations of cell culture (Suppl. Fig. 1). For Bmal1 knockdown, we used the following siRNA sequences: GAACTTCTAGGCACATCGT (siRNA#1) and GGGAAGCTCACAGTCAGAT (siRNA#2) (synthesized by Ribobio, Guangzhou, China). Transfection of the Bmal1 siRNA was carried out using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions and the cells were harvested 72h post-transfection for western blotting to verify and quantify the knockdown of Bmal1.

Immunohistochemistry (IHC)

IHC and the scoring of the Bmal1 staining intensity were performed as previously described (27-29). The Bmal1 antibody (1:200; Lifespan, Seattle WA, USA) was incubated with the CRC tissue sections at 4°C overnight. Two independent observers who were blinded to the clinical characteristics and outcomes of the patients assessed the IHC staining based on the proportion of positively stained tumor cells. The proportion of the stained cells and the extent of the staining were used as the evaluation criteria. The stained sections were evaluated at 200× magnification, and ten representative fields from each section were analyzed to determine the percentage of positively stained tumor cells. For each sample, the proportion of Bmal1-expressing cells varied from 0% to 100%, and the intensity of staining varied from weak to strong. The percentage of Bmal1-positive cells was scored as follows: \( \leq 10\% \) =
0, >10% to ≤ 25% = 1, >25% to ≤ 50% = 2, >50% to ≤ 75% = 3 and >75% = 4. The staining intensity was scored as follows: negative = 0, weak = 1, moderate = 2 and strong = 3. A final score was then calculated by multiplying these two scores. If the final score was ≤ 4, the tumor was considered to have low Bmal1 expression; whereas scores >4 indicated high Bmal1 expression.

**Western blotting**

Western blotting was performed according to standard methods, as described previously (30), using anti-Bmal1 (Lifespan, Seattle WA, USA), anti-ATM, anti-CHK2, anti-pCHK2\(^{\text{Thr68}}\), anti-p53, anti-MDM2 and anti-Wee1 antibodies (Cell Signaling, Danvers, MA, USA). An anti-α-tubulin mouse monoclonal antibody (Sigma, Saint Louis, MO, USA) was used as a loading control.

**MTT assay**

Cells were seeded in 96-well plates (NEST, Shanghai, China) at an initial density of 2×10\(^3\) cells/well and incubated with oxaliplatin for 72 h. Then, the cells were stained with 20 μL sterile MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) dye (5 mg/mL; Sigma, Saint Louis, MO, USA) for 4 h at 37°C followed by removal of the culture medium and the addition of 200 μL of dimethyl sulfoxide (Sigma, Saint Louis, MO, USA). The absorbance was measured at 570 nm, and 630 nm was used as the reference wavelength. All experiments were conducted in triplicate.

**Colony formation assays**

Cells were plated in 6-well plates (NEST, Shanghai, China) at 5×10\(^2\) cells/well and cultured for 10 days. The colonies were fixed with 10% formaldehyde for 5 min and then stained with 1% crystal violet for 30 sec.

**Flow cytometry**

Cells were harvested and washed with cold PBS, and the cell cycle distribution and apoptosis
rate were analyzed by flow cytometry. The protocol used for the cell cycle analysis was previously described (31), and $5 \times 10^4$ cells were analyzed using a Cytomics™ FC 500 instrument (Beckman Coulter, USA) equipped with CXP software. ModiFit LT 3.1 trial cell cycle analysis software was used to determine the percentage of cells in each phase of the cell cycle.

For apoptosis analysis, after washing with cold PBS, the cells were stained with annexinV-FITC and PI (Keygen, Nanjing, China), according to the manufacturer’s instructions.

**Statistical analysis**

All statistical analyses were performed using the SPSS version 16.0 statistical software packages. Statistically significant differences between groups were determined using a 2-tailed, paired Student’s t-test. Survival curves were plotted using the Kaplan-Meier method, and the different groups were compared using the log-rank test. A $p$-value of $< 0.05$ was considered to be statistically significant in all cases.
Results

Bmal1 inhibits CRC cell proliferation

To evaluate the role of Bmal1 in CRC cell proliferation, three CRC cell lines were chosen: HCT116 and THC8307, with low Bmal1 expression; and HT29, with high Bmal1 expression (Suppl. Fig. 2). We established the three cells stably expressing ectopic Bmal1, with Bmal1 expression more than twice as high as compared to control cell lines (Fig. 1A and Suppl. Fig. 3A). A siRNA specific for Bmal1 was transfected to knock down the expression of Bmal1 in HCT116, THC8307 and HT29 cells with more than 70% inhibition of Bmal1 expression (Fig. 1B and Suppl. Fig. 3B). A significant decrease in cell proliferation was observed in Bmal1-overexpressing cells compared with the vector control cells (Fig. 1C). These results were confirmed by colony formation assays, which showed a more than 45% decrease in the number of cell colonies in the Bmal1-overexpressing cells compared with control cells (Fig. 1D).

To further explore the significance of the proliferative function of Bmal1 in CRC cells, HCT116, THC8307 and HT29 cells were transiently transfected with specific siRNAs against Bmal1. Knockdown of Bmal1 significantly increased cell proliferation compared with control siRNA-transfected cells (Fig. 1C).

Overexpression of Bmal1 increases oxaliplatin sensitivity in vitro

To explore the effect of Bmal1 on the activity of the anticancer agent oxaliplatin in CRC cells, MTT and colony formation assays were performed in HCT116, THC8307 and HT29 cells. Cells overexpressing Bmal1 were more sensitive to oxaliplatin than cells expressing the control vector (Fig. 2A). The IC50s values for oxaliplatin in the Bmal1-overexpressing cells and the vector control cells were 1.17±0.04 and 3.94±0.28 μM, respectively, for the HCT116 cells; 2.17±0.05 and 9.77±0.89 μM, respectively, for the THC8307 cells; 6.37±0.66 and 15.93±2.19 μM, respectively, for the HT29 cells. Conversely, the Bmal1-knockdown cells
were more resistant to oxaliplatin (Fig. 2B). The IC50s values for the cells transfected with Bmal1 siRNA#1, Bmal1 siRNA#2 and the siRNA control were 9.16±1.04, 11.18±1.42 and 3.22±0.41 μM, respectively, for the HCT116 cells; 28.43±2.38, 32.11±3.12 and 8.91±0.74 μM, respectively, for the THC8307 cells; and 28.61±2.12, 34.44±3.14 and 14.82±1.52 μM, respectively, for the HT29 cells. These results were confirmed by colony formation assays using Bmal1-overexpressing cells (Fig. 2C and 2D).

**Overexpression of Bmal1 increases oxaliplatin sensitivity in vivo**

To further investigate whether Bmal1 overexpression affects oxaliplatin sensitivity in vivo, unsynchronized, log-phase Bmal1-overexpressing or control HCT116 cells were injected subcutaneously into Balb/c nude mice. To exclude differences in the cell proliferation rate between these cell lines, two experiments were designed: in experiment I, equal amounts of Bmal1-overexpressing or control cells (2×10^6) were injected subcutaneously; in experiment II, different amounts of Bmal1-overexpressing (3×10^6) or control (2×10^6) cells were injected. The tumors derived from the HCT116 cells overexpressing Bmal1 were significantly more sensitive to oxaliplatin compared with the HCT116 vector control tumors (p<0.001). In experiment I, the oxaliplatin-mediated growth inhibition rates of the Bmal1-overexpressing tumors and the control tumors were 81.5% and 39.6%, respectively (Fig. 3A); in experiment II, the oxaliplatin-mediated growth inhibition rates of the Bmal1-overexpressing tumors and the control tumors were 73.6% and 38.2%, respectively (Fig. 3B).

**Bmal1 protein expression correlates with the outcome of oxaliplatin-based first line chemotherapy in advanced CRC patients**

To further confirm the effect of Bmal1 expression on oxaliplatin activity, IHC staining for Bmal1 was performed on tumor tissues from 82 advanced CRC patients treated with FOLFOX or XELOX regimens. According to our scoring system, high Bmal1 expression was detected in 36/82 (43.9%) of the colorectal carcinomas, whereas low Bmal1 expression was
detected in 46/82 (56.1%) of the colorectal carcinomas. Kaplan-Meier survival curves and log-rank test survival analysis revealed that the OS of patients with high tumor Bmal1 expression was significantly longer than that of patients with low Bmal1 expression (median OS: 27 months vs 19 months, respectively; \( p = 0.043 \)). Strikingly, the PFS of patients with high Bmal1 expression was also dramatically longer than those with low Bmal1 expression (median PFS: 11 months vs 5 months, respectively; \( p = 0.015 \)) (Fig. 3C).

Based on the above observations, we reasoned that high Bmal1 expression might correlate with good clinical response to oxaliplatin-based chemotherapy and better survival. Thus, we retrospectively analyzed the relationship between Bmal1 expression and the response to FOLFOX or XELOX chemotherapy. Thirty-one out of 36 (86.1%) of the patients with high Bmal1 expression in their primary tumors benefited (CR+PR+SD) from chemotherapy, whereas only 28/46 (60.9%) of the patients with low Bmal1 expression benefited (Table 1). Furthermore, 31/59 (52.5%) patients who experienced a clinical benefit from chemotherapy expressed high Bmal1 levels, whereas only 5/23 (21.7%) of the PD patients expressed high Bmal1 levels (\( p = 0.012 \)). Moreover, if only the CR and PR patients were assumed to have benefited from treatment, then the results remained in agreement with the above finding (\( p = 0.027 \)). These results are consistent with our finding that Bmal1 overexpression increases oxaliplatin sensitivity \textit{in vitro} and \textit{in vivo}.

**Bmal1 enhances oxaliplatin induced apoptosis in CRC cells**

We further examined whether Bmal1 increase the sensitivity of CRC cells to oxaliplatin by enhancing the rate of apoptosis. An annexinV-PI assay was used to detect cellular apoptosis. Bmal1-overexpressing cells displayed increased cellular apoptosis and necrosis in response to oxaliplatin treatment for 48 h (Suppl. Fig. 4). However, these phenomena were observed at only high oxaliplatin concentrations. Furthermore, the difference in the apoptosis rate between the Bmal1-overexpressing cells and the control cells was not large enough to
explain the significant changes in the response to oxaliplatin treatment that were observed in *vivo* and *in vitro*. Thus, other mechanisms might be involved in this process.

**Bmal1 regulates oxaliplatin-mediated G2/M phase arrest in CRC cells**

To assess cell cycle distribution, flow cytometry was performed on Bmal1-overexpressing and control cells after oxaliplatin treatment. The cells were incubated with oxaliplatin for 6 h and then in drug-free medium for the next 42 h, and the cell cycle distribution was assessed. The Bmal1-overexpressing cells demonstrated a dramatically higher number of cells in G2/M phase compared with the control cells (Fig. 4A-C), suggesting that Bmal1 regulates G2/M phase arrest in response to oxaliplatin.

**Bmal1 is involved in the G2/M checkpoint**

Because Bmal1 regulates G2/M phase arrest in response to oxaliplatin, we hypothesized that Bmal1 is involved in the G2/M checkpoint. The ATM pathway activates DNA damage checkpoint proteins which results in G2/M phase arrest. Therefore, we assessed the expression of ATM, pCHK2^{Thr68}, CHK2, p53, MDM2 and Wee1 by western blotting in HCT116 and THC8307 cell lines. As expected, compared with the control cells, the expression levels of ATM, pCHK2^{Thr68}, p53 and Wee1 were significantly upregulated and MDM2 was downregulated in the Bmal1-overexpressing cells after oxaliplatin treatment (Fig. 5). These results suggest that the ATM pathway plays an important role in the ability of Bmal1 to increase sensitivity to oxaliplatin.
Discussion

In the present study, we found that Bmal1 inhibits the proliferation of CRC cells, and that Bmal1 overexpression increases the sensitivity of CRC to the anticancer drug oxaliplatin \textit{in vitro} and \textit{in vivo}. Furthermore, we demonstrated that Bmal1 protein expression correlates with the outcome of oxaliplatin-based first-line chemotherapy in advanced CRC patients, as patients with high tumor Bmal1 expression more frequently benefited from oxaliplatin-based chemotherapy when compared with patients with low Bmal1 expression. Additionally, we determined that the mechanism underlying the effects of Bmal1 is associated with the ability of Bmal1 to regulate G2/M phase arrest by activating the ATM signaling pathway. To the best of our knowledge, this study is the first demonstrating that Bmal1 is involved in the effect of oxaliplatin on CRC.

The current trend in chemotherapy is personalized medicine, and identifying novel biomarkers and therapeutic targets is a major focus of ongoing research. Herein, we systematically explored the effect of Bmal1 on oxaliplatin sensitivity in CRC in cells, mice and clinical samples. This study is the first to explore the role of Bmal1 in chemotherapy in conjunction with clinical features and prognostic data. We found that patients with high tumor Bmal1 expression levels had better outcomes compared with those with low Bmal1 expression (median OS: 27 months vs. 19 months, respectively). Impressively, the PFS of patients with high Bmal1 expression was also dramatically longer than that of patients with low Bmal1 expression (median PFS: 11 months vs. 5 months, respectively). Thus, our study provides strong evidence that Bmal1 is a potential prognostic biomarker and therapeutic target for treating human CRC.

Few studies have assessed the effect of Bmal1 on anticancer drugs. An earlier report demonstrated that the transactivation status of the Clock/Bmal1 complex correlates with the response to cyclophosphamide in mice (32). Another report showed that BMAL1-deficient
fibroblasts have reduced sensitivity to the DNA damage-inducing anticancer drugs, etoposide and daunorubicin (8). Our previous study also demonstrated that Bmal1 plays a role involved in the effect of cisplatin in mice (33). In the present study, we found that Bmal1 overexpression induces G2/M phase arrest in HCT116 and THC8307 cell lines after oxaliplatin treatment, indicating that the DNA damage-repair signaling pathway is involved in this process. Previous reports have suggested that Bmal1 affects the efficacy of DNA damage-inducing anticancer drugs and that this function is related to the circadian gating control of the cell cycle and cell proliferation (21, 34). The Bmal1/Clock heterodimer negatively controls the cell cycle and cell proliferation by repressing c-myc and p21 and activating p53 and Wee1, which results in DNA damage-induced cell cycle arrest or apoptosis (21, 35, 36). DNA double-strand breaks recruit and activate the ATM (ataxia telangiectasia mutated) protein, which then activates DNA damage checkpoint proteins, such as CHK2 and p53, leading to cell cycle arrest or apoptosis (37). Some clock genes that control the ATM pathway have been reported, including per1, per2 and timeless (38-41). We also demonstrated that the ATM signaling pathway plays an important role in G2/M phase arrest in CRC cells.

Oxaliplatin, a third-generation platinum analogue, functions by forming both inter- and intra-strand cross-links in DNA, thus preventing DNA replication and transcription and resulting in cell death (42). Although our study was a retrospective study in which the sampling time could not be controlled, the chemotherapy timing followed the regimens described in the Materials and Methods section, making our data comparable. Thus, we found that Bmal1 overexpression increases the sensitivity of CRC to oxaliplatin, which is consistent with previous observations indicating that circadian timing can significantly modify drug efficiency and tolerability in tumor models and cancer patients (22, 43). In future studies, shRNA should be used to test the effects of Bmal1 knockdown on tumor growth and drug sensitivity in animals because such experiments will further demonstrate the role of Bmal1 in
oxaliplatin sensitivity in the context of circadian timing.

Some translational studies have explored the relationship between Bmal1 and the clinical features of CRC patients. Previous reports have suggested that Bmal1 expression in CRC tissues is lower than that in matched healthy mucosa (44). Additionally the expression of Bmal1 and Per1 also correlates with liver metastasis and CRC outcomes (45). Our data support the hypothesis that Bmal1 has the potential to be a novel prognostic biomarker and a new therapeutic target in CRC. Future studies should focus on collecting blood samples at a specific time of the day to avoid complications due to the influence of circadian rhythms.

Our experiments suggest that Bmal1 inhibits the proliferation of CRC cells in vitro and in vivo. Our findings are consistent with those of multiple studies demonstrating the role of Bmal1 in the suppression of cell proliferation, indicating that Bmal1 is a potential tumor suppressor gene (13-15). In contrast to our findings, another recent study reported that Bmal1 promotes proliferation in malignant pleural mesothelioma (46). Based on this difference, we suspect that Bmal1 might play different roles in different tumor types, which further highlights the complicated mechanisms involved in tumorigenesis.

In conclusion, our results suggest that Bmal1 plays an important role in determining the sensitivity of human CRC to oxaliplatin. A complete understanding of the precise role of Bmal1 in advanced CRC may allow for the use of Bmal1 as a prognostic biomarker for patient response to oxaliplatin-based chemotherapy and aid in the development of novel therapeutic strategies.
Reference


319-26.


Cancer 2002;86: 999-1005.


Table 1. Colorectal cancer patients with high tumor Bmal1 expression are more sensitive to oxaliplatin treatment.

<table>
<thead>
<tr>
<th>Oxaliplatin</th>
<th>Bmal1 expression</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR+PR+SD</td>
<td>31</td>
<td>28</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>5</td>
<td>18</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p=0.012</td>
<td>Total</td>
<td>36</td>
<td>46</td>
<td>82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Response</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR+PR</td>
<td>20</td>
<td>20</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>5</td>
<td>18</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p=0.027</td>
<td>Total</td>
<td>25</td>
<td>38</td>
<td>63</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Bmal1 inhibits colorectal cancer cell proliferation. (A and B) Western blot analysis of Bmal1 protein expression in Bmal1-overexpressing HCT116 (left panel), THC8307 cell lines (middle panel) and HT29 cells (right panel) (A) and Bmal1-silenced HCT116 (left panel), THC8307 cell lines (middle panel) and HT29 cell lines (right panel) cells (B). α-Tubulin was used as a loading control. (C) MTT assays indicate that the growth rate of Bmal1 overexpressing cells was decreased (left panel) and that of the Bmal1-silenced cells was increased (right panel). (D) Colony formation assays indicated that the growth rate of the Bmal1-overexpressing cells was decreased.

Figure 2. Bmal1 increases cellular sensitivity to oxaliplatin in vitro. (A and B) Concentration-dependent growth inhibition in response to oxaliplatin in Bmal1-overexpressing and control cells (A), and in Bmal1-silenced cells (B). (C) Colony formation efficiency of HCT116, THC8307 and HT29 cells treated with oxaliplatin. Error bars represent the SD of three independent experiments. (D) Quantification of the colony formation efficiency in the three cell lines.

Figure 3. Bmal1 increases cellular sensitivity to oxaliplatin in vivo. (A and B) The same (2×10^6) (A) or different amounts of HCT116_Bmal1 (3×10^6) and control (2×10^6) cells (B) were injected subcutaneously into the flanks of nude mice (left panel). Representative images of tumor growth (left panel), tumor volume growth curves (middle panel) and mean tumor weights (right panel) are shown. (C) Immunohistochemical staining of Bmal1 protein expression in colorectal cancer tissues (left panel). The overall (middle panel) and progression-free survival (right panel) of patients with low and high Bmal1 expression are shown.

Figure 4. Bmal1 overexpression increases G2/M phase arrest in HCT116 (A), THC8307 (B)

and HT29 (C) cells. Prior to detection, the cells were incubated with oxaliplatin for 6 h and then incubated in the drug-free medium for the next 42 h.

Figure 5. Western blot analysis of ATM, pCHK2, CHK2, p53, MDM2 and Wee1 in Bmal1-overexpressing cells.
Figure 1

A

B

C

D

MTT Absorbance (%)

MTT Absorbance (%)

Days

Days

0 1 2 3 4 5

0 1 2 3 4 5

0 100 200 300 400 500

0 100 200 300 400 500

HCT116 EV

HCT116 EV

HCT116 EV

EV Bmal1

EV Bmal1

EV Bmal1

HCT116

HCT116

HCT116

THC3307

THC3307

THC3307

HT29

HT29

HT29

* EV

* Bmal1

Colony Relative Fold

Colony Relative Fold

HCT116 THC3307 HT29
Figure 2

(A) Inhibition (%) of OxaP at different concentrations for different cell lines.

(B) Inhibition (%) of OxaP at different concentrations for different cell lines.

(C) Colony formation assay showing the effect of Bmal1 expression on cell proliferation.

(D) Graph showing the colony formation efficiency under different conditions.
Figure 3

A

![Tumor Volume (cm³)](image1)

B

![Tumor Volume (cm³)](image2)

C

![Cumulative survival analysis](image3)

#
Overexpression of the circadian clock gene Bmal1 increases sensitivity to oxaliplatin in colorectal cancer

Zhao-lei Zeng, Hui-yan Luo, Jing Yang, et al.

*Clin Cancer Res* Published OnlineFirst November 25, 2013.

Updated version: Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-0171

Supplementary Material: Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2013/11/25/1078-0432.CCR-13-0171.DC1

Author Manuscript: Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts: Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions: To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions: To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.