Biochemical and Biophysical Research Communications 450 (2014) 1058–1062

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Silencing NPAS2 promotes cell growth and invasion in DLD-1 cells and correlated with poor prognosis of colorectal cancer

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ARTICLE INFO

Article history:
Received 15 June 2014
Available online 27 June 2014

Keywords:
Circadian gene
NPAS2
Colorectal cancer

ABSTRACT

Emerging evidences show that circadian rhythm disorder is an important factor of tumor initiation and development. Neuronal PAS domain protein2 (NPAS2), which is the largest circadian gene, has been proved to be a novel prognostic biomarker in breast cancer and non-Hodgkin's lymphoma. However, the potential functions of NPAS2 in colorectal cancer are still unknown. In our present study, we detected the mRNA expressions of NPAS2 in 108 CRC patients by RT-PCR, and found that NPAS2 expression was significantly down-regulated in tumor tissues than that in NATs. Clinicopathologic analysis revealed that low expression of NPAS2 was associated with the tumor size, TNM stage and tumor distance metastasis in colorectal cancer ($p < 0.05$). Furthermore, we effectively down-regulated NPAS2 mRNA expression by transfecting RNA interfere fragments into DLD-1 cells, and our results in vitro demonstrated that silencing NPAS2 expression could promote cell proliferation, cell invasion and increase the wound healing ability ($p < 0.05$). However, down-regulating NPAS2 expression did not influence the apoptotic rate in DLD-1 cells ($p > 0.05$). In conclusion, our study suggested that NPAS2, functioned as a potential tumor suppressor gene, could serve as a promising target and potential prognostic indicator for colorectal cancer.

1. Introduction

Colorectal cancer (CRC) is one of the most prevent malignancies leading to the cancer-related death in the world. The progression of CRC is a complex, multi-step and multi-factorial process, in which circadian rhythm disorder is an important factor of tumor initiation and development [1]. Although there is a growing body of evidence that disturbed circadian gene expression is involved in tumor development and tumor progression, studies focused on disruption of circadian rhythm in CRC are still limited [2].

Neuronal PAS domain protein2 (NPAS2), also known as MOP4, is the largest circadian gene located on chromosome 2 at 2q11.2. It is primarily expressed in the mammalian forebrain, as well as several peripheral tissues [3,4]. NPAS2 spans 177 kb, which can encode for a member of the basic helixloop-helix (bHLH)–PAS family of a transcription factors. NPAS2 dimerizes with BMAL1 (another core circadian protein) to control the transcription of two other circadian gene PER and CRY to maintain the biological rhythms [5,6]. Silencing NPAS2 has been demonstrated to cause some disruption of circadian system, such as the pattern of sleep [7,8]. Previous data also showed that NPAS2 could be a putative tumor suppressor gene to be involved in tumor related biologic pathways. For example, NPAS2 could regulate the circadian-related gene PER to suppress the transcription of the oncogene c-Myc [9]. It has been now identified to be a novel prognostic biomarker in breast cancer [10] and non-Hodgkin's lymphoma [11].

Circadian biological rhythm has also been showed to play an important role in diagnosis and prognosis in gastroenterology [12,13]. However, the potential functions of NPAS2 in colorectal cancer are still unknown. In our study, to evaluate the association between NPAS2 gene and CRC, we detected the mRNA expressions of NPAS2 in 108 CRC patients by RT-PCR, and analyzed its potential association with the clinicopathologic features. Moreover, we down-regulated NPAS2 mRNA expression by transfecting RNA interfere fragments into DLD-1 cells, and the effects of NPAS2 on cancer-related parameters, including proliferation, cell cycle, apoptosis, wound healing ability and cell invasion, were discussed.

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http://dx.doi.org/10.1016/j.bbrc.2014.06.104
0006-291X/

2. Materials and methods

2.1. Cell lines and tissues

Human colorectal cancer cells DLD-1 were obtained as a gift from Shanghai Key Laboratory of Gastric Neoplasia, Ruijin Hospital, School of medicine, Shanghai Jiao Tong University. Cells were cultured in RPMI-1640 (Invitrogen, USA) with 10% fetal bovine serum in a humidified incubator at 37 °C and 5% CO_2.

Tumor tissues with the corresponding NATs were got from 108 CRC patients at the Department of General Surgery, the First Affiliated Hospital of Soochow University, from 2004 to 2009. According to the clinicopathological criteria of the UICC, all the patients had an accurate histological diagnosis and underwent surgery without chemotherapy/radiotherapy before operation.

2.2. Quantitative reverse transcription-PCR (QRT-PCR)

A trizol reagent (Invitrogen, USA) was used to extract the total RNA from the tumor tissues, NATs or DLD-1 cells respectively. According to the manufacturer's instruction, the reverse transcription was performed in a 20-μl reaction system. The quantitative measurement of NPAS2 mRNA expression was performed using the 7500 real-time PCR system (Applied Biosystems, USA). The mRNA levels were determined by quantifying the PCR products versus β-actin. The sequences of primer for NPAS2 were ACACCCTTTCAAGACCTTGCC (Sense) and AGGTTCGTCAACTATGCACATTT (Antisense). The PCR conditions involved preliminary denaturation at 95 °C for 10 min to active Taq polymerase, and 40 cycles of 94 °C for 15 s, 57 °C for 30 s and 72 °C for 30 s, followed by a final elongation step at 60 °C for 10 min. All samples were processed in replicate and the NPAS2 mRNA expression was quantified as $1000 \times 2^{-\Delta Ct}$.

2.3. RNAi experiments

Non-specific sequences were used as a negative control (NC), which were GUAUGACAACAGCCUCAAGTT (Sense) and CUUGAGG CUGUUGUCAUACTT (Antisense). The sequences of SiRNA-1# were CCAAGAGAGCUUCUCGAAA (Sense) and UUUCGAGAAGCUCUCUU GG (Antisense); and the sequences of SiRNA-2# were GCUGAUGUU GGAGGCAUUA (Sense) and UAAUGCCUCCAACAUCAGC (Antisense) (Gene Pharma, Shanghai, China). DLD-1 cells and the corresponding transfected cells were divided into four groups: (1) parental DLD-1 group, (2) SiRNA-Con group, (3) SiRNA-1# group, (4) SiRNA-2# group. Firstly, parental DLD-1 cells in the logarithmic phase were seeded into a 6-well plate for 24 h prior to transfection, with 5×10^4 cells each well. Secondly, with the help of Lipofectamine 2000 (Invitrogen, USA), SiRNA-1# fragments, SiRNA-2# fragments or NC were transfected into DLD-1 cells respectively. Finally, QRT-PCR was used to detect the transfection effects of SiRNA-1# and -2# fragments.

2.4. Cell proliferation assays

The cell proliferation activity was assessed for 0, 24, 48, 72 and 96 h using the cell counting kit-8 (CCK-8) (Dojindo, Japan). After 24-h transfection, cells were digested and seeded into 96-well plates. Briefly, 10 μl of CCK-8 was added into each well. Then after incubation for 2 h at 37 °C, the optical density (OD) value of 450 nm was used to measure the activity by the Scan Reader (Labsystems, USA). The number of cells was examined by the relative absorbance at 450 nm, with each sample performed three times.

2.5. Flow cytometric analysis for cell cycle

In order to estimate cell cycle, cells of four groups were detected using the flow cytometry. After 48-h continuous culture, cells were harvested and fixed by 70% ethanol for 12 h at 4 °C. Then single cell suspensions were prepared to stain DNA based on the manufacturer's instructions (Beyotime, China). Cell cycle was measured and analyzed by FACS scan system (Beckman Instruments, USA).

2.6. Immunocytochemical analysis

The parental DLD-1 and the corresponding transfected cells were cultured in PRIM-1640 medium supplemented with 10% fetal bovine serum on cover slides. After continuous culture for 20 h, the slides were fixed in cold 4% paraformaldehyde for 30 min and treated with 1% Triton PBS solution for 10 min. Then PBS 1× solution with 10% FBS was used to block the cells for 45 min at room temperature. Cells of four groups were incubated with a Ki67 antibody (1:100, Santa Cruz Biotechnology, USA). Following PBS 1× washing, sections were incubated for 30 min using the secondary antibody (rabbit anti-mouse IgA–B, Santa Cruz Biotechnology, USA). Finally, the 3,3-diaminobenzine (DAB) was used to visualize the immunoreactive products. Results were carried out by the System Microscope IX71 (Olympus, Japan).

2.7. Tunnel assays

According to the manufacturer's instructions of TUNEL System Kit (Roche, Swiss), the terminal nucleotidyl transferase-mediated nick end labeling assay (TUNEL) was used to detect the programmed cell death. Cells of four groups were cultured on cover slides for 20 h in a humidified incubator at 37 °C and 5% CO_2. Firstly, the slides were fixed by the cold 4% paraformaldehyde for 30 min. Following PBS 1× washing, 3% H_2O_2 methanol solution was used to block the slides for 10 min at 20 °C. Then the slides were treated using the 1% Triton PBS solution for 2 min on ice after PBS washing. Avoiding the light, 50 μl of TUNEL reaction solution were applied to incubate the cells on slides for 60 min at 37 °C. Following PBS 1× washing, the signals of TUNEL were converted using the peroxidase (POD) for 30 min at 37 °C, and the sections were treated with DAB for 3 min at the room temperature. Results were examined by the light System Microscope IX71 (Olympus, Japan).

2.8. Scratch healing assays

For the scratch healing assays, cells were treated with 10 mg/ml mitomycin C (Sigma, USA) for 3 h. After that, cells were wounded using a pipette tip. Then PRIM-1640 with 10% FBS was added. The wound closure was observed for 72 h, with the light System Microscope IX71 (Olympus, Japan). The wound healing ability was calculated, compared to the width of wound closure for 0 h.

2.9. Cell invasion assays

According to the manufacture's recommends, the insert members were coated using the diluted Matrigel (BD Biosciences, USA) in 24-well Transwell Chambers (Corning Company, USA). Briefly, 600 μl of PRIM-1640 with 10% FBS was added to the lower chamber. Then 2×10^4 parental DLD-1 and the corresponding transfected cells in 200 μl FBS-free medium were added into the upper chamber separately. After cultured for 36 h at 37 °C and 5% CO_2, the non-invading cells were removed by PBS 1×. 0.1% Crystal violet (Beyotime, China) was used to stain the insert membranes, and the permeating cells were counted by the invert Microscope IX71 (Olympus, Japan).

2.10. Statistical analysis

All statistical analysis was performed by SPSS17.0 software (SPSS Inc., USA). The data were expressed as the mean ± standard deviation from experiments in replicate. The differences between groups were valued using Student's *t*-test and Chi-square test. $p < 0.05$ means that the differences were statistically significant.

3. Results

3.1. The expression of NPAS2 is down-regulated in CRC tissues

As shown in Fig. 1A, the NPSA2 mRNA level was significantly down-regulated in tumor tissues (120.50 ± 112.17) than that of the corresponding NATS (194.95 ± 178.00) ($p < 0.05$).

3.2. Relationship between NPAS2 expression levels and clinicopathological factors in CRC patients

In order to better understand the clinical relevance of NPAS2 expression in colorectal cancer, 108 CRC patients were classified into two groups, including NPAS2 high-expression group ($n = 54$) and NPAS2 low-expression group ($n = 54$). The relationship between the NPAS2 levels and clinicopathologic features was shown in Table. 1. Our data demonstrated that down-regulated NPAS2 mRNA expression was significantly associated with the tumor size ($p = 0.007$), TNM stage ($p = 0.001$) and distant metastasis ($p = 0.014$). However, no significant differences were detected including the age, sex, tumor location, differentiation, lymphatic metastasis and peritoneal dissemination ($p > 0.05$).

3.3. NPAS2 mRNA is effectively silenced by RNAi experiments

After cells were transfected with SiRNA-Con, SiRNA-1# or -2# for 24 h, we detected the NPAS2 mRNA expressions by QRT-PCR. As shown in Fig. 1B, the NPAS2 expression of SiRNA-1# group (23.43 ± 4.34) and SiRNA-2# group (18.65 ± 8.46) were significantly down-regulated, compared to that of SiRNA-Con group (111.35 ± 12.86) ($p < 0.05$).

Table 1
Relationship between NPAS2 expression level and clinicopathologic variables in 108 CRC patients.

Clinicopathologic parameters	NPAS2 mRNA expression		*p*
	Low ($n = 54$)	High ($n = 54$)	
Age (years)			
<60	23	26	0.562
⩾60	31	28	
Sex			
Male	25	34	0.082
Female	29	20	
Location			
Proximal colon	23	28	0.335
Distal colon and rectum	31	26	
Size			
<50 mm	17	31	0.007
⩾50 mm	37	23	
Differentiation			
Well, moderately	30	23	0.178
Poorly	24	31	
TNM stage			
I/II	15	33	0.001
III/IV	39	21	
Lymphatic metastasis			
Absent	24	29	0.336
Present	30	25	
Distant metastasis			
Absent	30	42	0.014
Present	24	12	
Peritoneal dissemination			
Absent	42	47	0.206
Present	12	7	

3.4. Inhibition of NPAS2 promotes cell growth and cycle progression, but do not influence apoptotic rates in DLD-1 cells

The CCK-8 assay was used to monitor the cell growth activities every 24 h. As shown in Fig. 1C, silencing NPAS2 expression by SiRNA-1# or -2# could significantly promote the cell proliferation at 72 and 96 h, compared to SiRNA-Con group ($p < 0.05$). In addition, immunocytochemical analysis for Ki-67 was also performed to monitor cell proliferation activities. In Fig. 2A–B, the positive rates of Ki-67 DLD-1 cells in SiRNA-1# group (88.24 ± 5.34%) and

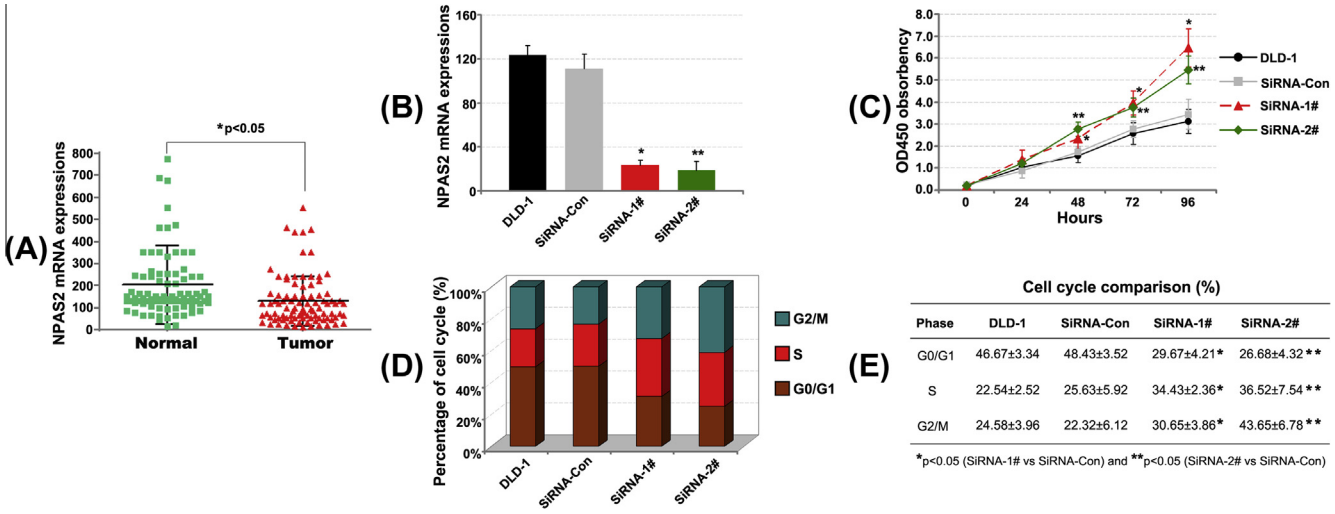

Fig. 1. (A) A relative expression of NPAS2 in colorectal cancer tissue and NATS. NPAS2 mRNA expression was significantly lower in the tumor tissues than that in NATS. (B) NPAS2 mRNA expression was effectively down-regulated by transfecting RNA interfere fragments into DLD-1 cells. (C) CCK-8 assay showed that silencing NPAS2 expression could promote the cell proliferation. (D) The histogram of cell cycle change after NPAS2-SiRNA transfection. (E) The percentage of cell cycle change after NPAS2–SiRNA transfection. (*$p < 0.05$ SiRNA-1# vs SiRNA-Con; **$p < 0.05$ SiRNA-2# vs SiRNA-Con).

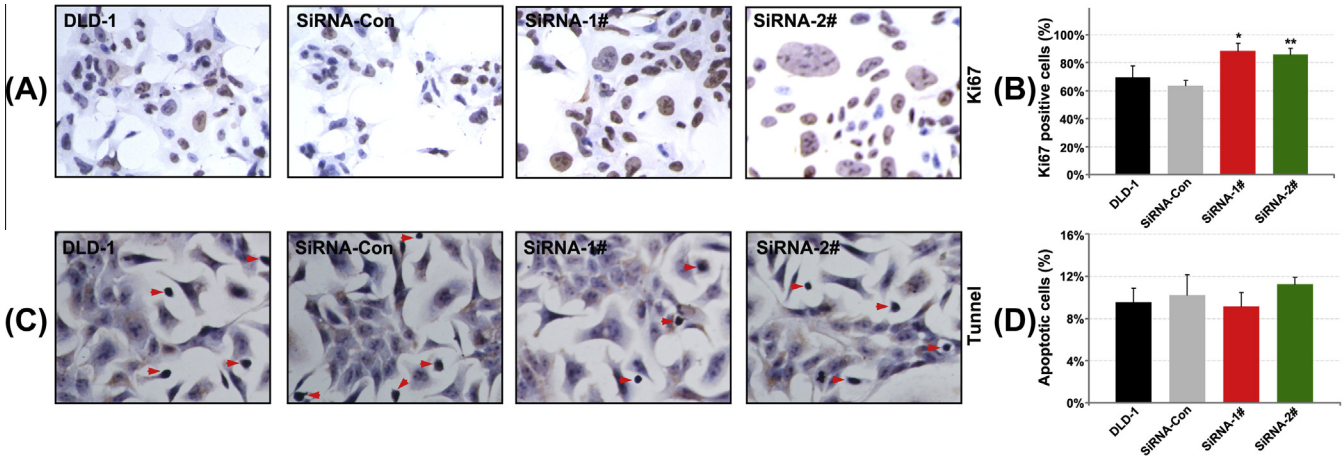

Fig. 2. (A) Immunocytochemical analysis for Ki67 after NPAS2–SiRNA transfection. (B) The changes of the percentages of Ki67 positive DLD-1 cells after NPAS2–SiRNA transfection. (C) TUNEL assay for cell apoptosis after NPAS2–SiRNA transfection. (D) The changes of the percentages of apoptotic DLD-1 cells after NPAS2–SiRNA transfection. (*$p < 0.05$ SiRNA-1# vs SiRNA-Con; **$p < 0.05$ SiRNA-2# vs SiRNA-Con).

SiRNA-2# group (85.71 ± 4.64%) were significantly higher than that of SiRNA-Con group (63.64 ± 3.92%) ($p < 0.05$).

Meanwhile, experiments for cell cycle and apoptosis were also carried out to evaluate the function of NPAS2 in colorectal cancer. As shown in Fig. 1D–E, cells in SiRNA-1# group (S: 34.43 ± 2.36%, G2/M: 30.65 ± 3.86%) or SiRNA-2# inhibitor group (S: 36.52 ± 7.54%, G2/M: 43.65 ± 6.78%) had a higher percentage of S or G2/M phase cells than that of SiRNA-Con group (S: 25.63 ± 5.92%, G2/M: 22.32 ± 6.12%) ($p < 0.05$). On the contrary, the percentage of G0/G1 phase cells by silencing NPAS2 expression in SiRNA-1# group (G0/G1: 29.67 ± 4.21%) or SiRNA-2# group (G0/G1: 26.68 ± 4.32%) was down-regulated, compared to the SiRNA-Con group (G0/G1: 48.43 ± 3.52%) ($p < 0.05$). We performed Tunnel assay to detect the apoptotic rate, and our data showed that there was no difference between SiRNA-1#, -2# group and SiRNA-Con group ($p > 0.05$) (Fig. 2C–D).

3.5. Inhibition of NPAS2 increases the wound healing ability in DLD-1 cells

The wound healing ability was examined using a scratch healing assay. NPAS2 down-expression cells had nearly closed the wound after 72-h, whereas SiRNA-Con cells were unable heal the wound (Fig. 3A). The percentage of wound healing in SiRNA-1# (78.34 ± 2.34%) and -2# group (84.56 ± 6.64%) were significantly higher than that of SiRNA-Con group (51.65 ± 4.42%) ($p < 0.05$) (Fig. 3B).

3.6. Inhibition of NPAS2 increases cell invasion in DLD-1 cells

As shown in Fig. 3C–D, after incubation for 36 h in 24-well plate, the number of invaded cells in SiRNA-1# group (44.85 ± 4.34 cells/field) or -2# group (37.56 ± 5.64 cells/field) was significantly higher than that of SiRNA-Con group (15.32 ± 8.42 cells/field) ($p < 0.05$).

4. Discussion

Disruption of circadian rhythm has been demonstrated to have broad consequence at the molecular level and pattern of behaviors [14–16]. Genetic variation of circadian genes has also been evaluated with tumor aggressiveness or patient outcome in glioma [17], hepatocellular cancer [18], prostate cancer [19], colon cancer [20], non-Hodgkin's lymphoma [11] and breast cancer [21,22]. Previous studies suggested that NPAS2, as the largest circadian gene, could regulate other genes of circadian regulatory system [23,24].

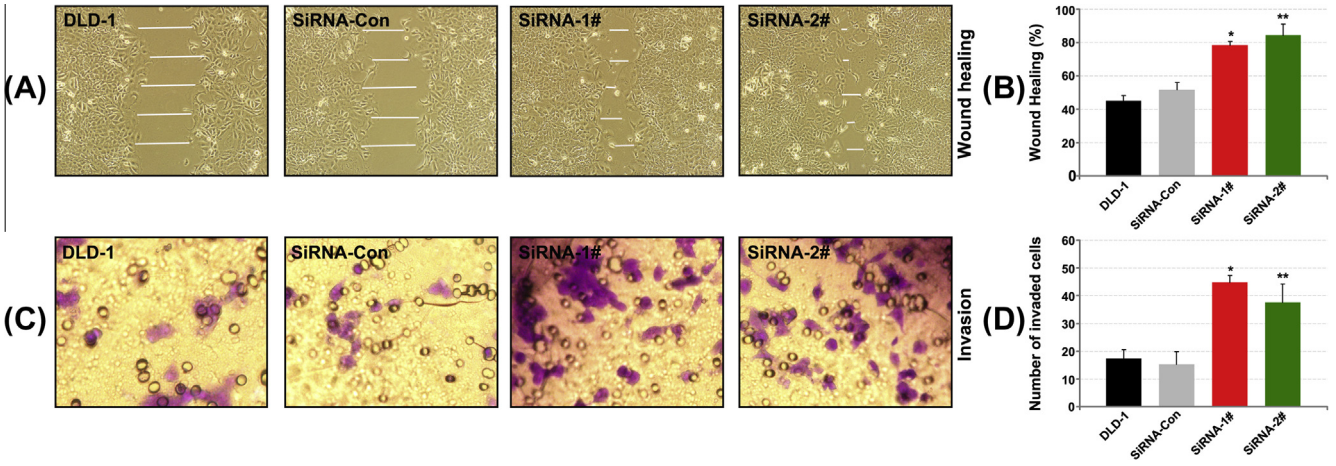

Fig. 3. (A) The scratch healing assay after NPAS2–SiRNA transfection at 72 h. (B) The changes of percentages of wound healing after NPAS2–SiRNA transfection. (C) Cell invasion assay at 36 h after NPAS2–SiRNA transfection. (D) Numbers of invaded cells. (*$p < 0.05$ SiRNA-1# vs SiRNA-Con; **$p < 0.05$ SiRNA-2# vs SiRNA-Con).

Hoffman et al. also demonstrated that NPAS2, which was involved in DNA-damage response, could be considered as a novel tumor suppressor. And knockdown of NPAS2 could significantly repress the expression of several cell cycle and DNA repair related genes [25].

In the present study, our data showed that NPAS2 mRNA expressions were significantly down-regulated in CRC tumor tissues compared with the NATs. To better understand the clinical relevance of NPAS2 expression in colorectal cancer, we also compared the relationship between NPAS2 and the clinicopathologic features. Clinicopathologic analysis revealed that low expression of NPAS2 was associated with the tumor size, TNM stage and tumor distance metastasis in colorectal cancer. But no significant differences were found in the age, sex, tumor location, differentiation, lymphatic metastasis and peritoneal dissemination. These findings indicated that NPAS2 might function as a putative tumor suppressor gene in CRC.

To further investigate the significance of NPAS2 in CRC, we knocked down NPAS2 mRNA expression using RNAi experiments in DLD-1 cells. Our immunocytochemical analysis for Ki67 and CCK-8 assay showed that silencing NPAS2 expression could markedly promote cell proliferation. Cell cycle analysis also confirmed that down-regulated NPAS2 expression led to the arresting cell cycle at S and G2/M phase in DLD-1 cells. These findings illustrated that NPAS2 might be involved in DNA damage response to inhibit the cell proliferation, which was consistent with the results of Hoffman's results [25]. Whereas, our results of tunnel assay showed that no differences were detected in cell apoptotic rate between siRNA groups and control group. Meanwhile, our data showed that silencing NPAS2 expression could increase the wound healing ability and cell invasion. As we know, metastasis is the most feared, most lethal, and least effectively treated characteristic of cancer, which presented the leading event about the majority of cancer fatalities, especially patients with gastrointestinal cancer [26,27]. These findings strongly suggested that NPAS2 could function as a potential suppressor of tumor growth and metastasis in CRC.

In conclusion, we have proved that NPAS2 mRNA expression was decreased in clinical colorectal cancer tissues, and low NPAS2 level was associated with the tumor size, TNM stage and tumor distance metastasis in CRC. Although aberrant NPAS2 expressions might not be associated with cell apoptosis in DLD-1 cells, our experiments in vitro demonstrated that silencing NPAS2 expression could promote cell proliferation, the wound healing ability and cell invasion. These results suggested that NPAS2, as a key circadian gene, might function as a novel and potential tumor suppressor gene in CRC, and could serve as a promising target or potential prognostic indicator for colorectal cancer.

Acknowledgments

This study was supported by a grant from the National youthful Science Foundation of China (Nos. 81101858, 81302145 and 81302147), the National Science Foundation of Jiangsu Province, China (No. BK20130268 and 20130270).

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