

hcrn81 is upregulated by rapamycin treatment in human colorectal adenocarcinoma cells

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Abstract. The aim of the present study was to determine the role of *hcrn81* in the regulation of the mammalian target of rapamycin (mTOR) pathway in human colorectal adenocarcinoma cells. The effect of rapamycin treatment on *hcrn81* expression was evaluated by examining the mRNA and protein expression of *hcrn81* in rapamycin-treated human colon carcinoma cell lines, SW480 and LoVo, using real-time PCR and western blot analysis, respectively. The results demonstrated that mRNA and protein levels of *hcrn81* were elevated following rapamycin treatment in these cell lines, indicating that *hcrn81* expression is upregulated by rapamycin treatment in human colorectal adenocarcinoma cells. Observations of the current study indicate that *hcrn81* may play a role in tumorigenesis by regulating the mTOR signaling pathway.

Introduction

Colorectal adenocarcinoma is the second leading cause of malignancy-related mortality worldwide (1,2), of which the prevalence has been increasing in recent years. Tumorigenesis of colorectal adenocarcinoma is a multi-step process that involves multiple factors and genes regulating a number of pathways. Therefore, it is important to investigate the roles of these factors and genes in colorectal adenocarcinoma for cancer prevention, early diagnosis and therapeutic development.

In a previous study using cDNA subtractive library construction and microarray analysis, 86 differentially expressed sequence tags (dbESTs) were identified in human colorectal adenocarcinoma tissues (3,4). Among these dbESTs, ES274081 (GenBank accession no. NM_001013649.3; gene name, *hcrn81*) was selected for further investigation. Using

qRT-PCR, mRNA levels of *hcrn81* in the colorectal cancer tissues were identified to be lower compared with normal colorectal tissues from patients with colorectal adenocarcinoma, indicating the involvement of *hcrn81* in the development of human colorectal adenocarcinoma (5).

The PI3K/Akt/mammalian target of rapamycin (mTOR) pathway is crucial in the development and progression of colorectal cancer by regulating cancer cell proliferation, resistance to apoptosis, angiogenesis and metastasis (6). The mTOR protein is a key kinase downstream of the growth factor receptor, PI3K and Akt signaling pathway, which is involved in cell growth, survival, metabolism and proliferation (7). In previous years, the role of mTOR in cancer development and progression has been elucidated. Activation of the mTOR signaling pathway often results from genetic alterations of a number of negative regulators of mTOR, including PTEN, tuberous sclerosis complex (TSC) 1 and TSC2 (8). It has been demonstrated that activation of the PI3K/Akt/mTOR pathway correlates with tumor progression and poor survival in a variety of tumor types (9,10), indicating that mTOR may be a promising molecular target for colorectal cancer. The mTOR inhibitor, rapamycin, is a natural macrolide antibiotic isolated from *Streptomyces hygroscopicus*. Rapamycin binds FKBP-12 (FK506-binding protein) and the resulting complex inhibits the protein kinase activity of mTOR. Rapamycin was originally used as an antifungal and immunosuppressive agent, however, the subsequent identification of the inherent antiproliferative properties of rapamycin led to the investigation of this compound as an anticancer agent (11). Therefore, to study the role of *hcrn81* in the tumorigenesis of colorectal cancer, the effect of rapamycin treatment on *hcrn81* expression was analyzed.

Materials and methods

Cell lines and culture conditions. Human colorectal carcinoma cell lines, SW480 and LoVo (both obtained from the American Type Culture Collection, Manassas, VA, USA), were cultured in Dulbecco's Modified Eagle's medium (DMEM; Hyclone Laboratories, Inc., Logan, UT, USA) containing 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were grown at 37°C in a humidified atmosphere with 5% CO₂. Experiments were performed using cells harvested from exponentially growing cultures.

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Drug. Rapamycin stock solutions (5 mg/ml; Fermentek Ltd., Jerusalem, Israel) were prepared in DMSO. These solutions were stored at -20°C prior to use and were diluted into six concentrations in DMEM for subsequent experiments.

In vitro cellular assays. Rapamycin stock solutions were diluted in DMEM at the concentrations of 0.05, 0.1, 0.2, 0.5, 1 and $10\ \mu\text{M}$. DMSO was used as the solvent control, of which the final concentration was 0.1%. Cells were treated with rapamycin for 48 h.

RNA isolation. TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used for total RNA isolation, according to the manufacturer's instructions. Total RNA yield was determined by absorbance at 260 nm using a spectrophotometer. The quality of RNA products was confirmed by sharp bands representing 28S and 18S rRNA molecules and the intensity ratio of 2:1 of these 2 bands (28S:18S) on 1% agarose gel.

First-strand cDNA synthesis. Total RNA isolated from each sample was treated with DNaseI to eliminate genomic DNA contamination prior to reverse transcription (RT). The RT reaction was performed in a $20\text{-}\mu\text{l}$ volume using the M-MuLV Reverse Transcriptase kit (Fermentas, Waltham, MA, USA) for first-strand cDNA synthesis under the recommended conditions. The synthesized cDNA product was immediately used for quantitative real-time PCR or stored at -20°C .

Quantitative real-time PCR. Quantitative real-time PCR was performed for cDNA amplification using SYBR Premix ExTaq (Takara Bio, Inc., Shiga, Japan) and primers listed in Table I, on a Bio-Rad C1000 real-time system (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions and applied international standards (12). For each PCR, $2\ \mu\text{l}$ cDNA obtained from $1\ \mu\text{g}$ RNA template was used. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 30 sec and 40 cycles of the following 3 steps: denaturation at 95°C for 5 sec, annealing at 57°C for 30 sec and elongation at 72°C for 30 sec. GAPDH was used as the internal control. The amplified cDNA product was quantified using the $2^{-\Delta\Delta\text{Ct}}$ method. Primers for *hcrn81* amplification were designed to target its open reading frame, using Primer Premier 5.0 software.

Western blot analysis. Cells were treated with $10\ \mu\text{M}$ rapamycin for 48 h. DMSO was used as the solvent control, of which the final concentration was 0.1%. Cell lysates were denatured in sample buffer containing SDS. The same amount of the denatured protein ($30\ \mu\text{g}$) was loaded on each lane and was separated on 12% SDS-PAGE and then the protein product was transferred to PVDF (Bio-Rad) membranes. Following blocking for 3 h in Tris-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin, membranes were incubated overnight at 4°C with primary antibody against *hcrn81* (1:500). Membranes were then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody and the corresponding protein product was visualized using ECL reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Table I. Primers for quantitative real-time PCR.

Gene	Primer sequence (5'→3')
<i>hcrn81</i>	F: ACGCAACCCAGACTATGAAGAG R: CACCTTCTCACTCACCTTTCCT
GAPDH	F: GGAAGGTGAAGGTCGGAGT R: TGAGGTCAATGAAGGGGTC

Statistical analysis. Statistical analysis was performed using the t-test and Fisher's exact test with SPSS version 19.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

As demonstrated in Fig. 1, mRNA expression of *hcrn81* was upregulated in rapamycin-treated cells, compared with cells treated with DMSO alone. Specifically, upregulation rates were 112.1, 115.3, 126.5, 119.6, 152.5 and 234.6% for the tested rapamycin concentrations ranging between 0.05 and $10\ \mu\text{M}$, respectively, in SW480 cells. In this case, only upregulation in response to the two highest concentrations, 1 and $10\ \mu\text{M}$, was found to be statistically significant ($P = 0.013$ and 0.036). The corresponding upregulation rates in LoVo cells were 110.2, 111.3, 121.4, 121.6, 122.5 and 159.7% for the tested rapamycin concentrations ranging between 0.05 and $10\ \mu\text{M}$, respectively. The upregulation in response to the highest concentration, $10\ \mu\text{M}$, was identified as statistically significant ($P = 0.011$).

As revealed in Fig. 2, following treatment with $10\ \mu\text{M}$ rapamycin for 48 h, the protein expression of *hcrn81* was upregulated in SW480 and LoVo cells lines tested with rapamycin, compared to that in the SW480 and LoVo cell lines. The upregulation was 1.269-fold ($p = 0.048$) and 2.789-fold ($p = 0.024$), respectively.

Discussion

In a previous study, we found that mRNA expression of *hcrn81* was downregulated in human colorectal carcinoma tissue samples by qRT-PCR. Specifically, among the 30 tested human colorectal carcinoma tissue samples, 5 revealed upregulated *hcrn81* mRNA expression, whereas 25 exhibited downregulated *hcrn81* mRNA expression, accounting for 83% of the tested samples. This observation indicated the potential involvement of *hcrn81* in the pathogenesis of colorectal carcinoma. In addition, the downregulation of *hcrn81* mRNA expression was observed in 91% of the moderately differentiated samples (21/23), but only 50% of poorly differentiated tissue samples (3/6). The significantly higher prevalence of *hcrn81* downregulation identified in moderately differentiated samples ($P < 0.05$) indicated a correlation of *hcrn81* expression with tumor stage at the mRNA level (5). In the present study, rapamycin treatment was demonstrated to induce *hcrn81* upregulation in human colorectal adenocarcinoma cell lines at the mRNA and protein level.

mTOR protein is a serine/threonine protein kinase involved in the nutrient-sensitive signaling pathway, which

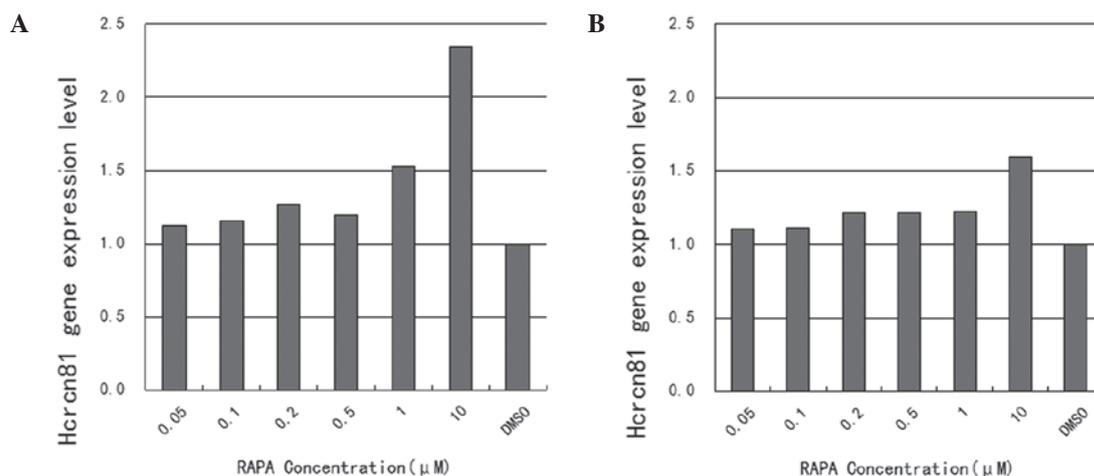


Figure 1. *hcrn81* mRNA expression in human colorectal carcinoma cell lines following 48-h rapamycin treatment. *hcrn81* mRNA expression in (A) SW480 and (B) LoVo cells following rapamycin treatment of various concentrations ranging between 0 and 10 μ M.

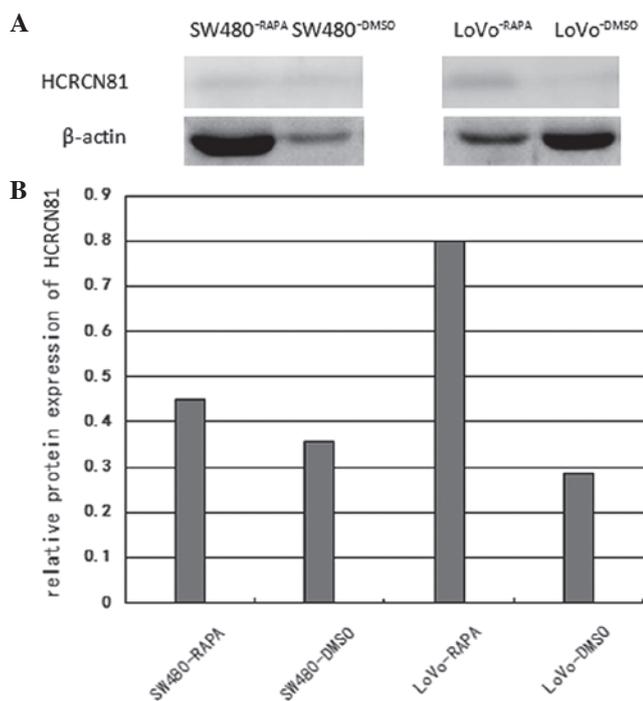


Figure 2. (A) *hcrn81* protein expression in human colorectal carcinoma cell lines following treatment with 10 μ M rapamycin for 48 h. Lanes 1, SW480 cells + rapamycin; 2, SW480 cells + DMSO; 3, LoVo cells + rapamycin; and 4, LoVo cells + DMSO. (B) Quantification of western blot analysis. *hcrn81* protein expression was upregulated in SW480 and LoVo cells treated with rapamycin, compared with corresponding DMSO-treated control cells.

is crucial for the regulation of cell growth and proliferation. The mTOR pathway is activated in various cell processes, including tumorigenesis, insulin resistance, adipogenesis, angiogenesis and T lymphocyte activation. In addition, the pathway is associated with various human diseases, including cancer, obesity and type 2 diabetes (7). The activity of mTOR is regulated by the concentration of amino acids, particularly leucine and the levels of energy, growth factors and oxygen. In addition to these key regulators, other cellular conditions and signals, including inflammation, Wnt signaling,

phosphatidic acid and genotoxic stress, have also been found to be involved in the regulation of the mTOR signaling pathway (7). Activation of the PI3K/Akt/mTOR pathway inhibits apoptosis induced by a number of types of stimuli, thereby promoting cell cycle progression, cell survival and proliferation, which are important for tumor invasion and metastasis. In addition, its role in neovascularization also promotes tumorigenesis. Akt has been found to be overexpressed in human colorectal carcinoma and Akt activation promotes cell proliferation and regulates cell survival by inhibiting apoptosis (13,14). In a previous study, Johnson *et al* found that the expression levels of several key components of the PI3K/Akt/mTOR pathway, including p85 α , Akt1, Akt2, phosphorylated-mTOR and phosphorylated-p70S6K, were significantly elevated in colorectal carcinoma tissue samples, compared with matched normal colorectal tissues from the same patient (14). Similarly, Vilar *et al* reported that the PI3K/Akt/mTOR pathway is of special relevance in mismatch repair-deficient colorectal cancer (15).

Rapamycin is known to induce apoptosis, indicating a potential role of the mTOR pathway in the regulation of cell survival (16). In addition, rapamycin has been revealed to be effective in the clinical treatment of several types of cancer. Boffa *et al* found that rapamycin treatment inhibited cancer cell growth and metastatic progression in non-small cell lung carcinoma (17). Medici and Olsen reported that rapamycin treatment inhibited the proliferation of hemangioma endothelial cells (18). Samkari *et al* demonstrated that rapamycin treatment induced expression of the anti-apoptotic protein, survivin, in neuroblastoma (19). Sun and Jin observed that rapamycin treatment repressed phosphorylation of 4E-BP-1 and p70-S6K induced by insulin in the human colorectal carcinoma cell line, HT29 (20).

In the present study, rapamycin treatment was demonstrated to induce *hcrn81* upregulation in the human colorectal adenocarcinoma cell lines, SW480 and LoVo, at the mRNA and protein levels. Specifically, upregulated mRNA expression of *hcrn81* was observed following rapamycin treatment at all concentrations ranging between 0.05 and 10 μ M. However, in SW480 cells, upregulation in response to the two highest

concentrations, 1 and 10 μM , was found to be statistically significant by Fisher's exact test ($P=0.015$ and 0.018). In LoVo cells, upregulation in response to the highest concentration, 10 μM , was identified as statistically significant by Fisher's exact test ($P=0.046$).

In summary, the effective concentration of rapamycin for significant *hcrn81* upregulation was 10 μM in the two cell lines. The dose-dependent relationship of *hcrn81* upregulation by rapamycin treatment indicated the potential involvement of *hcrn81* in the PI3K/Akt/mTOR pathway in colorectal adenocarcinoma cells, which may be by regulation of mTOR activity. It is possible that *hcrn81* is involved in the induction of cell apoptosis, blockage of cell cycle progression and inhibition of metastasis in cancer cells, similar to the effects of rapamycin treatment. However, further studies must be performed to comprehensively analyze the function of *hcrn81* in carcinogenesis.

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