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RNA Interference-Mediated Knockdown on DKC1 Gene in Chemosensitized Colorectal Cancer Cell Lines

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Abstract

Background

Dyskerin (DKC1) gene, a tolemerase ribonucleoprotein complex, has been reported to be up-regulated in various sporadic cancers, including colorectal cancer (CRC). This study was performed to investigate its potential as a therapeutic target for CRC.

Materials and Methods

The RNA interference (RNAi) technique was employed to down-regulate DKC1 expression in two human CRC cell lines, HCT116 and HT-29, and followed by 5-fluorouracil (5-FU) treatment. Functional assays were carried out. The efficacy of RNAi was assessed by quantitative polymerase chain reaction (qPCR) and Western blotting.

Results

RNAi targeting DKC1 reduced mRNA and protein levels significantly after 48 and 72 hours post-transfection respectively with concomitant decrease in cell viability (P<0.05) in both cell lines. HCT116 cells were arrested in G1 phase of the cell cycle. 5-FU treatment following silencing further reduced cell viability and arrested HCT116 cells in G2 phase. RNAi treatment also reduced HCT116 cell migration significantly.

Conclusion

Silencing of DKC1 in combination with 5-FU may represent a good strategy to inhibit the CRC growth.

Keywords: Colorectal cancer; RNA Interference; 5-Fluorouracil; DKC1 protein; Cell cycle

Introduction

Dyskerin is a highly conserved protein encoded by the *DKC1* gene in eukaryotes [1]. It is present in small nucleolar ribonucleoprotein particles that have been shown to have pleiotropic functions for all basic cellular events such as protein expression, cell growth and cell proliferation [2]. Dyskerin is an integral component of the telomerase ribonucleoprotein complex and is required for the stabilization of the telomerase RNA component, normal telomerase activity and telomere maintenance [3]. It is also essential in rRNA processing and normal ribosome biogenesis by converting the specific uridine residues of ribosomal RNA to pseudouridine [2]. Recently, its role in internal ribosome entry site (IRES)-mediated translation has also been reported [4].

Dyskerin expression is strongly correlated with active cell proliferation [5]. Its expression is up-regulated under experimental conditions that promotes cell growth and proliferation, and through oncogenic stimulation in breast [6] and colon cancers [7]. Recent studies have also identified up-regulation of the *DKC1* gene in association with hepatocellular carcinoma [8], oral squamous cell carcinoma [5] and prostate cancer [9]. Since up regulation of the *DKC1* gene is associated with cell proliferation, the *DKC1* gene can be a potential target for cancer therapy.

The fluoropyrimidine drug, 5-fluorouracil (5-FU), is widely used in CRC treatment since 1957 [10]. Its mechanism of action includes inhibition of thymidylate synthase, incorporation of its metabolites into RNA and DNA, and induction of cell cycle arrest and apoptosis [11]. However, the overall response rate for 5-FU in colorectal cancer (CRC) patients is low and depends on the DNA mismatch repair status [12]. Therefore, new treatment strategies to improve the efficacy of this drug as an anti-cancer agent are urgently needed.

RNA interference (RNAi) is a process of sequence-specific post-transcriptional gene silencing in a wide range of organisms and is initiated by double-stranded RNA that is homologous in sequence to the targeting gene [13]. To explore the potential of *DKC1* as a novel therapeutic target, we applied siRNA targeting *DKC1* to reduce its expression, followed by 5-FU treatment in CRC cell lines. The aim of this study is to determine the effects of siRNA and the combination of siRNA with 5-FU treatment on chemosensitivity of tumour cells.

Materials and Methods

Cell lines

Human adenocarcinoma cell line HT-29 and the HCT116 CRC cell lines used were purchased from American Type Culture Collection, Manassas, VA, USA. The cells were propagated in McCoy's 5A medium (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA).

Small interfering RNA (siRNA) transfection

Prior to the transfection, cells were trypsinized and counted. Cells were then diluted in antibiotic-free medium to a plating density of 5×10^4 cells/mL and 100 µL of cells were plated into each well of a 96-well plate and incubated overnight. Cells were transfected with 50 nM ON-TARGET plus SMART pool siRNA targeting *DKC1* (NM_001363) gene using DharmaFECT transfection reagent (Dharmacon, Lafayette, CO, USA) and incubated for two days according to the manufacturer's protocol. The siRNA targeting *GAPDH, a* housekeeping gene, was used as the positive control. RNA Induced Silencing Complex Free (RISC-free) siRNA was used as the negative control. The effects of siRNA silencing were then assessed using functional assays.

5-Fluorouracil (5-FU) treatment

5-FU (Sigma-Aldrich, St Louis, MO, USA) was dissolved in 100% dimethylsulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA) and then diluted in the media. Cells were treated with different concentrations (0-200 µM) of 5-FU for 24, 48 and 72 hours. The control cells were the siRNA-treated cells without the drug. The cytotoxic effect of 5-FU was assessed by obtaining the 50% inhibitory concentration (IC_{50}: inhibitory drug concentration that results in 50% cell survival) value. Cell lines treated with siRNA were further incubated with 5-FU in 1/10 of IC_{50} concentration for subsequent analysis.

Cell viability assay

Cell Titer-Glo Luminescent cell viability assay (Pr omega, Madison, WI, USA) was used to determine cell viability after siRNA transfection and 5-FU treatment of cells. The control wells containing medium but without cells were prepared to obtain a value for background luminescence. 100 µl of reagent was added to 100 µl of medium containing cells in each well for a 96-well opaque-walled plate. The contents were mixed for two minutes on an orbital shaker to induce cell lysis. The plate was incubated at room temperature for 10 minutes to stabilize the luminescent signal which was then measured using the Spectra Max L luminescence microplate reader (Molecular Devices, Sunnyvale, California, US) at the wavelength of 570 nm.

Cell migration and invasion assay

Cell migration was assessed using a QCM™ 24-well colorimetric cell migration assay kit (Millipore, Hamburg, Germany) while the cell invasion was assessed using a QCM 24-well colorimetric collagen cell invasion assay (Millipore, Hamburg, Germany) following manufacturers' instructions. Briefly, cells (1×10^4) in the serum free media were plated in the top chamber while the bottom chamber contained chemo-attractant (10% fetal bovine serum) media. After 48 hours of incubation, non-invasive cells were removed with a cotton swab. The cells that have migrated through the membrane and stuck to the lower surface of the membrane were stained and extracted. For quantification, the invading cells were detected on the Varioskan Flash microplate reader (Thermo Scientific, Waltham) at 560 nm. Assays were performed in triplicates.

Cell cycle analysis

The cells were processed using the Cycle TEST PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA, USA) based on manufacturers' instructions. After 48 hours of treatment, the cell suspension was placed into a 17 × 100-mm tube. The tube was centrifuged, aspirated and the cells were collected, washed, and suspended in 1 mL of the Buffer Solution. The staining procedure for DNA ploidy analysis requires 5.0×10^5 cells. The cell suspensions were centrifuged at 400x g for 5 minutes. All the supernatant were decanted. Then 250 µL of trypsin buffer was added and incubated for 10 minutes followed by adding 200 µL of trypsin inhibitor and RNase buffer and incubated for 10 minutes at room temperature. 200 uL of cold propidium iodide stain solution was added and incubated for 10 minutes on ice in the dark. The samples were filtered through 35-µm cell strainer cap into 12 × 75-mm tube. Flow cytometric determination of DNA content was performed using the FACS Aria II (BD Biosciences, San Jose, CA, USA). Data were analyzed using Mod fit Cell Cycle Analysis Software (Verity House Software, Topsham, ME, USA).

Validation of the siRNA knockdown

Efficiency of silencing of the *DKC1* gene was checked at mRNA level by qPCR using a Rotor-Gene RG-6000 Real-Time Thermal Cycler (Corbett Research, Sydney, Australia) utilizing the Solaris Human qPCR Gene Expression Assay (Thermo Scientific, Waltham) following manufacturer's protocols. The sequence for the forward and reverse primers for *DKC1* was 5′-GGACTATATCAGGACAGGTTTC-3′ and 5′-GAAGTATCCGTCGAATCCAG-3′ respectively. The probe sequence for this gene was TTCCCATGAGGTGGTAGCC. Expression of the siRNA-targeted gene was normalized to beta-actin (ACTB). In all transfection experiments, ΔC_T expression was normalized to untreated samples [14].

To ensure RNAi efficacy at the protein level, Western blot was performed 72 hours post-transfection. Cell lysates were harvested using RIPA buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SD Sand the complete protease inhibitor cocktail (Thermo Scientific). Lysates containing the equivalent of 30 µg protein were used and Western blot analysis was done following conventional protocols. In brief, the proteins were separated on 12% gels using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membrane. Antibodies and dilutions used included anti-DKC1 (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-beta-actin (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After being washed extensively, the membrane was incubated with horseradish peroxidase-conjugated mouse anti-rabbit (1: 5000 dilutions, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies for one hour at room temperature and developed with Super Signal West Pico Chemiluminescent Substrate (Pierce/Thermo Fisher Scientific Rockford, IL) according to the manufacturer's protocol. The Kodak Bio Max Light Film (Care stream Health, Woodbridge, CT) was used to expose the membrane for chemiluminescent band detection.

Statistical analysis

Statistical analysis was done using two-tailed Student's t test comparing mean values of treated and untreated samples using Microsoft Excel 2007 (Microsoft, Redmond, WA, and the results were considered significant for P value <0.05.

Results

RNAi against DKC1 downregulates RNA and protein expression

There was no significant difference in the mRNA level of targeted genes in cells that were transfected with RISC-free siRNA or transfection reagent only. After 48 hours post-transfection, knockdown of *DKC1* gene showed a significant reduction in mRNA levels compared with untreated

cells where the % knockdown (KD) of *DKC1* was 88.6% in HT-29 cells and 77.3% in HCT116 cells (n=6 each, P<0.05) (Figure 1A and Figure1B).

The reduction in DKC1 protein was confirmed by Western blot in both cell lines (n=3 each, Figure 2).

Silencing of DKC1 increased 5-FU sensitivity of HCT116 cells

The cytotoxic effect of 5-FU was assessed by obtaining the 50% inhibitory concentration (IC_{50}). The IC_{50} for HCT116 cells, which were incubated for 48 hours with 5-FU, was 100 µM (n=6 each, Figure 3A) while the IC_{50} for HT-29 cells, which were incubated for 72 hours with 5-FU, was 200 µM (n=6 each, Figure 3B). To determine the effect of RNAi and 5-FU sensitivity on HCT116 cell proliferation, cells were transfected with *DKC1* siRNA and subjected to 10 µM 5-FU treatment. Knockdown of *DKC1* showed a decrease in cell viability after 48 hours (n=6 each, P<0.05) compared to untreated cells. Further decrease in cell viability was observed when these cells were treated with 5-FU (n=6 each, P<0.05; Figure 4A).

A similar decrease in cell viability was observed with *DKC1* knockdown in HT-29 cells after 48 hours (n=6 each, P<0.05). However, 5-FU treatment following RNAi did not cause significant reduction in HT-29 cell viability (n =6 each, Figure 4B).

Silencing of DKC1 reduced cell migration

Cell migration was reduced 55.9 ± 10% (P<0.05) in HCT116 cells following RNAi targeting *DKC1*, as compared to control cells (n=6 each, Figure 5) while RNAi targeting *DKC1* had no effect on HT-29 cells after 48 hours of transfection (n=6 each, Figure 5). Knockdown of *DKC1* gene also showed no significant difference in cell invasion in both cell lines (n=6 each, data not shown).

Silencing of DKC1 arrested the HCT116 cells in the G1 phase of cell cycle

For HCT116 cells, knockdown of *DKC1* increased the percentage of cells in G1 phase (66 ± 3.4%) (P<0.05) when compared to control cells (57.2 ± 3.3%) after 48 hours post-transfection (n=6 each, Figure 6A). 5-FU treatment following silencing of *DKC1* arrested HCT116 cells in G2 phase (48.3 ± 1.7 %) when compared to control cells (32.7 ± 4.7 %) (n=6 each, Figure 6B) whereas HT-29 cells were unaffected by either knockdown or further treatment with 5-FU (n=6 each, data not shown).

Discussion

The siRNA technology has been applied to develop new treatments for cancer. For this study we hypothesized that the use of RNAi against *DKC1* gene together with 5-FU could reduce the dose of chemotherapeutic

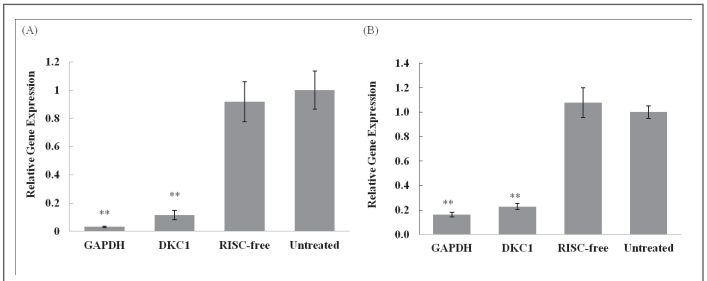

Figure 1: Histogram showing the suppression of *DKC1* mRNA in HT-29 (A) and HCT116 (B) cells following *DKC1* siRNA transfection. The gene expressionof *DKC1* was normalized to the reference gene i.e. beta-actin (*ACTB*). *GAPDH* was used as the positive control to confirm the specificity of siRNA silencing. Data shown in mean ± SD from three separate experiments.***P<0.01** compared with the untreated cells and RNA Induced Silencing Complex Free (RISC-free) cells.

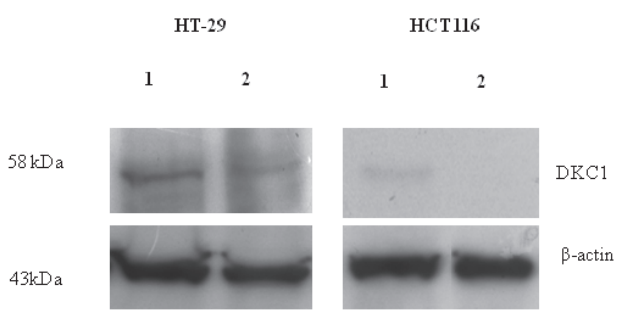

Figure 2: Western blot analyses of DKC1 protein expression in HT-29 and HCT116 cells. Equal amount of total proteins was used for lane (1) Untransfected cells and lane (2) siRNA against DKC1, as demonstrated by the same blot probed with antibodies against DKC1 and ACTB (beta actin) as the control.

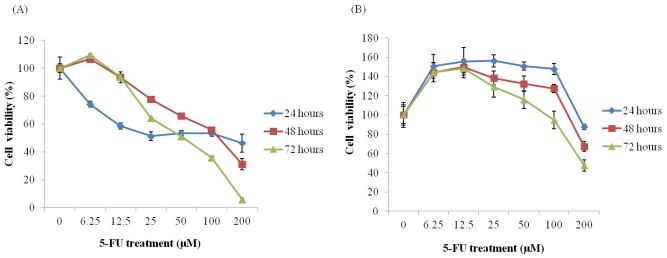

Figure 3: Line graph showing the growth inhibition by 5-fluorouracil (5-FU) in HCT116 (A) and HT-29 (B) cells. The cells were treated with 0, 6.25, 12.5, 25, 50, 100 and 200 µM 5-FU for 24, 48 and 72 h and its viability was determined using a Cell Titer-Glo Luminescent assay. The cytotoxic effect of 5-FU was assessed using IC_{50}. The concentration of the drug that causes the 50% cell survival for HCT116 cells was 100 µM while the IC_{50} for HT-29 cells, which were incubated for 72 hours with 5-FU, was 200 µM. The data presented as the mean of three separate experiments, each performed in triplicate; *bars*, SD.

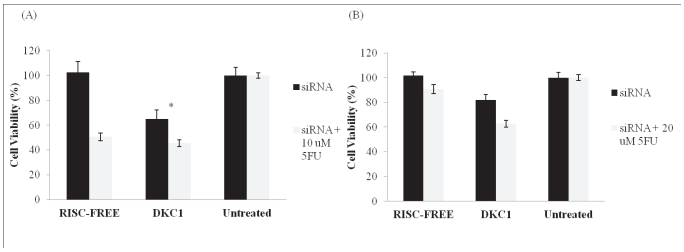

Figure 4: Histogram showing the growth inhibition by combining siRNA and 5-FU treatment in (A) HCT116 and (B) HT-29 cells. The cells were transfected with *DKC1* siRNA for 48 h, with or without the addition of 5-FU and the viability was determined using a Cell Titer-Glo Luminescent assay. Cells transfected with RISC-free siRNA were served as negative controls. Significant suppression of cell viability after 48 h was observed after knockdown of *DKC1* gene followed by 5-FU treatment as compared to the untreated cells. Columns are presented as the mean of three separate experiments, each performed in triplicate; *bars*, SD. *P<0.05 compared with control cells.

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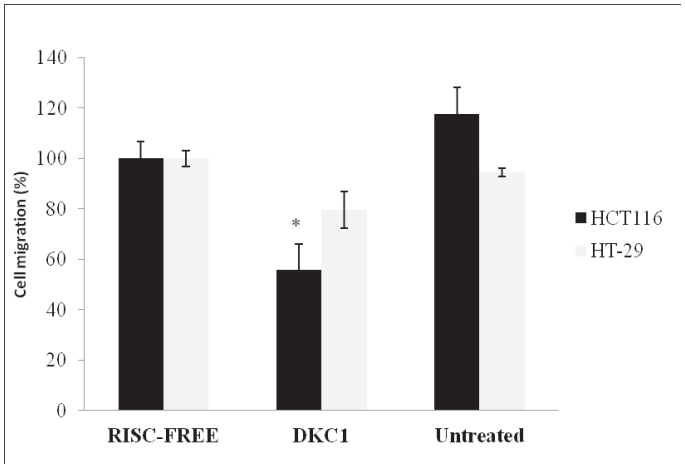

Figure 5: Histogram showing the effect of *DKC1* siRNA on cell migration of HCT116 and HT-29 cells. Cell migration was significantly reduced (P<0.05) in HCT116 cells following RNAi targeting *DKC1,* as compared to control cells while RNAi targeting *DKC1* had no effect on HT-29 cells after 48 hours of transfection. Columns are presented as the mean of three separate experiments, each performed in triplicate; *bars*, SD. *P<0.05 compared with control cells.

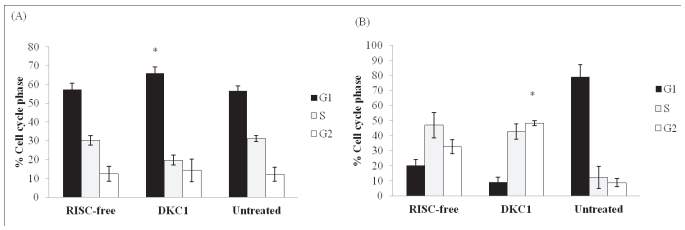

Figure 6: Histogram showing the effect of *DKC1* siRNA on HCT116 cell cycle in the absence (A) or presence (B) of 5-FU. Cells were transfected with siRNA for 48 hand then cultured with or without the addition of 5-FU. (A) Knockdown of *DKC1* significantly increased the percentage of HCT116 cells in G1 phase (P<0.05) when compared to control cells after 48 hours post-transfection. (B) 5-FU treatment following silencing of *DKC1* arrested HCT116 cells in G2 phase when compared to the control. Columns are presented as the means of three separate experiments, each performed in triplicate; *bars*, SD. *P<0.05 compared with control cells.

drugs used known for their debilitating side effects. We successfully demonstrated the effectiveness of using the siRNA technology to suppress the function of specific molecular targets and investigate the downstream effects *in vitro*.

The potential of *DKC1* as a therapeutic target was shown and the knockdown of *DKC1* suppressed HCT116 and HT-29 cell viability 48 hours post-transfection. The results obtained concurred with that of a previous study which reported that the expression of *DKC1* correlated with the rate of cell proliferation [5].The critical function of *DKC* in colon cancer cells is more likely to rely in protein biosynthesis which mainly affects the cell viability and proliferation [5]. Our study showed a reduced percentage of cell migration in the HCT116 cells following the silencing of *DKC1* gene. However, suppression of *DKC1* did not cause any significant effect on cell migration in the HT-29 cell line.

The different effects observed is probably due to the cell lines having different mutations. For HT-29, there is a G -> A mutation in codon 273 of the p53 gene resulting in an Arg -> His substitution while HCT116 cell presented a wild type cell line [15]. *TP53* is a tumor suppresor gene which maintains the genome integrity and induces apoptosis in cells damaged beyond repair [16]. HT-29 cells also harbored mutation in *BRAF* gene while HCT116 cells harbored mutation in *KRAS* gene. These two genes are proto-oncogenes in the RAS–RAF–mitogen-activated protein kinase pathway relaying pro-proliferative signaling [15]. Besides, HCT116 cells are derived from a poorly differentiated primary colon cancer with microsatellite instability that makes them prone to accumulate mutations throughout the genome [17]. In contrast, HT-29 cells are derived from a moderately differentiated colon cancer which has a microsatellite stable characteristic [17]. The different of the genetic features may confer different sensitivity responses to chemotherapeutic drugs such as 5-FU.

Our study showed that silencing of DKC1 by RNAi resulted in enhanced chemosensitivity in HCT116 cells by further reduction in cell viability. 5-FU has been used clinically for over 30 years and is known to exert its effect on proliferating cells by interfering with DNA synthesis [11]. Furthermore, 5-FU induced cell cycle arrest [18,19].

We also demonstrated that 5-FU treatment with or without *DKC1* silencing resulted in HCT116 cells accumulating in G2 and S phases respectively. For the HT-29 cells, RNAi targeting *DKC1* with 5-FU treatment as well as the negative control induced a marked increase in the relative cell numbers in the S phase of the cell cycle. These findings are consistent with a previous study which showed that 5-FU is an S phase-active chemotherapeutic agent, with no activity when cells are in G_0 or G_1 [20]. 5-FU treatment causes DNA damage, specifically double-strand (and single-strand) breaks during the S phase due to the misincorporation of FdUTP into DNA [21]. However, damage to DNA can occur in all phases of the cell cycle in proliferating cells, and the repair mechanisms involved vary in the different phases of the cell cycle [22]. Inhibition of DNA synthesis by 5-FU is manifested in the S phase and incorporation of 5-FU into RNA occurs in the G1 phase [23]. Based on a previous report, the DNA- or RNA-directed cytotoxicity by 5-FU resulted in the disappearance of the early S phase cells or accumulation of the G1/S phase cells in human colon cancer cells [23,24].We showed that there was no significant difference in the cell cycle arrest between cells treated with RNAi and 5-FU treatment compared to cells treated with 5-FU treatment alone. This suggests that 5-FU treatment alone is capable of inducing remarkable changes in the cell cycle regulation in CRC cells.

In conclusion, silencing of *DKC1* has potential to be used in combination with 5-FU to further decrease the viability of HCT116 cells and HT29 cells.

Acknowledgements

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