Anti-proliferative effect of PRKAR1A gene suppression by RNA interference in cholangiocarcinoma cell line
ผลการแทรกแซงการแสดงออกของยีน PRKAR1A ต่อการยับยั้งการเจริญเติบโตในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี

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Abstract

Cholangiocarcinoma (CCA) induced by liver fluke (*Opisthorchis viverrini*, Ov) infection is one of the most common and serious diseases in northeast Thailand. We have previously reported significant overexpression of c-AMP dependent protein kinase regulatory subunit A (PRKAR1A) mRNA in Ov and N-nitrosodimethylamine (NDMA)-induced CCA in hamsters, suggesting that PRKAR1A is strongly involved in CCA carcinogenesis. The present work aimed to elucidate whether PRKAR1A is suitable to be the potential target for inhibiting CCA cell growth. To prove this hypothesis, the RNA interference (siRNA) technique was used to determine the anti-proliferative effect of PRKAR1A gene suppression on a CCA cell line. The siRNA treatment leaded to the suppression of the PRKAR1A mRNA and protein expression resulting in the reduction of CCA cell proliferation ($p < 0.01$). The inhibition of PRKAR1A expression leading to reduction of CCA cell growth suggests that this molecule may be used as a drug target for CCA therapy.

บทคัดย่อ

มะเร็งท่อน้ำดี เป็นปัญหาทางสาธารณสุขที่สำคัญของประเทศไทยโดยเฉพาะในภาคตะวันออกเฉียงเหนือ อันเนื่องมาจากในภูมิภาคนี้มีอุบัติการณ์เกิดโรคสูงที่สุดในโลก จากการศึกษาที่ผ่านมาพบว่ามีการแสดงออกที่เพิ่มสูงขึ้นของยีน PRKAR1A ในเนื้อเยื่อตับของหนูแฮมสเตอร์ที่ถูกชักนำให้เป็นมะเร็งท่อน้ำดีด้วยการทำให้ติดเชื้อพยาธิใบไม้ตับ *Opisthorchis viverrini* (Ov) ร่วมกับการให้สารก่อมะเร็งชนิด *N-nitrosodimethylamine* (NDMA)

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งานวิจัยนี้ได้ศึกษาผลของการยับยั้งการแสดงออกของยีน PRKAR1A ต่อการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีพบว่า การยับยั้งการแสดงออกของยีน PRKAR1A โดยวิธี RNA interference สามารถยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี ซึ่งจากผลดังกล่าวแสดงให้เห็นว่า PRKAR1A น่าจะมีบทบาทสำคัญในกระบวนการก่อมะเร็งและเป็นประโยชน์ในการใช้เป็นเป้าหมายของยาในการรักษามะเร็งท่อน้ำดีได้

คำสำคัญ: มะเร็งท่อน้ำดี
Keywords: Cholangiocarcinoma, PRKAR1A, RNA interference

Introduction

Cholangiocarcinoma (CCA), a malignant tumor arising from bile duct epithelium, is a common cancer and a major public health problem in northeast Thailand. Chronic infection by a liver fluke, *Opisthorchis viverrini* (Ov) has been postulated to be a risk factor for CCA (Parkin, 1991). We have previously used a fluorescence differential display technique to determine the differential gene expression in Ov metacercariae and *N*-nitrosodimethylamine (NDMA) induced hamster cholangiocarcinogenesis and found significant overexpression of PRKAR1A, the regulatory subunit of cyclic AMP (cAMP)-dependent protein kinase (PKA), in liver bearing tumor when compared with normal liver and gall bladder epithelia (Loilome, 2006). Protein kinase A (PKA or cyclic adenosine monophosphate [cAMP]-dependent protein kinase) plays a critical role in numerous cellular processes including the regulation of cell growth, gene transcription, and metabolism (Cho-Chung, 1995). PKA is a heterodimer composed of two catalytic and two regulatory subunits. The regulatory subunits, PRKAR1A or PRKAR2B, determine the isotype of PKA (type I or type II, respectively) (Krebs, 1972). PKAI expression has been associated with cell proliferation and neoplastic transformation, whereas expression of PKAII predominates in normal, non-proliferating tissues (Cho-Chung, 1995). Moreover, it has been demonstrated that treating different tumor cell lines with antisense oligodeoxynucleotides targeting PRKAR1A causes inhibition of cell proliferation in vitro (Cho-Chung, 1999; Nesterova, 2000) and tumor growth *in vivo* (Cho-Chung, 1997; Cho-Chung, 1995; Wang, 1999). Therefore, the present study aimed to elucidate the effect of PRKAR1A gene suppression on CCA cell proliferation by RNA interference technique.

Materials and Methods

Cell line and cell culture

Human cholangiocarcinoma cell line, M214 was cultured in HAM-F12 media (Gibco/BRL, Grand Island, NY) containing 100 U/ml penicillin and 100 μg/ml streptomycin with 10% fetal bovine serum at 37 °C with 5% CO2. The presence of *Mycoplasma* contamination was periodically checked.

PRKAR1A RNA interference

M214 cell line which showed highest PRKAR1A/PRKAR1B ratio (data not shown) was used as a model for PRKAR1A suppression. PRKAR1A validated siRNA (siRNA ID 42857) and validated non-targeting siRNAs (scramble controls) were purchased (Ambion, Foster City, CA) and transfected using siPOR™ *NeoFX* Transfection Agent (Ambion, Foster City, CA), according to the manufacturer's instructions. Briefly, 1 hr before

transfection, we trypsinized healthy, growing, M214 cells using the routine trypsin-EDTA procedure. Inactivated trypsin was formed by resuspending the cells in HAM-F12 with 10% FBS without antibiotic. The cells were set aside at 37°C in a water bath. Then, they were diluted with 5 µl siPORT NeoFX Transfection Agent in 100 µl OPTI-MEM I medium (Invitrogen, Carlsbad, CA), mixed gently and incubated for 10 min at room temperature. Next, they were diluted in 3.75 µl of 20 µM stock concentration siRNA into 100 µl OPTI-MEM® I medium, and mixed gently. Diluted siPOR™ *NeoFX* Transfection Agent and diluted siRNA were combined and mixed by pipetting up and down. The mixture was incubated for 10 min at room temperature to allow transfection complexes to form and then RNA/ siPOR™ *NeoFX* Transfection agent complexes dispensed into 6-wells of a clean culture plate. After that, 2.3 ml (2×10^5) cell suspensions were overlayed onto the transfection complexes and the plate gently tilted to mix. The final concentration of siRNA was 30 nM. Cells were cultured for 48 h before being collected. Cell growth was determined by trypan blue counting and the levels of PRKAR1A mRNA and protein were determined by relative quantification real time PCR assay and western blot analysis.

RNA extraction and cDNA synthesis

Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Reverse transcription reaction consisted of 2.5 µg total RNA and random hexamer (2.5 µM) which were mixed together and then heated at 70°C for 10 min. After that, the reaction mixture contained the first-stranded cDNA synthesis buffer (1x; 75 mM KCl,50 mM Tris-Cl pH 8.3, 3mM $MgCl_2$), 10 mM DTT, 0.5 mM each dNTPs and 200 units reverse transcriptase (Promega, Medison, WI). Reverse transcription was carried out using a DNA thermocycler (GeneAmp PCR system 2400, Applied Biosystems, Foster City, CA). The thermal conditions were 25 °C for 10 min, 37°C for 1 hr, and 95°C for 5 min.

Real time PCR and relative quantification of PRKAR1A mRNA

Real time PCR was performed using TaqMan® Gene Expression assay kit and the ABI 7500 real time PCR system (Applied Biosystems, Foster City, CA). TaqMan PCR primers and probes for PRKAR1A (Hs00267597_m1) and GAPDH (4326317E) were used in this experiment (Applied Biosystems, Foster City, CA). PCR reactions were performed using TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA) following the manufacturer's protocol in duplicate for each sample. The relative quantification of PRKAR1A gene expression was done using the comparative cycle threshold (C_T) method (Livak, 2001) using GAPDH as endogenous control as in the following formula.

Relative Quantification (RQ)

$$= 2^{-\text{average ddCT}}$$

Where

$$d C_t = \text{Average } C_t \text{ (target)} - \text{Average } C_t \text{ (endogenous)} \quad \ldots\ldots(1)$$

so $dd C_t$ = Average d Ct (target) - Average d Ct (calibrator).....(2)

and Target= PRKAR1A, Endogenous = GAPDH, Calibrator = non-transfected sample.

Western blot analysis

Ten µg of the protein extract from the cell lysates were solubilized in SDS buffer and boiled for 5 min. Samples were separated on 12.5% polyacrylamide

gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% skim milk in Tris buffered saline (TBS) at room temperature for 1h, and then incubated with polyclonal antibody to human PRKAR1A (dilution 1:1000, Calbiochem, Gibbstown, NJ) for 1h at room temperature. After washing with TBS containing 0.1% polyoxyethylene sorbitan monolaurate (Tween-20 or TBS-T), membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biochemical, Santa Cruz, CA, USA) at room temperature for 1 h. After rinsing with TBS-T, membranes were exposed to the ECL Plus Western Blotting Detection System (GE Healthcare, UK) for 5 min. Human GAPDH was used as a loading control. The immunoblot and intensity were analysed by ImageQuant analysis system (GE Healthcare, UK).

Results and Discussion

siRNA directed against PRKAR1A decreased its mRNA and protein expression

To determine whether RNA interference could be used for inhibiting the expression of PRKAR1A, M214 cell line (Figure 1) was used as a model. Cells were transfected with validated siRNA sequence against exon 2 and exon 3 of PRKAR1A mRNA (NM_002734). After 48 hours of transfection, siRNA reduced approximately 55% of PRKAR1A mRNA and protein expression, respectively (Figures 2, 3). Although, we have increased the final concentration of siRNA to be 100 nM, a similar result for decrease of PRKAR1A mRNA and protein expression was found. Therefore, the stable PRKAR1A knockdown CCA cell lines using viral transfection system might be needed to obtain total PRKAR1A knockdown.

Decreased expression of PRKAR1A with siRNA reduces M214 cell growth

The contribution of PRKAR1A/PKA in CCA cell proliferation was further confirmed using trypan blue counting. The near-total abrogation of PRKAR1A protein was associated with significant inhibition of proliferation in both cell lines. We observed a significant ($p<0.01$) growth reduction in cells transfected with siRNA directed against PRKAR1A as compared with those transfected with scramble control siRNA (Figure 4). The result is consistent with previous studies that suppression of PRKAR1A expression by antisense oligodeoxynucleotides as well as siRNA produced growth inhibition in human cancer cells both in vitro and in vivo (Cho-Chung, 1997; Cho-Chung, 1999; Farrow, 2003; Mantovani, 2008; Nesterova, Cho-Chung, 2000; Nesterova, Cho-Chung, 2004). In addition, others have shown that PRKAR1A can be a potential target for cancer therapy by which several selective inhibitors of PRKAR1A/PKA I showing potential effect to inhibit tumor growth are available. (Chen, 2000; Cho-Chung, 1997; Cho-Chung, 1995; Tortora, 2000; Tortora, 2001a; Tortora, 2001b; Tortora, 2003; Tortora, Ciardiello, 2000).

Taken together, the present work shows that the suppression of PRKAR1A expression in cholangiocarcinoma cell inhibits cell growth, therefore targeting PRKAR1A may have a possible therapeutic indication to CCA patients. Further study regarding the alteration pattern of PRKAR1A/PKAI signaling pathway in CCA when the protein target was knocked-down by siRNA is under investigation.

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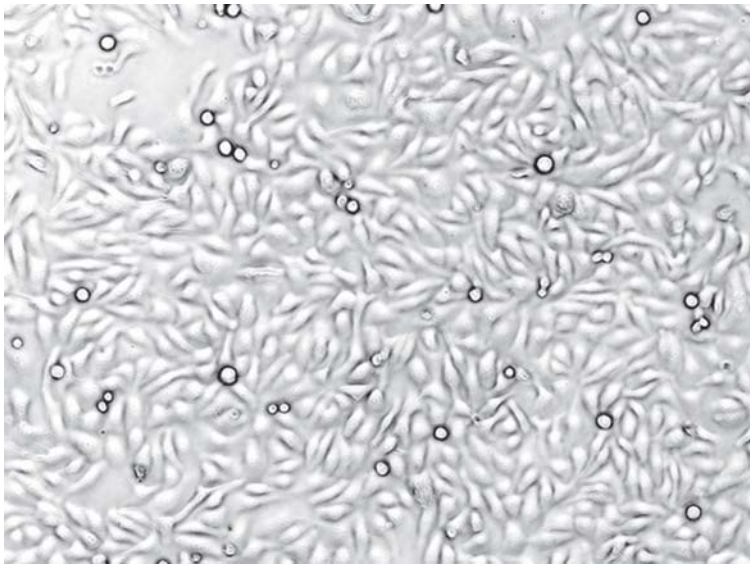

Figure 1. M214 CCA cell line grown in 6-well plate (2x105 cells). Magnification is 10X.

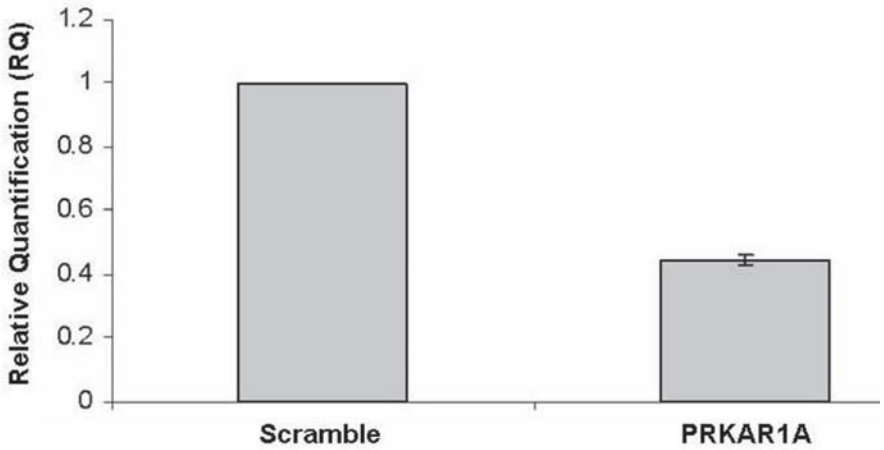

Figure 2. Relative quantification of PRKAR1A mRNA in M214 cells after being transfected with PRKAR1A siRNA or nontargeting control siRNA (scramble control) for 48 h. PRKAR1A mRNA was approximately 55% reduced when adding 30 nM of siRNA compared with scramble control.

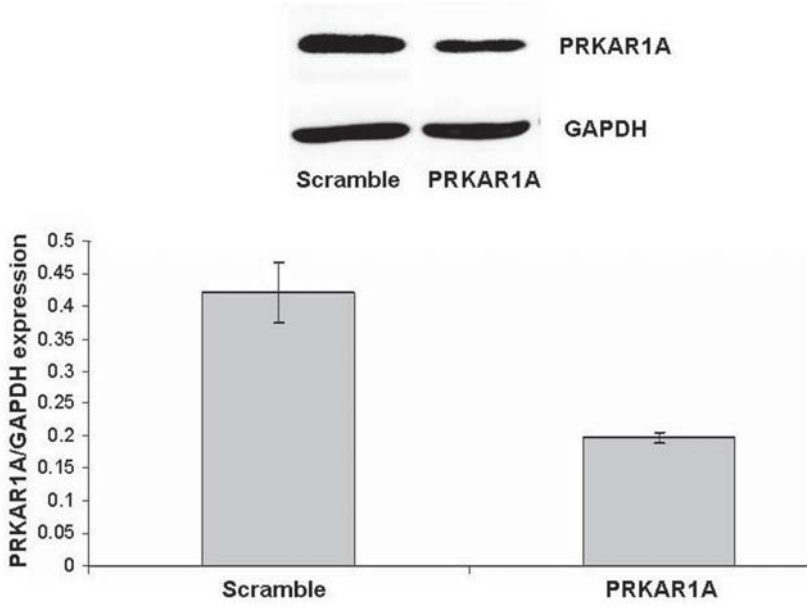

Figure 3. Western blot analysis of PRKAR1A in M214 cells after being transfected with PRKAR1A siRNA or nontargeting control siRNA (scramble control) for 48 h. PRKAR1A protein was approximately 55% reduced when adding 30 nM of siRNA compared with scramble control ($p < 0.01$).

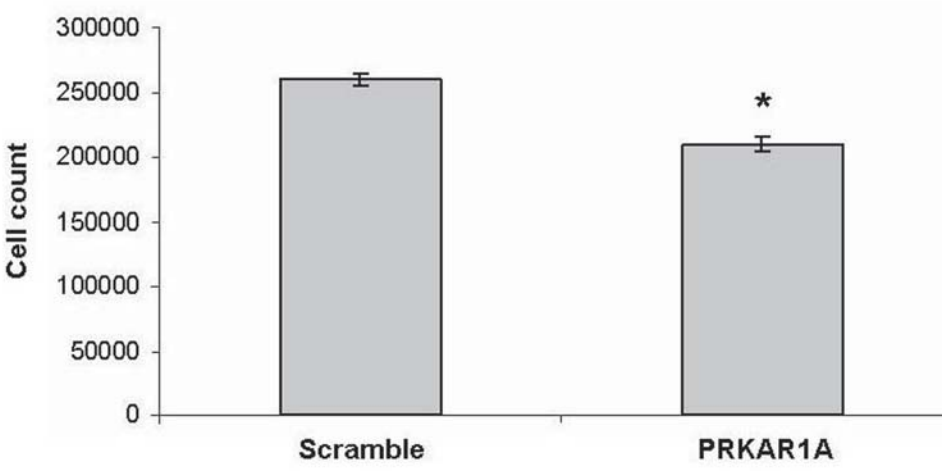

Figure 4. Transfection of M214 cell line with PRKAR1A siRNA reduced cell growth ($p < 0.01$) in comparison with scramble control.