Contribution of RIZ1 to Regulation of Proliferation and Migration of a Liver Fluke-Related Cholangiocarcinoma Cell

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Abstract

Purpose: Retinoblastoma-interacting zinc finger gene (RIZ1) is a tumor suppressor gene which is highly inactivated by promoter hypermethylation in patients with liver fluke-related cholangiocarcinoma (CCA). Epigenetic aberration of this gene might withdraw the ability to restrain tumor cell proliferation and migration. We aimed to define the role of RIZ1 on cell proliferation and migration in CCA cell line. Materials and methods: Small interference RNA (siRNA) was used to knock down the expression of RIZ1 in a CCA-derived cell line in which cell proliferation and cell migration were performed. Results: A predominant nuclear localization of RIZ1 was observed. Reduction of RIZ1 by siRNA augmented cell proliferation and migration. Conclusion: The result suggested that RIZ1 might play a role in regulating cell proliferation and migration in CCA. Reduction of RIZ1 expression may aggravate the progression of CCA.

Keywords: RIZ1 - tumor suppressor - cholangiocarcinoma - siRNA - cell migration - cell proliferation

Introduction

Liver and bile duct cancer is one of the top ten diseases with high mortality in Thai population (Ministry of Public Health, 2002). Cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) are the most common cancer in this group (Khuhaprema et al., 2007). The incidence of these cancers is varied by the region in which CCA is the majority of liver cancer in northeast Thailand (Khuhaprema et al., 2007). Patients with CCA are often diagnosed clinically and cholangiographically but mostly are in advanced stage and are difficult to cure successfully. The prognosis of CCA is extremely poor. Chronic infection with the liver fluke, Opisthorchis viverrini (OV), is the major risk factor for the development of CCA in northeastern Thai (Sripa et al., 2007; Aljiffry et al., 2009). Prolonged inflammation induced by parasitic infection enhances the susceptibility of bile duct epithelium to carcinogenic chemicals leading to genetic and epigenetic damages in the cells. Abnormal growth and the accumulation of genetic and epigenetic aberrations eventually result in malignant transformation of the bile duct epithelium (Sripa et al., 2007; 2008).

Our previous study showed the correlation of MSI at D1S228 with poor patient survival (Limpaiboon et al., 2006). This microsatellite marker is adjacent to RIZ (PRDM2) locus. Although RIZ has been discovered more than a decade (Muraosa et al., 1996; Liu et al., 1997), its molecular biology is not well understood and has not been characterized properly. RIZ has been proposed into 4 isoforms (Genbank accession number NC_000001.10) (Figures 1 and 2), in which only 2 isoforms, RIZ1 (isoform a; Genbank accession number NM_012231.4; NP_036363.2) and RIZ2 (isoform b; Genbank accession number NM_0010007257.2; NP_001007258.1), have been usually mentioned in almost all studies regarding RIZ genes.

RIZ1 acts as a bona fide tumor suppressor while RIZ2 acts as an oncogene (Huang, 1999). The most important role of RIZ1 is histone methyltransferase (HMT) activity which catalyzes methylation of lysine 9 of histone H3 (H3K9) resulting in the modulation of chromatin structure and inhibition of transcriptional process. RIZ1 is also a downstream effector of estrogen receptor (ER) pathway which regulates cell growth and proliferation (Abbondanza et al., 2000). Aberration of RIZ1 by both genetic and epigenetic mechanisms has been reported in cancers (Carling et al., 2003; Tokumaru et al., 2003). A few studies proposed the existence of other 2 isoforms, MTB-ZF (isoform c; Genbank accession number NM_015866.4; NP_056950.2) (Muraosa et al., 1996) and
antibiotic-free Dulbecco’s Modified Eagle’s medium/ nutrient mixture Ham’s F-12 (Wako, Osaka, Japan) with 10% fetal bovine serum supplement and incubated at 37°C with humidified atmosphere of 5% CO₂. Cells were harvested and prepared for total RNA and nuclear protein by using RNasea Mini kit (QIAGEN, Valencia, CA) and 2-D Sample Prep for Nuclear Proteins (Peirce, Rockford, IL), respectively.

**Knockdown assay**

The small interfering RNA (siRNA) duplex (Invitrogen, Carlsbad, CA) was designed for specific silencing of RIZ1 covered the region coding for amino acid residues 97-103 of RIZ (obtained from Gazzzerro et al., 2006), within the PR domain (sense 5’-AACUGGCUGCAUAUGUGAU-3’). Cells (1x10⁴) were seeded in a 24-well plate containing 500 µl antibiotic-free medium and incubated at 37°C overnight. Transient transfection of siRNA was performed using Lipofectamine™ 2000 according to the manufacturer’s instructions (Invitrogen). The transfected cells were incubated at 37°C in a 5% CO₂ incubator. After 6 h incubation, the medium was replenished by the complete medium and incubated further at 37°C in a 5% CO₂ incubator. After 48 h transfection, cells were processed for determination of RIZ expression using immunofluorescence, Western blot, and real-time RT-PCR. Cell proliferation assay and wound healing assay were also performed.

**Immunofluorescence staining**

Cells (5x10⁴) were grown in a 24-well plate under standard condition for 18-24 h to get 70-90% confluence. After washing with cold phosphate buffer saline (PBS), cells were fixed at room temperature for 15 min with 4% paraformaldehyde. Cell permeabilization was performed using 0.2% Triton-X/PBS at room temperature for 5 min and non-specific proteins were blocked overnight by 3% bovine serum albumin (BSA) in PBS. Antibody reaction was performed for 1 h, then cells were washed three times with PBS for 5 min. Nuclei were stained with 1:1000 diluted Hoechst 33342 (Molecular Probes, Eugene, OR) for 5 min. After washing with cold phosphate buffer saline (PBS), cells were incubated at 37°C in a 5% CO₂ incubator. After 48 h transfection, cells were processed for determination of RIZ expression using immunofluorescence, Western blot, and real-time RT-PCR. Cell proliferation assay and wound healing assay were also performed.

**Time-lapse observation**

Cells (5x10⁴) were seeded into a collagen type I coated 24-well glass-bottom plate (EZView™ LB Culture Plate, IWAKI, Tokyo, Japan) and cultured for 24 h under standard condition before time-lapse analysis. Cellular

**Materials and Methods**

**Cell line and cell culture**

CCA-derived cell line M213 was maintained in an
morphological changes were monitored by a time-lapse microscope IX81 equipped with an autofocus multi-well flat table and CO₂ incubation chamber system (Olympus, Tokyo, Japan). The differential interference contrast (DIC) image acquisition was performed every 15 min for 24 h using MetaMorph device controller and automation system Ver. 7.5.5.0 (Molecular Devices, CA). The movie was created from the recorded image stacks using MetaMorph offline imaging system software (Universal Imaging, West Chester, PA).

**Proliferation assay**

Proliferation assay was determined by a water-soluble tetrazolium salt (WST) method using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Cells were seeded in a 96-well plate at 1,250 cells per well. Triplicate wells were assigned to each condition. Cells were grown under standard condition for 12 h, 24 h and 48 h incubation. WST was assayed by adding CCK-8 reagent and the absorbance at 450 nm (OD450) was measured after 2 h reaction. Mean and standard error of the mean (SEM) of each triplicate were presented in the graph and subjected to unpaired Student t-test. Statistical significance was accepted at P<0.05.

**Wound healing assay**

Cells (1x10⁵) were seeded in a collagen type I coated 24-well glass-bottom plate (EZView™ LB Culture Plate, IWAKI, Japan) and cultured for 24 h under standard condition. Cell monolayer was then scratched with a sterilized 200 ml pipet tip (Greiner bio one, Tokyo, Japan). Cell migration was monitored under time-lapse microscope as described above.

**Results**

**Determination of RIZ1 expression in M213 cholangiocarcinoma cell line**

In order to investigate the contribution of RIZ1 in cell proliferation and cell migration in cell line M213, baseline expression of RIZ1 mRNA and protein were determined. Due to the lack of cell line which does not express RIZ1, siRNAs were used to knockdown RIZ1 expression in M213 using Lipofectamine 2000™. After 48 h transfection, the efficiency of siRNA was evaluated by real-time RT-PCR, Western blot, and immunofluorescence staining. Because of non-specific amplification of real-time RT-PCR and non-specific reaction of the antibody detected by Western blot, we were unable to observe the difference between siRIZ1 and siControl transfected cells (data not shown). However, RIZ1 expression could be determined by immunofluorescence staining. The high expression of RIZ1 was found in nuclei but low in cytoplasm (Figure 3).

**Effect of RIZ1 knockdown on cell proliferation in M213**

The role of RIZ1 on cell proliferation was determined by WST assay after 24, 48 and 72 h siRNA transfection (Figure 4). RIZ1-knockdown significantly increased the...
proliferation of M213 after 24 and 48 h transfection (P=0.037 and P<0.01, respectively). However, there was no statistical difference between RIZ1-knockdown and control after 72 h transfection (P=0.981). To monitor cell proliferation, time-lapse video observation was performed after 48 h transfection. Transient transfection of siRIZ1 showed more number of mitotic cells than siControl transfected cells (Figure 5). This result was accordant with WST suggesting that reduction of RIZ1 by siRNA increased the proliferation of M213.

Effect of RIZ1 knockdown on M213 cell migration

To address whether RIZ1 mediated tumor cell migration, M213 was transiently transfected with siRIZ1 for 48 h, wound was created by scratch assay, and cell migration was monitored under time-lapse microscope. siRIZ1 transfected M213 took a shorter time to heal the wound than control suggesting that tumor cell migration was mediated by RIZ1 (Figure 6).

Discussion

In this study we could not determine RIZ1 expression by real-time PCR and Western blot. This might be because of the misunderstanding about the characteristics of RIZ isoforms. We have figured out that the primer set commonly used for determining RIZ1 expression is targeted to exons IV and VII which is shared between RIZ1 and MTB-ZF; in such a case, the detected transcript not only represents RIZ1 but also MTB-ZF. However, the existence of MTB-ZF isoform was described only once by Muraosa et al (Muraosa et al., 1996). This isoform was obtained from a cDNA synthesized from THP-1 cell by using random hexamers and cloned into λgt11 expression vector. The proximal part (5’- end) of MTB-ZF is homologous to exons I - VI of RIZ1 whereas the distal part (3’- end) is homologous to the entire sequence of RIZ2 isoform. This may result from the false generating of a concatamer combining 5’ fragment of RIZ1 and entire RIZ2 molecule in which MTB-ZF isoform is never presented naturally. In this case, the RT-PCR would represent only RIZ1 transcript. While MTB-ZF isoform remains controversial and requires further study to warrant its existence, the primer set targeting exons VIII and IX should be assigned for more specifically detecting RIZ1 expression.

The increase in RIZ1 mRNA expression has been reported at late stage of several cancers (Pastural et al., 2007; Sun et al., 2011). This may be due to the non-specific reaction of RIZ1 RT-PCR primers targeting PR domain which also bind to isoform d and MTB-ZF. However, these studies speculated that the increase in mRNA expression may not reflect the level and biological function of RIZ1 protein.

In particular contexts, for example, in the presence of 17β-estradiol (E2), RIZ1 could express but its histone methyltransferase activity is terminated and forced to act as a co-activator of estrogen receptor which augments growth and metastasis of tumor cells (Pastural et al., 2007). Interestingly, increased expression of both E2 and ER were found in CCA patients (Alvaro et al., 2006). However, both functions of RIZ1 might be decreased after transient knockdown by siRIZ1.

The molecular mechanisms which demonstrate the biological functions of RIZ1 are limited. In this study we demonstrated the effect of RIZ1 expression on cell migration and cell proliferation. These functions might be mediated by the role of RIZ1 to suppress the activation of some growth factors such as IGF-1 which regulates several cellular functions including cell proliferation, apoptosis, and the resistance against oxidative stress and secretory process (Kahlert et al., 2000; Alvaro et al., 2006; Pastural et al., 2007). IGF-1 promotes cell migration, for example, by induction of CC chemokine ligand 5 (CCL5) expression in adjacent cells and this paracrine secretion promotes metastasis of cancer cells (Karnoub et al., 2007; Makinoshima et al., 2009). Phosphatidylinositol-3-kinase (PI3K)/Akt and extracellular-regulated kinase (ERK) pathways were reported as downstream mechanisms mediated by IGF1 in induction of cell proliferation and survival in cancers including intrahepatic cholangiocarcinoma (Schmitz et al., 2007; Alvaro et al., 2008; Ma et al., 2010). A recent study showed that RIZ1 could enhance p53 expression resulting in suppression of cell proliferation of monocytic leukemia cells (Shadat et al., 2010).

Our study suggested the critical role of RIZ1 in regulation of cancer behaviors in CCA. More appropriate protocol in detection of this molecule should be considered according to biological facts we mentioned in here. Subcellular localization and expression of RIZ1 interacting molecules, such as IGF-1 and ER, are also the important factors directing the role of RIZ1 and should be investigated in this cancer.

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References


