

## RESEARCH ARTICLE

# 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

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## Introduction

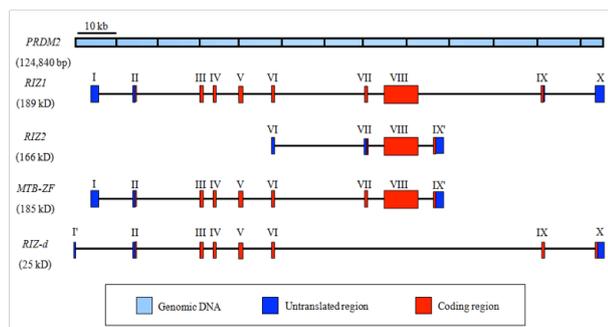
Liver and bile duct cancer is one of the top ten diseases with high mortality in Thai population (Ministry of Public Health, 2002). Cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) are the most common cancer in this group (Khuhaprema et al., 2007). The incidence of these cancers is varied by the region in which CCA is the majority of liver cancer in northeast whereas HCC is more predominant in other parts of 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 (Limpai boon et

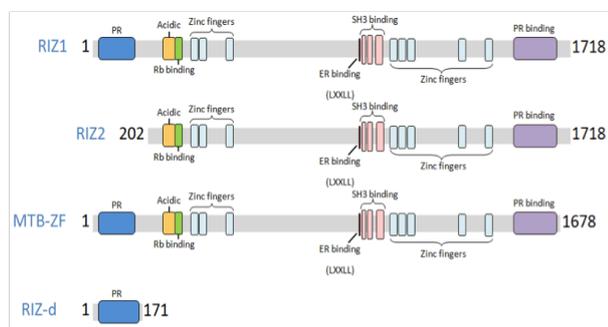
al., 2006). This microsatellite marker is adjacent to *RIZ* (*PRDM2*) locus. Although *RIZ* has been discovered more than a decade (Muraosa et al., 1996; Liu et al., 1997), its molecular biology is not well understood and has not been characterized properly. *RIZ* has been proposed into 4 isoforms (Genbank 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

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**Figure 1. Schematic of PRDM2 or RIZ.** Genomic DNA is presented in cyan bar which is divided into 10 kb in each compartment. Four isoforms are shown based on the location utilized from the same genomic DNA. The predicted molecular weight is shown under the name of each isoform on the left side of its gene structure. The exon numbers are shown in Roman characters. Blue and red boxes represent untranslated and coding regions, respectively (Based on the information from www.ncbi.nlm.nih.gov).



**Figure 2. The Functional Domains of RIZ Family Protein.** Schematic was illustrated based on the information from www.uniprot.org. The numbers shown are the amino acid residues of each RIZ isoform. PR = PR domain; ER = estrogen receptor; Rb = retinoblastoma protein; SH3 = Src homology 3.

isoform d (Genbank accession number NM\_001135610.1, NP\_001129082.1) (Buyse et al., 1995). *MTB-ZF* acts as a transcription activator of human heme oxygenase-1 promoter. The function of isoform d of *RIZ* has not yet been defined.

Our previous study detected genetic and epigenetic alterations of *RIZ1* in about 52% of liver fluke-related CCA subjects (Khaenam et al., 2010). Promoter hypermethylation is the major event accounting for 38% of CCA subjects while loss of heterozygosity (LOH) and frameshift mutation are quite rare. Despite the known frequency of *RIZ1* alterations in liver fluke-related CCA patients, the biological function of this molecule in CCA has not been defined yet. This study attempts to demonstrate the role of *RIZ1* in regulating tumor progression including cell proliferation and cell migration. M213, an adenocarcinoma CCA-derived cell line, was used as the model. Our study found that reduction of *RIZ1* in CCA cell line resulted in an increase of tumor cell proliferation and migration suggesting the important role of *RIZ1* in the progression of CCA.

## Materials and Methods

### Cell line and cell culture

CCA-derived cell line M213 was maintained in an

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% CO<sub>2</sub>. Cells were harvested and prepared for total RNA and nuclear protein by using RNeasy 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 Gazzero et al., 2006), within the PR domain (sense 5'-AACUGGCUGCGAUUAUGUGAAU-3'). Cells (1x10<sup>4</sup>) 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% CO<sub>2</sub> incubator. After 6 h incubation, the medium was replenished by the complete medium and incubated further at 37°C in a 5% CO<sub>2</sub> 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 (5x10<sup>4</sup>) 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. *RIZ1* protein was detected by 1:500 diluted rabbit anti-*RIZ1* (ab9710, Abcam, Cambridge, UK) in 0.2% BSA/PBS. Antibody reaction was performed for 1 h, then cells were washed three times with PBS for 5 min. *RIZ1* staining was monitored by using 1:1000 diluted Alexa Flour 488 labelling goat anti-rabbit antibody (Molecular Probes, Eugene, OR) in 0.2% BSA/PBS. Reaction was incubated for 1 h and 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. Cells were rinsed three times with PBS for 5 min, maintained in PBS and stored in the dark at 4°C until inspected under fluorescence microscope. The images were recorded and analyzed using a fluorescent microscope (IX71) equipped with DP Controller and DP Manager (Olympus, Tokyo, Japan).

### Time-lapse observation

Cells (5x10<sup>4</sup>) 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

morphological changes were monitored by a time-lapse microscope IX81 equipped with an autofocus multi-well flat table and CO<sub>2</sub> 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  $P < 0.05$ .

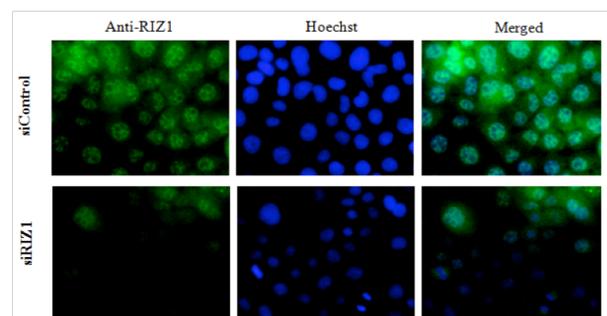
#### Wound healing assay

Cells ( $1 \times 10^5$ ) 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

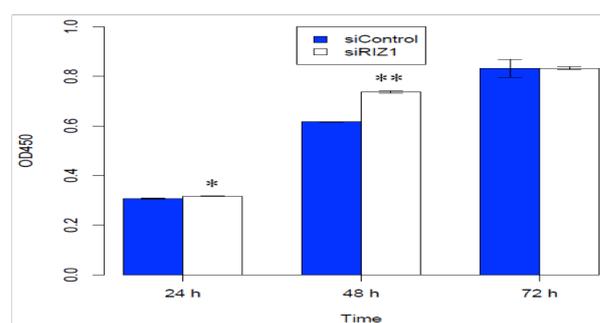


**Figure 3. Reduction of *RIZ1* Expression After 48 h siRIZ1 Transfection in M213.** Nuclear localization of *RIZ1* in siControl treated M213 cells (upper panel) showed the endogenous level of *RIZ1* protein in this cell line. The expression of *RIZ1* was decreased after siRIZ1 transfection (lower panel).

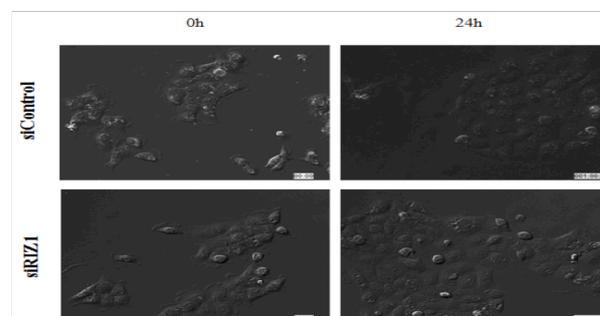
real-time RT-PCR, Western blot, and immunofluorescence staining. Because of non-specific amplification of real-time RT-PCR and non-specific reaction of the antibody detected by Western blot, we were unable to observe the difference between siRIZ1 and siControl transfected cells (data not shown). However, *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

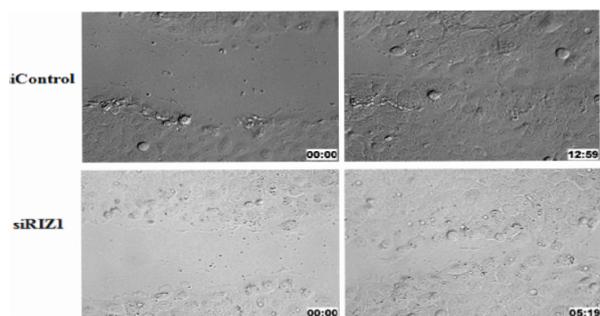
The role of *RIZ1* on cell proliferation was determined by WST assay after 24, 48 and 72 h siRNA transfection (Figure 4). *RIZ1*-knockdown significantly increased the



**Figure 4. WST Assay Illustrated the Increase of Cell Proliferation in siRIZ1 Treated M213.** WST assays were performed after 24, 48 and 72 h transfection. Bar height represents mean of triplicate assays as shown above each bar. The error bar represents standard error of the mean (SEM). \* $P < 0.05$ , \*\* $P < 0.01$  compared to control at the same time.



**Figure 5. Suppression of *RIZ1* Expression by siRIZ1 Increased Cell Proliferation of M213 (Lower Panel) Compared to siControl Transfected Cells (Upper Panel).** Time presented in day:hour:minute format is shown at the right lower corner of each frame.



**Figure 6. Decreased *RIZ1* expression by siRIZ1 increased cell migration by reducing time for wound healing in M213.** Time presented in hour:minute is shown at the right lower corner of each frame.

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 si*RIZ1* 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 si*RIZ1* for 48 h, wound was created by scratch assay, and cell migration was monitored under time-lapse microscope. si*RIZ1* 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  $\lambda$ gt11 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 concatemer 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  $17\beta$ -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 si*RIZ1*.

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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