RESEARCH NOTE

SMALL RNA INTERFERENCE INHIBITION OF UBIQUITIN-SPECIFIC PROTEASE 14 GENE EXPRESSION IN INTRAHEPATIC CHOLANGIOCARCINOMA PRIMARY CELLS

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Abstract. Studies on genomic instabilities of intrahepatic cholangiocarcinoma (ICC) from Thai patients have shown 52% variations in a specific region of ubiquitin-specific protease 14 gene (*USP14*) located on 18p11.32. Inhibition of *USP14* expression in three different primary cells cultured from resected ICC tissues by transfecting with double-strand RNA interference targeting exon 16 region of *USP14* mRNA resulted in 6-50% reduction in mRNA levels, with cells with low *USP14* expression being more affected. These findings lend support to a potential in developing strategies against USP14 in ICC.

Keywords: intrahepatic cholangiocarcinoma, primary cell culture, RNAi, ubiquitin-specific protease 14

INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor of bile duct epithelium, categorized into three subtypes according to its anatomical location, namely, intrahepatic, perihilar and distal (DeOliveira *et al*, 2007). Each subtype has a unique epidemiology, prognosis and clinical management. As CCA can be misdiagnosed as hepatocellular carcinoma, a biopsy for histopathological assessment is necessary (Rizvi *et al*, 2018). CCA is a more common malignancy in Asia, but its occrence in Europe and North America has significantly increased in the past decade (Blechacz, 2017). Chronic inflammation from primary sclerosing cholangitis can trigger cell differentiation, especially in perihilar region (Bergquist *et al*, 2002; Claessen *et al*, 2009; Chapman *et al*, 2010; Chapman *et al*, 2012). Patients with bile duct cystic disorder (Caroli's disease) have a relatively higher risk for CCA early in life (Söreide *et al*, 2004; Welzel *et al*, 2007; Tyson and El-Serag, 2011). However, the highest global prevalence of CCA is found in northeastern Thailand, a suspected

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etiology being liver fluke *Opistochorchis viverrini* infection common in this region of the country owing to the local practice of consuming raw fermented infected fish (Hughes *et al*, 2017).

Target genes for chemotherapy and/or gene disruption in proliferative and inflammatory CCA include fused *FGFR2* and mutant *IDH1* and *IDH2* (Razumilava and Gores, 2014), DNA repair genes (*GSTO1*, *MTHFR*, *TYMS*, and *XRCC1*), toxin protective genes (*ABCC2*, *CYP1A2* and *NAT2*), and immunological surveillance genes (*KLRK1*, *MICA* and *PTGS2* (Tyson and El-Serag, 2011). Expression of *ubiquitin-specific protease 14* (*USP14*) is related to cholangiocarcinoma cell differentiation (Chuensumran *et al*, 2011).

Here, the effects of inhibiting by means of RNA interference (RNAi) of *USP14* expression in primary cells derived from intrahepatic cholangiocarcinoma (ICC) of a Thai patient were investigated as a promising target for ICC therapy.

MATERIALS AND METHODS

Primary cell cultures

Primary cell cultures were prepared from specimens of human ICC obtained from patients undergoing surgical resection as previously described (Tengchaisri *et al*, 1995). Cell cultures were maintained in Ham's F12 medium (Gibco/BRL, Life Technologies, Milan, Italy) supplemented with 15% fetal bovine serum, 10 ng/ml epithelial growth factor, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml gentamycin at 37°C in a humidified atmosphere of 5% CO_2.

The research protocol was approved by The Joint Research Ethics Committees (JREC), Army Medical Department, Thailand (no. S007q).

Double-strand (ds)RNAi construction and transfection

DsRNAi targeting exon 16 (coding amino acids: SIVTPEDILR LSGGGDWHIA YVLLYGPRRV EIMEEESEQ) of human *USP14* mRNA (Stealth-USP14) employed was USP14-HSS113437 (sense) (5′-AUAUCUUCUGGUGUUACGAUG-CUGA-3′) and USP14-HSS113437 (antisense) (5′-UCAGCAUCGUAA-CACCAGAAGAUAU-3′) (Invitrogen Thermo Fisher Scientific Inc, Carlsbad, CA), and a dsRNAi with low GC-content (cat. no. 12935-200; Invitrogen Thermo Fisher Scientific Inc, Carlsbad, CA) was used as negative control. These dsRNAi constructs were transfected into primary ICC cells in a 12-well plate (1×10^5 cells/well) by means of Lipofectamine® LTX reagent (Invitrogen Thermo Fisher Scientific Inc, Carlsbad, CA), which then were incubated as described above for 24 hours.

Quantitative assessment of *USP14* mRNA level in transfected cells

Quantification of transfection efficacy was conducted by measuring *USP14* mRNA level in transfected cells. RNA was extracted using a TRI REAGENT (Sigma-Aldrich, St Louis, MO) and cDNA synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Life Technologies Corporation/Thermo Fisher Scientific, Carlsbad, CA) containing SuperScript® III Reverse Transcriptase (M-MLV RT), Platinum® *Taq* DNA polymerase and random primers. Levels of *USP14* cDNA was quantified using quantitative (q)PCR as previously described (Chuensumran *et al*, 2011), employing USP14 forward (5′-ACCCTCAGCCAAAACTGTC-3′) and reverse (5′-AGGGCTGCAGTAATATA-CTG-3′) primers, and β-actin forward (5′-AGCGAGCATCCCCCAAAGTT-3′)

and reverse (5′-GGGCACGAAGGCTCATCATT-3′) primers as internal control. In brief, reaction mixture (20 µl) contained 18.0 µl of LightCycler FastStart DNA SYBR Green I Master Mix (Roche Diagnostics, Mannheim, Germany), 3.2 µl of $MgCl_2$ (4 mM), 2 µl each of forward and reverse primer (0.5 µM), 2 µl of LightCycler FastStart Enzyme (Roche Diagnostics, Mannheim, Germany), 2 µl of cDNA (40 ng), and 8.8 µl of distilled water. Thermocycling was performed in a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) as follows: 15 cycles of 95˚C for 15 seconds, 67-57˚C (step delay touchdown) for 5 seconds and 72˚C for 20 seconds. Relative quantification of *USP14* cDNA level was determined using $2^{-\Delta\Delta Ct}$ method (Ct, cycle threshold) normalized to β-actin cDNA (Livak and Schmittgen, 2001). Each experiment was conducted in triplicate.

RESULTS

Primary ICC cells prepared from three resected tissues were characterized as moderately differentiated cells--primary ICC cells #1, #2 and #4. Transfection of the three primary ICC cells with Stealth-USP14 dsRNAi targeting *USP14* mRNA using Lipofectamine® (Invitrogen, Life Technologies Corporation/Thermo Fisher Scientific, Carlsbad, CA) resulted in relative reduction in *USP14* expression ranging from 56-95% (Table 1). A significant result simply means that the designed RNAi had substantially blocked *usp14* gene. Although primary cells usually have a limited lifespan, they offer a huge number of advantages compared to cell lines that do not represent the original tissue/gene, resulting in false negative or false positive findings.

DISCUSSION

USP14 inhibits proteasome activity thereby reducing cellular protein turnover (Lee *et al*, 2010). USP14 is up-regulated in non-small-cell lung carcinoma (NSCLC) tumor tissues of patients with poor prognosis (Wu *et al*, 2013) and its expression is associated with androgen-responsive prostate and breast cancers (Liao *et al*, 2017; Liao *et al*, 2018). Knockdown of *USP14* expression inhibits *in vitro* proliferation and tumorigenesis of esophageal squamous carcinoma cells (Zhang *et al*, 2017). The present study shows *USP14* expression in primary ICC cells was amenable to inhibition by RNAi targeting exon 16, but results were variable among the three primary ICC cell tested, but primary ICC cells with low levels of *USP14* mRNA were more affected (Table 1). Further studies are needed to understand how reduction in *USP14* expression affects primary ICC cell properties, *eg* migration and invasion assays.

In conclusion, the study suggests USP14 might be a promising target for novel antitumor therapies.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Table 1
Relative *ubiquitin-specific protease 14* (*USP14*) mRNA expression levels in human intrahepatic cholangiocarcinoma primary cells transfected with double-strand RNA interference (dsRNAi) determined by quantitative PCR.

Primary ICC cells	Ct *β-Actin* (internal control) mean ± SD		ΔCt *β-Actin* mean ± SD	Ct *usp14* mean ± SD		ΔCt *usp14* mean ± SD	ΔΔCt (Δ*usp14* - Δ*β-Actin*)	Relative *usp14* expression equal to $2^{-\Delta\Delta Ct}$	Percent *usp14* expression
	N	T		N	T				
s+_1	19.27±0.24	18.92±0.37	-0.35±0.35	26.65±0.28	30.56±0.46	3.91±2.17	4.26	0.05	5.22
s-_1	19.27±0.00	19.27±0.00	0.00±0.00	26.65±0.00	26.65±0.00	0.00±0.00	0.00	1.00	100.00
s+_2	20.55±0.32	20.03±0.36	-0.52±0.42	34.27±0.34	34.92±0.44	0.65±0.50	1.17	0.44	44.44
s-_2	20.55±0.00	20.55±0.00	0.00±0.00	34.27±0.00	34.27±0.00	0.00±0.00	0.00	1.00	100.00
s+_4	33.83±0.32	33.34±0.27	-0.49±0.38	13.51±0.53	16.37±0.22	2.86±1.61	3.35	0.10	9.81
s-_4	33.83±0.00	33.83±0.00	0.00±0.00	13.51±0.00	13.51±0.00	0.00±0.00	0.00	1.00	100.00

ICC: Intrahepatic cholangiocarcinoma; s+_1: primary ICC cell #1; s+_2: primary ICC cell #2; s+_4: primary ICC cell #4; s-_1: negative case control for primary ICC cell #1; s-_2: negative case control for primary ICC cell #2; s-_4: negative case control for primary ICC cell #4; N: non-transfected cells; T: transfected with Stealth-USP14 dsRNAi for s+ cells and dsRNAi with low GC-content for s- cells; SD: standard deviation; Ct: Cycle threshold; ΔCt *β*-Actin: Ct of transfected cells minus Ct of non-transfected cells using the *β*-Actin primers; ΔCt *usp14*: Ct of transfected cells minus Ct of non-transfected cells using the *usp14* primers; ΔΔCt: ΔCt *usp14* minus ΔCt *β*-Actin.

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