incubation, the filters were fixed and stained with hematoxylin and counted in five random high-power fields under a light microscope.

Cell invasion assay

The invasion of cholangiocarcinoma cells was assayed in 24-well Biocoat Matrigel invasion chamber (8μm; Becton Dickinson, Franklin Lakes, NJ). $5x10^4$ cells were seeded in the upper chamber. The bottom chamber contained 40 ng/ml of HGF. After 24 h of incubation, the invading cells at the lower surface of Matrigel-coated membrane were fixed with 70% ethanol, stained with hematoxylin and counted in five random 100x power fields under a light microscope.

Detection of actin cytoskeleton

Cholangiocarcinoma cells were treated with siRNA specific to c-Met or control, seeded on coverslips and incubated for 24 h. Then the cells were incubated in serum-free medium containing with 40 ng/ml of HGF for 4 h. The cells were fixed with 4% paraformaldehyde, permeabilized in 1% Triton X-100 for 15 min and blocked with 1% BSA. The cells were exposed to rhodamine phalloidin for 30 min and washed with TTBS. Then coverslips were mounted on the slide-glass using 50% glycerol in PBS. The cells were examined under a fluorescent microscope (Olympus).

Statistical analysis

The experiments were all performed in triplicate and identical results were obtained. Values were expressed as the mean and SD. The student's *t*-test was used for analysis of the cell proliferation and invasion assay. The *p* value of less than 0.05 was considered significant.

Results

Expression of c-Met in cholangiocarcinoma cells

The expression of c-Met in two cholangiocarcinoma cell lines (RMCCA1 and HuCCA1) was investigated. Western blot analysis demonstrated definite expression of c-Met in both cholangiocarcinoma cell lines (Fig.1).

The effect of HGF on cholangiocarcinoma cell proliferation

Since the activation of c-Met with HGF was known to play an important role in cell proliferation in many kinds of cancer cells, we investigated the role of HGF in cholangiocarcinoma cell proliferation. Cell proliferation assay was performed in HuCCA1 and RMCCA1 cells treated with HGF at concentrations of 0, 20, 40 and 100 ng/ml. After 24 h of incubation, the results showed that HGF had no effect on cholangiocarcinoma cell proliferation (Fig. 2).

The effect of HGF on cholangiocarcinoma cell migration and invasion

To study the mechanism by which HGF induced the migration of cholangiocarcinoma cells, cell migration assay was performed. We found that HGF induced the migration of RMCCA1 and HuCCA1. Their maximum effect was identified at 40 ng/ml of HGF. The maximal migration indices were 250 ± 20%. To test whether HGF induced cholangiocarcinoma cell invasion, standard invasion assay was performed with RMCCA1 and HuCCA1 cells treated with HGF at the concentration of 40 ng/ml. HGF enhanced cholangiocarcinoma cell invasion more than 2 folds compared with untreated cells (Fig. 3).

The effect of HGF on the phosphorylation of MEK1/2 in cholangiocarcinoma cells

We attempted to evaluate the signaling pathways relevant to HGF-induced invasion of cholangiocarcinoma cell. The phosphorylation of signal molecules, which was previously demonstrated as c-Met, mediated signaling molecules, was assayed by western blot analysis. RMCCA1 and HuCCA1 cells were treated with HGF and then the cell lysate was used for detection of the phosphorylation of MEK1/2, MAPK and Akt. HGF-treated cells demonstrated a higher extent of the phosphorylated MEK1/2 and MAPK than untreated cells. However, the phosphorylated Akt was detected only in HGF-treated RMCCA1 cells (Fig. 4).

The effect of c-Met siRNA on the expression of c-Met and the effect of c-Met siRNA on HGF-induced phosphorylation of MEK1/2 in cholangiocarcinoma cells

To determine whether the activation of c-Met and its signal transduction MEK1/2 are necessary for cholangiocarcinoma cell invasion, cells were transfected with siRNA targeted to c-Met. The expression of c-Met mRNA was detected by real time RT-PCR and western blot analysis. The significant suppression of c-Met was identified in HuCCA1 and RMCCA1 cells transfected with c-Met siRNA at 48 hours comparing with control siRNA (Fig.5). The HGF-induced phosphorylation of MEK1/2 was also investigated after treated the cells with c-Met siRNA or control siRNA. The phosphorylation of MEK1/2 in c-Met siRNA treated cells was extensively lower than in control siRNA treated cells (Fig.6).

The effect of c-Met siRNA or MEK1/2 inhibitor U0126 on HGF-induced cholangiocarcinoma cell proliferation and cell invasion

Activation of c-Met with HGF had no effect on HuCCA-1 and RMCCA1 cell proliferation. Furthermore, the treatment of U0126 or c-Met siRNA to cholangiocarcinoma cells did not show inhibitory effect on cell proliferation (Fig.7).

suppression of c-Met expression. The findings provided several data about the significant molecules that promote cholangiocarcinoma cell invasion. We identified that cholangiocarcinoma cells express c-Met both RNA level and protein level and stimulation of c-Met with HGF promotes cancer cell migration and invasion. Our studies suggested that these events involved the activation of the extracellular signal-regulated kinase (ERK) cascade, a central pathway that transmits signals from many extracellular agents to regulate cellular processes. The latter assertion is based on the finding that inhibition of c-Met expression by siRNA or inhibition of MEK1/2 by its specific inhibitor U0126 suppressed the phosphorylation of MEK1/2 and also inhibited the invasiveness property of cholangiocarcinoma cell. MEK1/2 is dual specificity protein kinases that function in a mitogen activated protein kinase cascade controlling cell growth and differentiation [12]. Activation of MEK1/2 occurs to phosphorylation of two serine residues at position 217 and 221 (in the activation loop of subdomain VIII) by Raf-like molecules. MEK1/2 is activated by a wide variety of growth factors and cytokines, and also by membrane depolarization and calcium influx [13]. Although, some reports suggested that HGF was a potent stimulator for Akt phosphorylation and cancer cell proliferation [14], other reports including this study made exactly opposite conclusions [4]. Stimulation of c-Met with HGF has neither effect on Akt phosphorylation nor cell proliferation in HuCCA1 cells. Evidence existed that the effect of c-Met to enhance cancer cell proliferation may need the activation of PI3-kinase pathway which was not defined in HuCCA1 cells. The different biological functions may come from different activations of the multifunctional docking site of c-Met. In cancer cells, high levels of actin polymerization are important for the formation of pseudopodia, which in turn are implicated in the enhancement of cancer cell migration and

invasion [15]. This study showed that the treatment of cholangiocarcinoma cells with HGF resulted in the increase in actin polymerization. In addition, inhibition of c-Met expression with c-Met siRNA resulted in a dramatic decrease in action polymerization. These findings suggested that HGF and c-Met plays an important role in the invasion as well as the metastasis in cholangiocarcinoma.

In conclusion, this experiment showed that the stimulation of c-Met plays an important role in cholangiocarcinoma cell invasion. Inhibition of c-Met and its pathway could become one of the potential approaches for cholangiocarcinoma therapy.

Acknowledgements

We acknowledge support from the Thailand Research Fund (2005) and grants from medical biotechnology center department of medical science, Thailand. The sequence of primers and the condition of real-time PCR were kindly provided by Dr. Kazuhiro Mizumoto, Department of Surgery and Oncology, Graduate school of Medical Sciences, Kyushu University.

References

1. Chamberlain, R.S., and Blumgart, L.H. Hilar cholangiocarcinoma. a review and commentary. Ann Surg Oncol. 7(1): 55, 2000
2. Washburn, W.K., Lewis, W.D., and Jenkins, R.L. Aggressive surgical resection for cholangiocarcinoma. Arch Surg. 130(3): 270, 1995
3. Ayhan, A., Ertunc, D., Tok, E.C, and Ayhan, A. Expression of the c-Met in advanced epithelial ovarian cancer and its prognostic significance. Int J Gynecol Cancer 15(4): 618, 2005
4. Coltella, N., Manara, M.C., Cerisano, V., Trusolino, L., Di Renzo, M.F., Scotlandi, K. , and Ferracini, R. Role of the MET/HGF receptor in proliferation and invasive behavior of osteosarcoma. FASEB J. 17(9): 1162, 2003
5. Olivero, M., Rizzo, M., Madeddu, R., Casadio, C., Pennacchietti, S., Nicotra, M.R, Prat, M., Maggi, G., Arena, N., Natali, P.G., Comoglio, P.M., and Di Renzo, M.F. Overexpression and activation of hepatocyte growth factor/scatter factor in human non-small-cell lung carcinomas. *Br J Cancer*. 74: 1862, 1996
6. Ren, Y., Cao, B., Law, S., Xie, Y., Lee, P.Y., Cheung, L., Chen, Y., Huang, X., Chan, H.M., Zhao, P., Luk, J., Vande Woude, G., and Wong, J. Hepatocyte growth factor promotes cancer cell migration and angiogenic factors expression: a prognostic marker of human esophageal squamous cell carcinomas. Clin Cancer Res 11(17): 6190,2005
7. Aishima, S.I., Taguchi, K.I., Sugimachi, K., Shimada, M., Sugimachi, K., and Tsuneyoshi, M. c-erbB-2 and c-Met expression relates to cholangiocarcinogenesis and progression of intrahepatic cholangiocarcinoma. Histopathology 40(3): 269, 2002

8. Christensen, J.G, Burrowsc, J., and Salgia, R. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. Cancer Lett 225: 1, 2005
9. Zhang, Y.W., and Vande Woude, G.F. HGF/SF-met signaling in the control of branching morphogenesis and invasion. J Cell Biochem 88(2): 408, 2003
10. Gao, C.F., and Vande Woude, G.F. HGF/SF-Met signaling in tumor progression. Cell Res 15(1): 49, 2005
11. Leelawat, K., Ohuchida, K., Mizumoto, K., Mahidol, C., and Tanaka, M. All-trans retinoic acid inhibits the cell proliferation but enhances the cell invasion through up-regulation of c-met in pancreatic cancer cells. Cancer Lett. 224(2): 303, 2005
12. Ohren, J.F., Chen, H., Pavlovsky, A., Whitehead, C., Zhang, E., Kuffa, P., Yan, C., McConnell, P., Spessard, C., Banotai, C., Mueller, W.T., Delaney, A., Omer, C., Sebolt-Leopold, J., Dudley, D.T., Leung, I.K., Flamme, C., Warmus, J., Kaufman, M., Barrett, S., Tecle, H., and Hasemann, C.A. Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel noncompetitive kinase inhibition. Nat Struct Mol Biol. 11(12): 1192, 2004
13. Gomez, E., Pritchard, C., and Herbert, T.P. cAMP-dependent protein kinase and Ca2+ influx through L-type voltage-gated calcium channels mediate Raf-independent activation of extracellular regulated kinase in response to glucagon-like peptide-1 in pancreatic beta-cells. J Biol Chem. 277(50): 48146, 2002
14. Maulik, G., Madhiwala, P., Brooks, S., Ma, P.C., Kijima, T., Tibaldi, E.V., Schaefer, E., Parmar, K., and Salgia, R.. Activated c-Met signals through PI3K with dramatic effects on cytoskeletal functions in small cell lung cancer. J Cell Mol Med 6(4): 539, 2002

15. Kirfel, G., Rigort, A., Borm, B., and Herzog, V. Cell migration: mechanisms of rear detachment and the formation of migration tracks. Eur J Cell Biol 83(11-12): 717, 2004

FIGURES

Figure 1: Expression of c-Met in cholangiocarcinoma cells.

HuCCA1 and RMCCA1 were cultured in HAM 's F12 media supplemented with 10% FBS for 48 h and The cell lysate was assayed for c-Met expression by Western blot. The c-Met protein, characterized as paralleled bands, a precursor form (p170) and a β subunit (p145b) was identified in both cell lines.

Figure 2: Effect of HGF on the proliferation of cholangiocarcinoma cells.

HuCCA1 and RMCCA1 were treated with HGF at various concentrations (0-100 ng/ml). After 24 h, cell proliferation assay was performed by using WST-1. The absorbance at 450 nm, against a reference wave length of 650 nm, was determined.

Figure 3: Effect of HGF on the migration and invasion of cholangiocarcinoma cells

A. Migration of cholangiocarcinoma cells was assayed as described in the Materials and Methods. HGF induced the migration of cholangiocarcinoma cells. The maximum effect was observed at 40 ng/ml of HGF.
B. Invasion of cholangiocarcinoma cells was assayed using a Biocoat Matrigel invasion chamber, which consists of 8-μm pore filters that has been overlaid with Matrigel. HGF induced the invasion of cholangiocarcinoma cells. *, $p<0.05$ compared with control.

Figure 4: Western blot analysis of MEK1/2, MAPK and Akt phosphorylation in HGF-treated cholangiocarcinoma cells

RMCCA1 and HuCCA1 cultured in serum-free medium for 48 h were treated with indicated doses of HGF for 15 min and the cell lysate was obtained. MEK1/2, MAPK and Akt phosphorylation were determined by western blot.

Figure 5: The suppression of c-Met expression in cholangiocarcinoma cells by c-Met siRNA.

(A) Cells were transfected with c-Met siRNA or control siRNA. After 48 h of incubation, the expression of c-Met was detected by real time RT-PCR. c-Met was significantly suppressed in c-Met siRNA treated cells comparing with control siRNA treated cells. *, $p<0.05$ compared with control.

(B) c-Met expression was detected by western blot. The significant suppression of c-Met expression was clearly detected in RMCCA1 cholangiocarcinoma cells treated with c-Met siRNA.

Figure 6: The effect of HGF and c-Met siRNA on the phosphorylation of MEK1/2

(A) RMCCA1 and HuCCA1 were transfected with the c-Met siRNA and control siRNA for 48 h and then treated with 40 ng/ml of HGF for 15 min. MEK1/2 phosphorylation (P-MEK) was determined by western blot. c-Met siRNA transfection significantly inhibited the phosphorylation of MEK1/2 induced by HGF.

(B) Results from three biologically separate experiments, showing mean levels of P-MEK expression ± SD resulting from c-Met siRNA transfection relative to levels from cells transfected with control siRNA

Figure 7: The effect of HGF and MEK1/2 inhibitor (U0126) on cell proliferation in HuCCA1 cells transfected with c-Met siRNA and control siRNA

RMCCA1 and HuCCA1 transfected with the c-Met siRNA and control siRNA for 48 h were treated with and without U0126 and then stimulated with 40 ng/ml of HGF for 24 h. Cell proliferation assays were determined by WST-1. The data represent the average results from 3 individual experiments.

Fig 8: The suppression of migration and invasion activity of cholangiocarcinoma cells after treatment with c-Met siRNA, control siRNA and MEK inhibitor (U0126)

(A) RMCCA1 and HuCCA1 transfected with the c-Met siRNA and control siRNA for 48 h then were treated with and without U0126. $5x10^4$ cells were seeded in the 8-μm pore filters (Transwell, 24-well cell culture, Coster, Boston, MA). The bottom chamber contained 40 ng/ml of HGF. After 24 h, the cells on the lower surface were counted under a microscope at five random 100x power fields. The experiment was repeated for 3 times and the data represent the average results from 3 individual experiments.

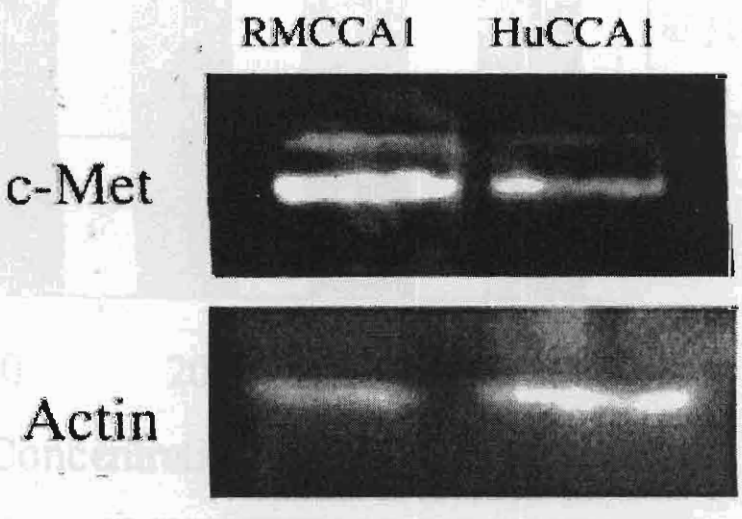
(B) RMCCA1 and HuCCA1 transfected with the c-Met siRNA and control siRNA for 48 h then were treated with and without U0126. $5x10^4$ cells were seeded in

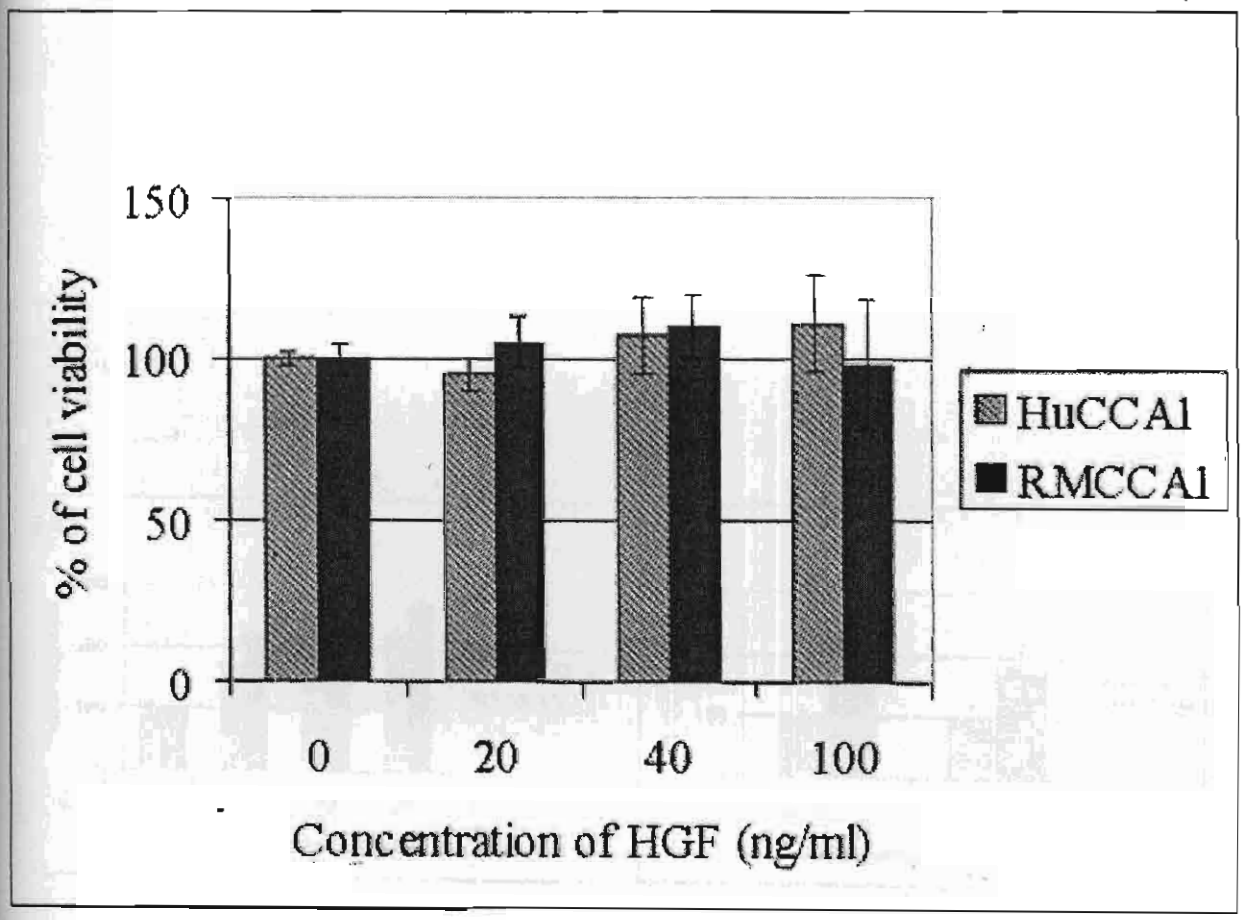
the 24-well Biocoat Matrigel invasion chamber. The bottom chamber contained 40 ng/ml of HGF. After 24 h, the cells on the lower surface were assayed as previously described.

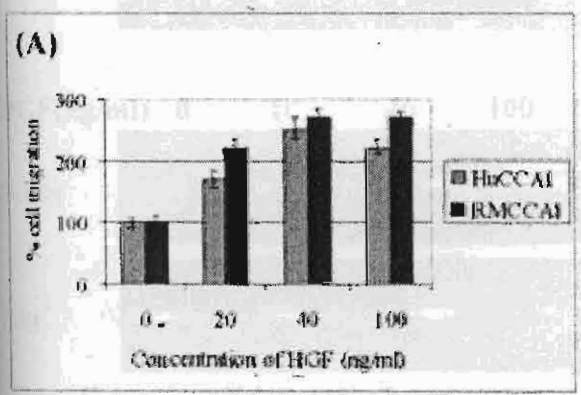
Figure 9: *Effect of HGF and c-Met on actin cytoskeleton in RMCCA1 cells*

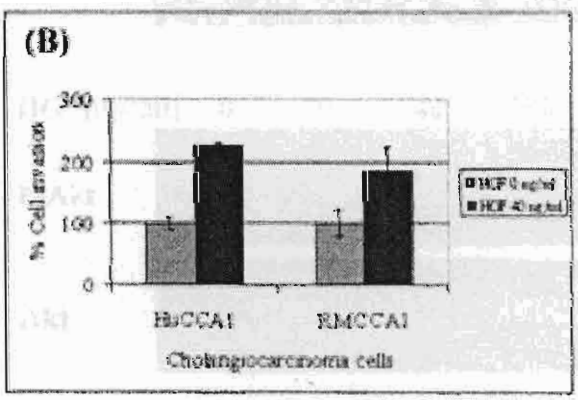
RMCCA1 cells were treated with 40ng/ml HGF and incubated for 2 h. Then cells were stained with rhodamine-phalloidin to visualize actin cytoskeleton. Cells were transfected with control siRNA or c-Met siRNA and then treated with HGF

re to download high resolution image

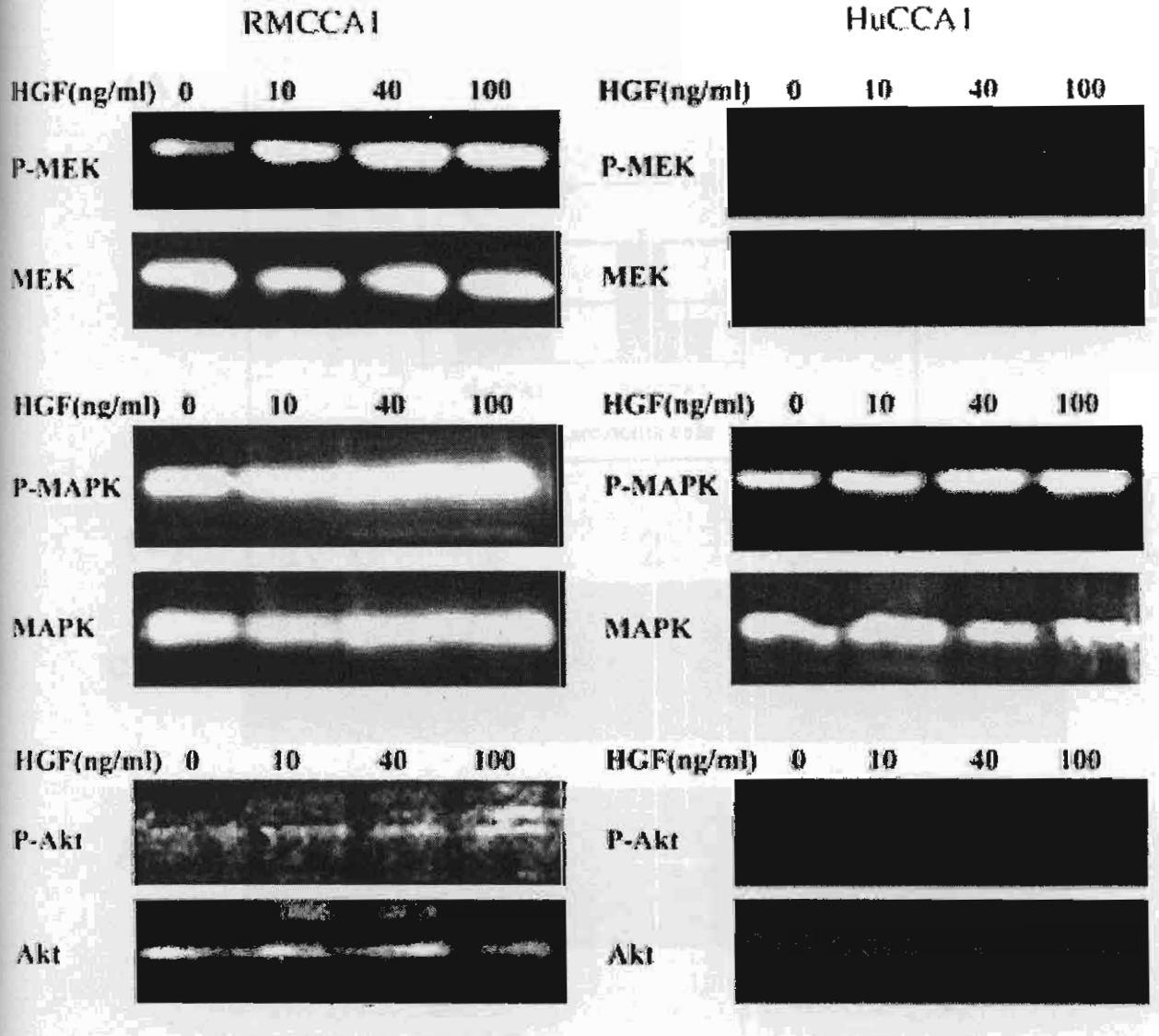




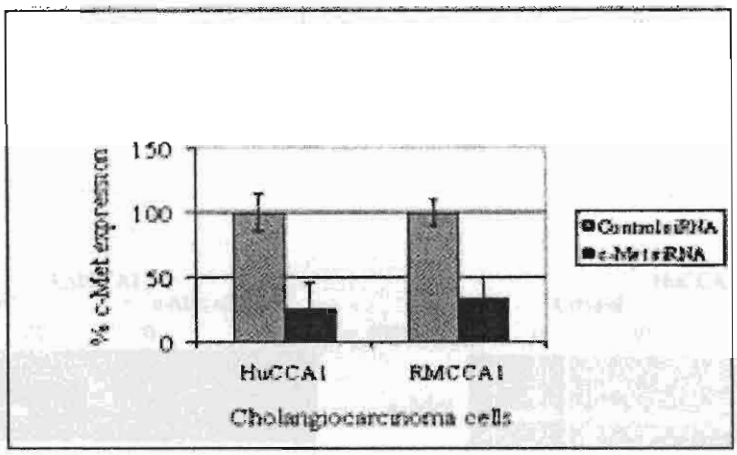




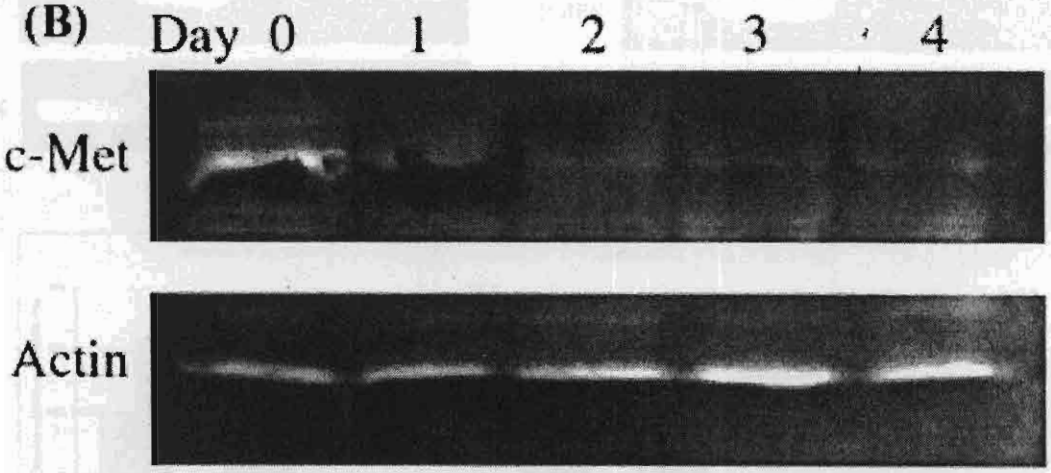
e 4
here to download high resolution image

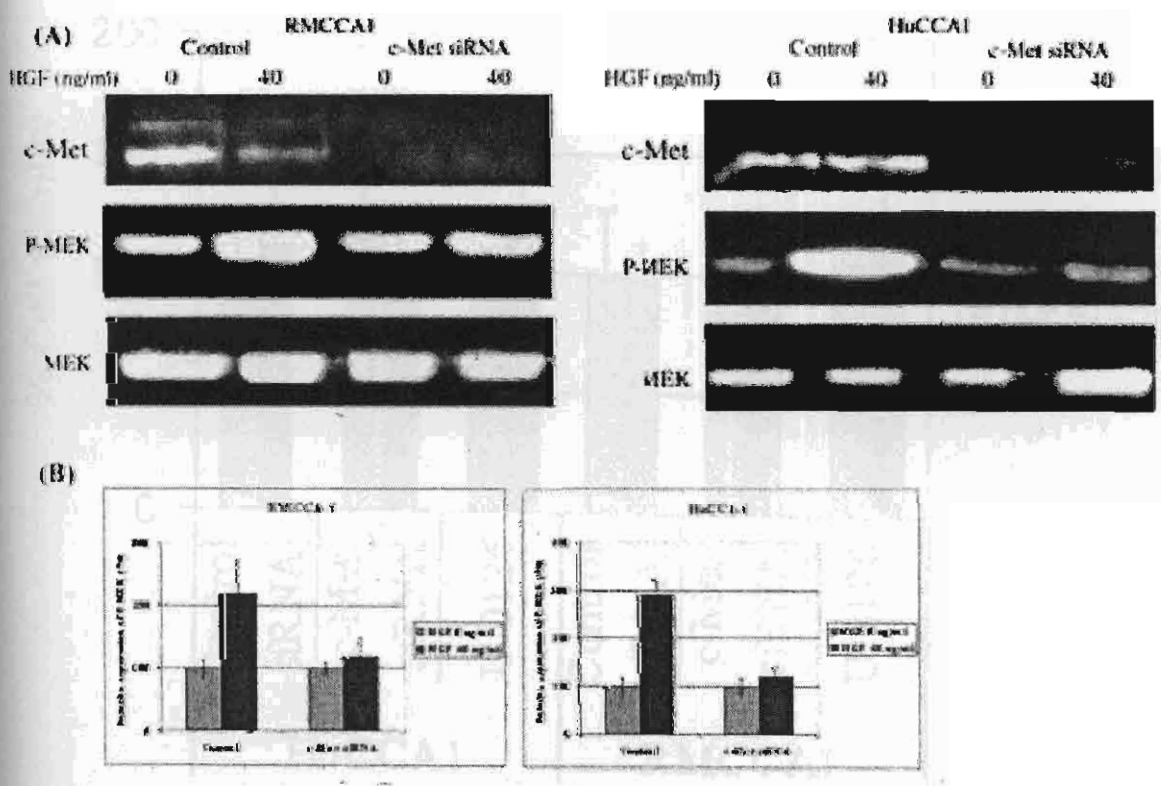


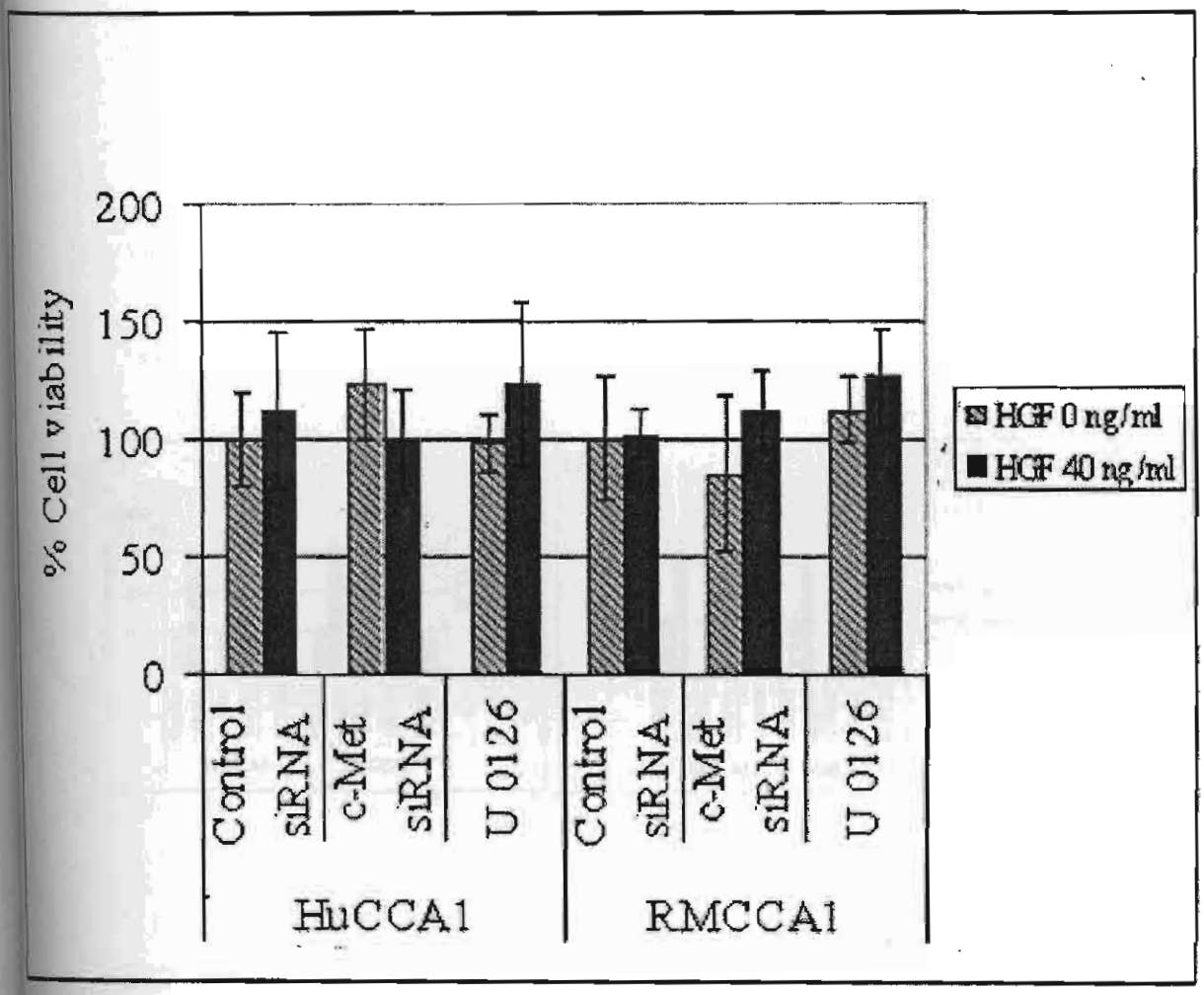
(A)



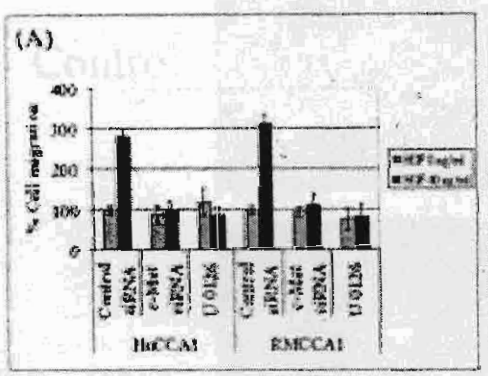
(B)

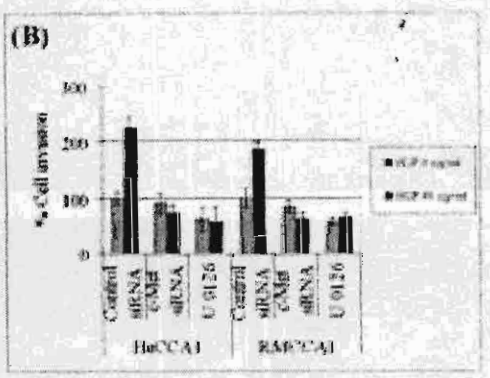




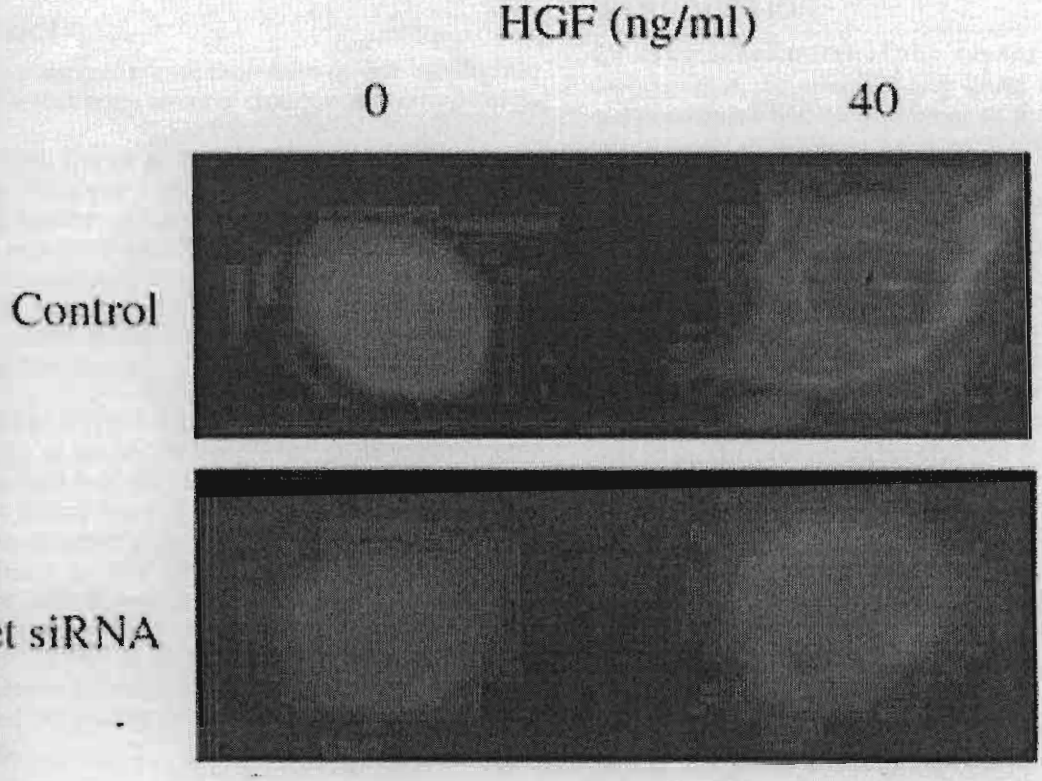


re to download high resolution image





g
here to download high resolution image



PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 July 14; 12(26): 4195-4198
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Circulating hTERT mRNA as a tumor marker in cholangiocarcinoma patients

Kawin Leelawat, Surang Leelawat, Thawee Ratanachu-Ek, Somboon Trubwongchareon, Jerasak Wannaprasert, Saad Tripongkaruna, Suchart Chantawibul, Panadda Tepaksorn

Kawin Leelawat, Thawee Ratanachu-Ek, Somboon Trubwongchareon, Jerasak Wannaprasert, Saad Tripongkaruna, Suchart Chantawibul, Department of Surgery, Rajavithi Hospital, Bangkok, 10400, Thailand
Kawin Leelawat, Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, 10400, Thailand
Surang Leelawat, Faculty of Pharmacy, Rangsit University, Patumthani, 12000, Thailand
Panadda Tepaksorn, Medical Biotechnology Center, National Institute of Health, 13000, Thailand
Supported by Thailand Research Fund and Rajavithi Hospital Fund
Correspondence to: Kawin Leelawat MD, PhD, Department of Surgery, Rajavithi Hospital, Rajavithi Rd, Rajathevi, Bangkok, 10400, Thailand. kawin_leelawat@hotmail.com
Telephone: +66-2-3548080 Fax: +66-2-3548080
Received: 2006-01-24 Accepted: 2006-02-28

Abstract

AIM: To investigate human telomerase reverse transcriptase (hTERT) mRNA in the serum of cholangiocarcinoma patients.

METHODS: The serum of thirty three cholangiocarcinoma patients, forty one benign biliary tract disease patients and ten healthy volunteers were collected and analyzed for the expression of hTERT mRNA by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). We then examined the correlation between values of serum hTERT mRNA and the pathological staging of cholangiocarcinoma.

RESULTS: hTERT mRNA was detected in 28 of 33 (84.85%) of serum obtained from cholangiocarcinoma patients and 9 of 41 (21.9%) of serum obtained from benign biliary tract disease patients. hTERT mRNA was not detected in any serum obtained from healthy volunteers. on the other hand the common tumor marker, CA19-9 was detected in 20 of 33 (60.6%) of serum obtained from cholangiocarcinoma patients and 8 of 41 (19.5%) of serum obtained from benign biliary tract disease patients. However, no correlation was found between the present of serum hTERT mRNA and tumor staging.

CONCLUSION: These results indicate that the detection of circulating hTERT mRNA was identified in almost all cholangiocarcinoma patients. It offers a novel tumor marker, which can be used as a complementary study for diagnosis of cholangiocarcinoma.

Key words: Cholangiocarcinoma; Telomerase; hTERT; Real-time reverse transcriptase-polymerase chain reaction

Leelawat K, Leelawat S, Ratanachu-Ek T, Trubwongchareon S, Wannaprasert J, Tripongkaruna S, Chantawibul S, Tepaksorn P. Circulating hTERT mRNA as a tumor marker in cholangiocarcinoma patients. *World J Gastroenterol* 2006; 12(26): 4195-4198

http://www.wjgnet.com/1007-9327/12/4195.asp .

INTRODUCTION

Cholangiocarcinoma is the cancer arising from cholangiocyte, the epithelial cells lining the intrahepatic and extrahepatic bile ducts. It is one of the most common liver cancers in the population of Northeast Thailand and responsible for approximately one in five cancer-related deaths among Thai patients[1]. Three-year survival rates of 40%-60% have been reported only in a few number of patients resected for cure[2]. Diagnosis of cholangiocarcinoma is often difficult. It requires multiple complementary studies including evaluation of clinical symptoms, imaging, and tumor markers. Tissue biopsy and cytology have poor sensitivity and are positive only in about 30% of cases of cholangiocarcinoma. Recently, the percentages of positive serum obtained from the common marker (CA19-9) are only less than 70%[3]. In addition, CA19-9 can be elevated in cholestasis in the absence of malignancy, and following liver injury. Thus, their accuracy for the diagnosis of cholangiocarcinoma is limited. It is necessary to find novel markers to use in diagnosis and treatment.

The human telomerase, which composed of two subunits including telomerase RNA template (hTR) and telomerase transcriptase protein (hTERT), functions as a reverse transcriptase enzyme in the process of telomere synthesis[4]. Telomerase activity was detected in 85%-100% of cancer patients whereas normal somatic cells have low or undetectable[4,5]. In addition, previous results demonstrated that circulating tumor-related RNA

including telomerase is frequently found in the plasma and serum of cancer patients[6-8].

Consequently, telomerase activity is possibly used as a common molecular tumor marker in the serum. Previous studies also found a good correlation between the telomerase activity and the expression of hTERT subunit. The aim of this study is to test the usefulness of hTERT mRNA detection in the serum of cholangiocarcinoma patients by using real-time reverse transcriptase polymerase chain reaction.

MATERIALS AND METHODS

Cell lines

The human cholangiocarcinoma cell line HuCCA1 (kindly provided by Prof. Sirisinha, Department of Microbiology, Mahidol University) and RMCCA1 (established from Department of Surgery, Rajavithi Hospital) were grown in Ham's F12 medium supplemented with 100 mL/L fetal bovine serum at 37℃ in a 5% (50 mL/L) CO_2 humidified atmosphere.

Patients and sample preparation

Thirty-nine informed and consenting patients undergoing surgery for cholangiocarcinoma at the Rajavithi Hospital, Thailand, between July 2003 and April 2006 were included in this study. Tumor samples were collected at the time of surgery and histopathologically characterized to confirm the diagnosis. Pathological data, including tumor staging was also collected. Fifty patients undergoing surgery for benign biliary tract disease were included in this study. Ten normal subjects were studied as negative controls.

Sample Collection

Blood was collected prior to surgery in plain tubes for serum sampling. After clotting, tubes were centrifuged at 1000 r/min for 15 min at room temperature, and serum was collected. This was followed by a second 15-min centrifugation at 1000 r/min to remove cellular debris. Serum samples were aliquoted and stored at -70℃ until use. Serum CA19-9 level was measured in Clinical Laboratory of Rajavithi Hospital. The cut-off level was 100 IU/mL.

RNA Extraction

RNA from cell lines and serum was extracted using a commercially available kit (High Pure RNA Kit; Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Only fresh or once-freeze thawed serum was used.

Real time RT-PCR for hTERT mRNA

Quantitative detection of hTERT mRNA was performed with the TeloTAGGG hTERT Quantification Kit (Roche Diagnostics GmbH, Mannheim, Germany), using the LightCycler system (Roche Diagnostics, Mannheim, Germany) for real-time PCR according to the manufacturer's instructions. For the reaction mixtures, 2 μL of hTERT reaction mix, 0.1 μL of reverse transcriptase, 2 μL of hTERT or PBGD mix, 13.9 μL of H_2O and 2 μL of standard RNA template or RNA from serum samples was prepared. The reaction conditions were reverse transcription at 60℃ for 10 min, followed by initial denaturation at 95℃ for 30 s and 40 cycles of denaturation at 95℃ for 0.5 s, annealing at 60℃ for 10 s, and extension at 72℃ for 10 s, respectively. The standard curve was established by determination of the five standards hTERT mRNA provided by the kit. The samples were normalized on the basis of the content of PBGD. Serum samples with more than 150 copies of PBGD suggesting an appropriate quality of RNA were used for the analysis of telomerase. Serum samples in which hTERT mRNA were detected were assigned to the hTERT-positive group.

Table 1 The patient demographic data and blood chemistry data

	Benign biliary tract disease	Cholangiocarcinoma	Healthy volunteers
Sex (Male:Female)	21:20	19:14	6:4
Age (year), (median)	53.68 (22-82)	56.40 (35-85)	49.50 (26-60)
SGOT (IU/dL)	105.32 ± 52.26	112.52 ± 44.54	30.20 ± 12.20
SGPT (IU/dL)	96.04 ± 45.42	73.41 ± 44.30	28.50 ± 9.22
Total Bilirubin (mg/dL)*	3.8 ± 2.21	12.8 ± 5.24	1.05 ± 0.25
Alkaline Phosphatase (U/dL)*	309 ± 67.53	550 ± 24.44	98 ± 10.60

*$P < 0.005$.

Statistical analysis

Values were expressed as mean ± SD. Mean values were measured by the Student's t test. Correlations between serum hTERT mRNA and stage of cholangiocarcinoma were assessed using the chi-square test (χ^2). Sensitivity, specificity, positive predictive value, and negative predictive value were measured. $P < 0.05$ was considered as statistically significant.

RESULTS

Detection of hTERT mRNA in cholangiocarcinoma cells

The expression of hTERT mRNA in two cholangiocarcinoma cell lines (HuCCA1 and RMCCA1) was investigated. Quantitative real-time RT-PCR demonstrated definite expression of hTERT mRNA in both cholangiocarcinoma cell lines (Figure 1). This evidence confirmed the existence of telomerase activity in cholangiocarcinoma. Therefore, we attempted to detect the circulating hTERT mRNA in the serum of cholangiocarcinoma patients.

Detection of serum hTERT mRNA

Thirty-nine patients who were confirmed diagnosis as cholangiocarcinoma, fifty benign biliary tract disease patients and ten healthy volunteers were included in this study. Their serum was collected and extracted for total RNA. Only RNA samples, that could be detected by the expression of porphobilinogen deaminase (PBGD) as a housekeeping gene were included in this study. The thirty-three serum samples from cholangiocarcinoma patients, fortyone serum samples from benign biliary tract disease patients and ten serum samples from healthy volunteers detected for PBGD were as-

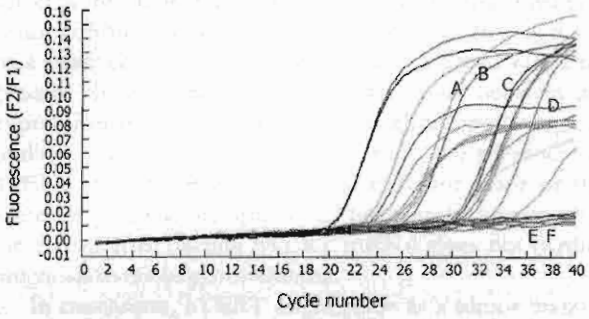


Figure 1 Continuously monitoring the development of signals in parallel standards and samples results in a series of amplification curves. The amplification of a 198 bp fragment of the generated hTERT cDNA was identified. (A: Signal from RMCCA1; B: Signal from HuCCA1; C, D: Signals from serum of cholangiocarcinoma patients; E, F: Signals from serum of benign biliary tract patients).

sayed for the expression of hTERT mRNA (Figure 1). The patients' demographic data was demonstrated in Table 1. There were no differences in sex, age, serum SGOT and serum SGPT between benign and cancer patients. However, total bilirubin and alkaline phosphatase were significantly high in cancer groups.

Serum hTERT mRNA was recognized in 28 of 33 cholangiocarcinoma patients (84.85%) and 9 of 41 benign biliary tract disease patients (21.9%). However, serum hTERT mRNA was not detected in any healthy volunteers. The efficiency of serum hTERT was compared with the serum CA19-9 as shown in Table 2. Serum hTERT was higher in sensitivity for detection of cholangiocarcinoma than serum CA19-9.

Detection of serum hTERT mRNA in relation to tumor stage

We also evaluated for the association between serum hTERT mRNA and the histopathological staging of cholangiocarcinoma in the surgical specimens resected from these patients. The result showed that serum hTERT mRNA could be detected in all stages of cholangiocarcinoma patients. However, it did not correlate with the staging of cholangiocarcinoma (Table 3).

Table 2 Comparative analysis of serum hTERT mRNA and serum CA19-9 in diagnosis of cholangiocarcinoma

	Serum hTERT (%)	Serum CA19-9 (%)
Sensitivity	84.85	60.6
Specificity	78.05	80.49
Positive predictive value	75.68	71.43
Negative predictive value	86.49	71.74
False Negative	13.51	28.26
False Positive	24.32	28.6

Table 3 Detection of serum hTERT mRNA in relation to tumor staging

Tumor stages	Serum hTERT mRNA positive	negative
Stage I	2	1
Stage II	17	3
Stage III	9	1

DISCUSSION

Eukaryotic chromosomal ends consist of repeating DNA sequences (TTAAGG) termed telomeres. An enzyme that adds telomeric repeats onto chromosomal ends is telomerase. This enzyme is composed of two subunits; hTR and hTERT[6]. Accordingly, both hTR and hTERT are necessary for telomerase activity, yet the catalytic activity of the enzyme is generally regulated through the presence and activity of hTERT. Therefore, the detection of hTERT mRNA is a guarantee for the present of telomerase activity[8]. In most somatic cells in which telomerase activity is undetectable, telomeric sequences are lost with each cell division because of the end-replication problem. Unlike healthy cells, most malignant human cells are capable of escaping senescence and sustaining infinite proliferation through the activation of telomerase to stabilize their telomere length[4,8-12].

Traditionally, telomerase activity has been assessed based on a biochemical primer extension assay, the inefficiency and low sensitivity of which, together with the low amounts of telomerase activity, greatly limit the application of the assay in primary human tumors[9]. Therefore, the detection of hTERT by real-time RT-PCR was used in this study. This assay determined the expression of telomerase by measuring the amount of the mRNA encoding its catalytic subunit hTERT. The relative telomerase expression levels were determined by comparing them to the expression levels of the housekeeping gene porphobilinogen deaminase (PBGD). hTERT-encoding mRNA from the serum was reverse transcribed and 198 bp fragments of the generated cDNA was amplified with specific primers in a one step RT-PCR reaction. The amplicon was detected by fluorescent emission using a specific pair of hybridization probes. In a separate RT-PCR, mRNA encoding for porphobilinogen deaminase (PBGD) was processed. The reaction product served as both a control for RT-PCR performance and as a reference for relative quantification.

Our study showed that hTERT mRNA was detected in almost all of the cholangiocarcinoma patients (84.85% of cases). However, in benign biliary tract disease patients, hTERT mRNA was also detectable (21.9% of cases). According to the previous report, telomerase activity has been reported in normal lymphocytes[8-12]. This result suggested the contamination of lymphocytes in the serum specimens. Comparison with the common tumor marker, CA19-9 was detected in only 60.60% of cases. This data suggested that hTERT mRNA should be a candidate tumor marker in cancer patients.

hTERT mRNA is not specifically detected in serum of cholangiocarcinoma patients but it was also significantly found in several types of cancers such as breast cancer, malignant melanoma and thyroid cancer[10]. Certainly, we have detected hTERT mRNA in the serum of five patients with hepatocellular carcinoma and three patients with pancreatic cancers (data not shown). This indicates that the

detection of circulating extracellular tumor-derived mRNA is not confined to any one cancer type, but may actually be a relatively ubiquitous finding across a broad range of cancers. However, no relationship was found between the expression of hTERT mRNA and clinicopathological findings. According with previous study, the presence of hTERT was unrelated to tumor size, tumor grade or the presence of nodal metastasis[12]. These results suggest that the detection circulating hTERT mRNA does not predict prognosis in cholangiocarcinoma.

In conclusion, hTERT might serve as a tumor marker, which early identified circulating specific RNA originating from tumor cells. However, further examinations using more cholangiocarcinoma cases are necessary to evaluate the usefulness of this marker.

REFERENCES

1 **Sriplung H**, Sontipong S, Martin N, Wiangnon S, Vootiprux V, Cheirsilpa A, Kanchanabat C, Khuhaprema T. Cancer incidence in Thailand, 1995-1997. *Asian Pac J Cancer Prev* 2005; **6**: 276-281

2 **Chamberlain RS**, Blumgart LH. Hilar cholangiocarcinoma: a review and commentary. *Ann Surg Oncol* 2000; **7**: 55-66

3 **Olnes MJ**, Erlich R. A review and update on cholangiocarcinoma. *Oncology* 2004; **66**: 167-179

4 **Bachand F**, Triki I, Autexier C. Human telomerase RNA-protein interactions. *Nucleic Acids Res* 2001; **29**: 3385-3393

5 **Yan P**, Coindre JM, Benhattar J, Bosman FT, Guillou L. Telomerase activity and human telomerase reverse transcriptase mRNA expression in soft tissue tumors: correlation with grade, histology, and proliferative activity. *Cancer Res* 1999; **59**: 3166-3170

6 **Johnson PJ**, Lo YM. Plasma nucleic acids in the diagnosis and management of malignant disease. *Clin Chem* 2002; **48**: 1186-1193

7 **Fleischhacker M**, Beinert T, Ermitsch M, Seferi D, Possinger K, Engelmann C, Jandrig B. Detection of amplifiable messenger RNA in the serum of patients with lung cancer. *Ann N Y Acad Sci* 2001; **945**: 179-188

8 **Novakovic S**, Hocevar M, Zgajnar J, Besic N, Stegel V. Detection of telomerase RNA in the plasma of patients with breast cancer, malignant melanoma or thyroid cancer. *Oncol Rep* 2004; **11**: 245-252

9 **Mabruk MJ**, O'Flatharta C. Telomerase: is it the future diagnostic and prognostic tool in human cancer? *Expert Rev Mol Diagn* 2005; **5**: 907-916

10 **Chen XQ**, Bonnefoi H, Pelte MF, Lyautey J, Lederrey C, Movarekhi S, Schaeffer P, Mulcahy HE, Meyer P, Stroun M, Anker P. Telomerase RNA as a detection marker in the serum of breast cancer patients. *Clin Cancer Res* 2000; **6**: 3823-3826

11 **Blasco MA**. Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet* 2005; **6**: 611-622

12 **Li YR**, Wu JM, Wang L, Huang X, Shi J, Hu LH. Human telomerase reverse transcriptase expression and its clinical significance in laryngeal squamous cell carcinoma. *Acta Otolaryngol* 2005; **125**: 409-414

S- Editor Wang J **L- Editor** Olaleye SB **E- Editor** Liu Y

Establishment and characterization of a cholangiocarcinoma cell line (RMCCA-1) from a Thai patient

Panthip Rattanasinganchan, Kawin Leelawat, Saad Tripongkaruna, Chintana Tocharoentanaphol, Somboon Trubwongchareon, Tuangporn Suthiphongchai, Rutaiwan Tohtong

Panthip Rattanasinganchan, Rutaiwan Tohtong, Tuangporn Suthiphongchai, Kawin Leelawat,
Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, 10400, Thailand
Kawin Leelawat, Saad Tripongkaruna, Somboon Trubwongchareon, Department of Surgery, Rajavithi Hospital, Bangkok, 10400, Thailand
Chintana Tocharoentanaphol, Kawin Leelawat
Cancer Cytogenetic Unit, Chulabhorn Cancer Center, Chulabhorn Research Institute, Patumthani, 12000, Thailand

Supported by Thailand Research Fund (The Royal Golden Jubilee Ph.D. Program), Thailand Research Fund (New Researcher Program) and Rajavithi Hospital Fund
Correspondence to: Rutaiwan Tohtong Asst. Prof. Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, 10400, Thailand
scrth@mahidol.ac.th
Telephone: :+66-2-201-5458 **Fax:** +66-2-354-7174

ABSTRACT

AIM: To establish and characterize a new cell line derived from peripheral cholangiocarcinoma of a Thai patient.

METHODS: The peripheral cholangiocarcinoma specimen surgically obtained from the patient was aseptically processed by washing and mincing before culturing in Ham's F12 medium containing 10% fetal bovine serum. After 3 months, when the cell line has become homogeneous and stabilized, several features were investigated, including growth characteristics, immunocytochemistry for cytokeratins, expression of tumor markers, chromosomal analysis by G-banding and multicolour fluorescence in situ hybridization (MFISH), *in vitro* migration and invasion characteristics.

RESULTS: The RMCCA-1 cell line has been established. These cells proliferated as a monolayer with a population doubling time of 48 hours. Immunocytochemistry showed positive staining for human cytokeratin 7 and cytokeratin 19, verifying the biliary epithelial origin. RMCCA-1 secreted carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), but not α-fetoprotein (AFP). Chromosome analysis identified aneuploidy karyotypes with a modal chromosome number of 59. RMCCA-1 exhibited a high level of *in vitro* migration. The cell line exhibited a significant number of chromosomal aberrations as shown by MFISH and G-banding methods.

CONCLUSION: A new cell line derived from peripheral cholangiocarcinoma of a Thai patient has been established. This cell line is shows *in vitro* invasiveness and a high degree of motility. It will serve as a valuable tool for further studies on tumor biology, molecular pathogenesis, metastatic mechanism and response to therapeutic drugs of cholangiocarcinoma.

Key words: Cholangiocarcinoma; Cell line; Establishment; MFISH; Invasion; Migration

INTRODUCTION

Cholangiocarcinoma is a highly malignant epithelial neoplasm that arises within the intrahepatic and extrahepatic biliary tract [1]. The pathogenesis of this disease has been strongly associated with chronic inflammation and cellular injury within bile ducts, as well as partial obstruction of bile flow, manifested by various high risk conditions such as PSC (primary sclerosing chloangitis), hepatolithiasis and infestation by liver fluke (*Ophisthorchis viverrini* or *Clonorchis sinensis*) [2,3]. Although considered as a rare disease, cholangiocarcinoma occurs at a particularly high rate in Northeastern Thailand, with 84.6:100,000 males and 36.8:100,000 females affected by the disease. This is the area where the incidence rate of cholangiocarcinoma is the highest in the world, largely accounted by the habit of consuming uncooked cyprinoid fish, which are infected with the liver fluke [4].

Cholangiocarcinoma has become a serious threat to public health due to increasing worldwide incidence and mortality rates associated with lack of early detection and limited therapeutic options. At diagnosis, most patients are presented with advanced disease, possibly with undetected metastasis, resulting in less than 12 months survival. Even those with operable tumor, the recurrence rate was extremely high, with a 5-year survival rate of less than 40% [2, 5]. Various routes of tumor spreading have been reported in cholangiocarcinoma, including direct invasion, infiltration along the biliary tree, vascular and lymphatic permeation and perineural or intraneural invasion [6].

Progress in understanding the molecular mechanisms governing cholangiocarcinoma invasion and metastasis has been limited by the lack of suitable cell lines and experimental models. Here, we described an establishment and preliminary characterization of a human cell line originated from a Thai patient presented with peripheral cholangiocarcinoma. This new cell line, which we named RMCCA-1, exhibits various characteristics typical of the biliary epithelial cells, as well as invasiveness and motility as shown by the *in vitro* assay. Thus this cell line will be useful for the studies

of not only the tumor biology, molecular pathogenesis and drug response, but also the molecular mechanisms governing the metastatic spread of cholangiocarcinoma.

MATERIALS AND METHODS

Clinical specimen

A 40-year-old male patient was admitted to Rajavithi Hospital, Bangkok, Thailand with a professional diagnosis of peripheral cholangiocarcinoma. Significant laboratory analysis at admission showed elevated serum level of alkaline phosphatase (ALP) (374 U/L, normal 39-117 U/L), total bilirubin (1,973 g/L, normal 0-150 g/L), direct bilirubin (1,545 g/L, normal 0-50 g/L), CA19-9 (0.08 U/L, normal 0.00-0.04 U/L) and carcinoembryonic antigen (CEA) ($7.56x10^6$ g/L, normal 0.000-$3.4x10^6$ g/L) while the serum α-fetoprotein (AFP) ($2.24\ x10^3$ U/L, normal 0.000-$5.8\ x10^3$ U/L), and CA125 ($1.718\ x10^4$ U/L, normal 0.00-$3.5\ x10^4$ U/L /ml) were normal. Computed tomography (CT) scans showed an ill defined hypo-density mass occupying the whole left lobe of the liver. At laparotomy, no ascites was found, and the surface of liver was smooth. Intraoperative ultrasonography revealed a solitary mass at left lobe of liver with moderate dilatation of left intrahepatic duct. The patient was then subjected to left hepatectomy and lymph node dissection. The tumor specimen was removed and subjected to histopathological study and to tissue culture under the approval of the Ethics Committee of Rajavithi Hospital.

Tumor histopathology

The lesion was classified as a well-differentiated peripheral cholangiocarcinoma at stage T2N0M0 according to the UICC standardization (Figure 1).

Primary culture

After the tumor tissue was surgically removed from the patient, it was immediately suspended in transfer medium (Ham's F12 containing antibiotics) at 4 °C. The tissue was quickly washed in PBS pH 7.4 several times before being minced. Later the cell suspension was placed in a 100 mm x 20 mm plastic tissue culture dish (CORNING, New York, USA) containing 10 ml of growth medium (HAM's F12, 200mL/L of fetal bovine

serum and $1x10^7$ g/L epidermal growth factor (EGF) (Pacific Science, Peprotech, New Jersey, USA), 0.1 U/L penicillin G sodium, 0.1 g/L streptomycin sulfate and $2.5x10^4$ g/l amphotericin B). The cell cultures were, then, incubated at 37 °C in a humidified 50 mL/L CO_2 atmosphere and observed daily. Tumor cells were separated from the contaminating fibroblast cells by manually dropping 2.5 mL/L trypsin and 0.2 mL/L EDTA in PBS onto an isolated tumor colony. Subsequently the detached tumor cells were transferred by pipettes to a new culture dish under a phase contrast microscope. After about 1 month, a homogeneous layer of epithelial tumor cells with sustained growth pattern was established.

Growth Kinetics

A suspension of $2x10^3$ cells was cultured in triplicates in 100 µl of Ham's F12 medium supplemented with 100mL/L of FBS in a 96-well plate (CORNING, New York, USA). At time intervals, 10 µL of MTT solution was added to the individual wells, followed by incubation for 4 h at 37 °C in a humidified 50 mL/L CO_2 atmosphere. MTT converted to insoluble formazan dye in live cells was then dissolved by addition of 200 µL DMSO before the absorbency was read at 540 nm. The doubling time of the cell population was determined from the exponential phase of the growth curve.

Immunofluorescence staining

The monoclonal antibody mixture AE-1/AE-3 (DAKO, Denmark) recognized all known basic and most acidic keratin, thus it was used as a general marker of epithelial cells. Cytokeratin 7 (monoclonal mouse Anti-Human Cytokeratin 7, DAKO, Denmark) and cytokeratin 19 (monoclonal mouse Anti-Human Cytokeratin 19, DAKO, Denmark) were used to specifically distinguish biliary epithelial cells from hepatocytes [7, 8]. RMCCA-I cells were grown on sterile coverslips until confluent before being fixed in methanol for 15 min, blocked in 10 mL/L bovine serum albumin (BSA), and incubated with primary antibody for 60-min. After that, a secondary antibody conjugated with fluorescein isothiocyanate (DAKO, Denmark) was added, and the incubation was allowed to proceed

for 60 min at 37 °C. After washing, the coverslips were examined under a fluorescent microscope (Nikon Eclipse TE 2000-U, Kanagawa, Japan).

Chromosome preparation and G-banding analysis

The established tumor cells at 12th passage were subjected to chromosomal analysis. The cells were treated with 10 μmol/L Colchicine (Sigma, St. Louis, USA) for 30 min and resuspend in hypotonic solution, 0.075 mol/L KCl, for 7 min, fixed in Carnoy's fixative and spreaded onto cold glass slides. The cells were stained with Giemsa and the representative chromosome sets were photographed for karyotype analysis. Interpretation of the karyotype was based on ISCN (1995). The modal chromosome number was determined from 20 cells.

Multicolour Fluorescence in situ Hybridization (MFISH) analysis

A duplicate slide, prepared from the same culture used for G-banding, was subjected to MFISH analysis according to the manufacturer's protocol (Metasystem, Germany). Five fluorophors were used for hybridization, including FITC, Spectrum Orange, Texas Red, Cy5 and DEAC. After hybridization, the slides were evaluated under a fluorescence microscope (Axioimage, Zeiss, Germany), and the images were captured and analyzed using the Program Isis (Metasystem, Germany).

Tumor marker detection

A suspension of 10^5 tumor cells was cultured in serum-free Ham's F12 medium for 24 h before the conditioned medium was collected and centrifuged at 1800 r/min for 10 min. Then the supernatant was collected for detection of CA 19-9, CEA and AFP by ELISA method.

In vitro invasion assay

Cancer cell invasiveness was determined using transwell chamber (Costar, Cambridge, MA, USA) coated with 0.3 g/L matrigel (Collaborative Research Inc., Bedford, MA, USA). Approximately, $1x10^5$ cells in culture medium containing FBS were added into the upper

compartment of the transwell, and incubated at 37^{O}C in a humidified atmosphere containing 50 mL/L CO_2 for 6 h. The lower compartment contained culture medium plus FBS. The filters were fixed with methanol and stained with 0.5% crystal violet in 25% methanol for 1 h, before rinsing with tap water several times. The cells on the upper surface of the filters were gently removed using cotton swabs and the cells that have invaded into the lower surface were counted under a microscope. The numbers of invaded cells in five random X10 microscopic fields were counted and expressed as number of cells per well. The results shown represented mean±SE of the number of invaded cells from three independent experiments, each carried out in triplicates.

In vitro motility assay

Motility assay was performed in a similar fashion to the invasion assay, except no matrigel coating was applied to the upper surface of the transwell filters. The numbers of migrating cells in five random X10 microscopic fields were counted and expressed as number of cells per well. The results shown represented mean±SE of the number of migrating cells from three independent experiments, each carried out in triplicates.

Gelatin zymography assay

Cells were starved by culturing in the medium without FBS for 24 h before collection of the conditioned medium. The conditioned medium was mixed with 5X SDS-sample buffer before separation in a 120 g/L SDS-PAGE containing 1mg/ml gelatin. After electrophoresis at 200V for 1 h, the gel was washed in a 2.5% Triton X-100 solution twice. The gel was then incubated in buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 1 mmol/L $ZnCl_2$, 10 mL/L Triton X-100 and 0.2 g/L NaN_3 for 16–18 h, after which the gel was stained with 5 g/L Coomassie blue in 300 mL/L methanol and 100 mL/L acetic acid. After destaining, the clear band of gelatinolytic activity was quantitated using a Biorad GS700 gel scanner [9].

RESULTS

Primary cell culture

The human CCA tissue fragments adhered to the dish after plating for 6h. After three weeks, a layer of epithelial cells appeared, from which contaminating spindle-shaped fibroblasts could be readily distinguished and separated from the epithelial tumor cells by differential trypsinization. After successive 16 passages, a homogeneous immortalized culture of tumor cells was established and was named RMCCA-1. The cells from passages 16^{th} and 30^{th} were then used for morphological analysis. Both exhibited circular to spindle shape with many processes and ornamental fringes. The nucleus and cytoplasm appeared granulated. Some cells were unusually large and contained multinuclei (Figure 2).

Growth Kinetics

RMCCA-1 cells were in lag phase until day 5, after which they entered a logarithmic growth phase. The doubling time determined from the slope of the growth curve was 48 h (Figure 3).

Immunofluorescence staining

All RMCCA-1 cells showed positive staining with the AE-1/AE-3 monoclonal antibody mixture (Figure 4A), which recognizes the human epidermal cytokeratins, the signature of epithelial cells that distinguishes them from fibroblasts. Furthermore specific markers for the adenocarcinoma and transitional cell carcinomas, cytokeratin 7 (Figure 4B) and 19 (data not shown), were also positive.

Chromosome analysis

The G-banded analysis demonstrated aneuploidy karyotype with marked structural abnormalities of the chromosomes (Figure 5A). Although this cell line was established from a male patient, it lacks a Y chromosome and, instead, possesses 2 X chromosomes. The number of chromosomes ranges between 54 and 61, with a modal chromosome number of 59. The final karyotype was determined by consolidating the G-banding and MFISH results. Sixteen structural rearrangements were found, including 9 unbalanced translocations and 2 balanced translocations. In addition, the absence of Y

chromosome in this cell line was also confirmed by both G-banding and MFISH techniques.

Tumor markers in Spent Media

The level of CA19-9 was significantly higher than that of the normal serum, whereas the levels of CEA and AFP were normal (Table 1).

Invasiveness and Motility

An important characteristic of metastatic cancer is the ability to migrate and invade the underlining basement membrane, the surrounding tissues and the blood vessels. We thus examine the invasiveness and motility of the RMCCA-1 cells, compared with those of three other established Cholangiocarcinoma cell lines from Thai patients (KKU-100, KKU-213 and HuCCA-1), using *in vitro* invasion and motility assay, respectively [10]. RMCCA-1 exhibited relatively higher migration rate (1,688 $\pm$ 207 cells/well) compared with the other 3 cell lines (1,514 $\pm$ 152 cells/well, 1,123 $\pm$ 163 cells/well, 40 $\pm$ 17 cells/well for KKU-213, KKU-100 and HuCCA-1, respectively). Surprisingly, the invasiveness of RMCCA-1 was significantly lower than those of KKU-213 and KKU-100 (181 $\pm$ 54 cells/well for RMCCA-1 vs.1021 $\pm$ 5 cells/well for KKU-213 and 765 $\pm$ 244 cells/well for KKU-100), while slightly higher than that of HuCCA-1 (38 $\pm$ 21 cells/well) (Figure 6).

Gelatin zymography

Most metastatic cells secrete proteinases to facilitate its invasion through tissue barriers. One of the most characterized families of tissue-degrading enzymes from cancer is the matrix-metalloproteinases (MMPs). Here we examined the ability to secrete the MMPs from RMCCA-1 cells by gelatin zymography. The result shows an intense band at 72 ku in FBS (fetal bovine serum), corresponding to the gelatinase activity known to be present in the FBS. However, the conditioned medium of RMCCA-1 lacks the activity (data not shown).

DISCUSSION

Cholangiocarcinoma (CCA) is a cancer arising from the bile duct epithelium. It is a lethal disease of which little is known about its biology, pathogenesis and behavior. Up to present, there has been no effective early detection protocol or curative strategy for the disease; most patients seek curative treatment at advanced stage with poor prognosis. Therapeutic options for cholangiocarcinoma have been limited due to poor response to chemotherapy and radiation therapy. Surgery is perhaps the only effective cure although the 5-year survival after surgical treatment is less than 40% [2, 5]. Therefore, it is urgently required that the molecular markers for early detection are mapped and molecular targets for effective treatment are deciphered. One way to understand the nature of such disease is by studying the behavior of cell lines derived from the tumor *in vitro*. Here we describe the establishment and preliminary characterization of a cell line derived from a peripheral cholangiocarcinoma of a Thai patient which we have named RMCCA-1.

The population doubling time of this cell line was about 48 h, where those of other cholangiocarcinoma cell lines originated from Thai patients were 55 h for HuCCA1 [7] and 72 h for KKU100 [8]. Analysis by ELISA of the spent medium from RMCCA-1 cells showed that these cells have retained some functional characteristics of the original tumor, including over expression of the tumor marker CA19-9, corresponding to the abnormally high level of CA19-9 in the patient's serum at admission. CA19-9 was often detected in patients with malignant cholangiocarcinoma and pancreatic cancer [11]. Many kinds of cancer cell lines also secrete CA19-9, including those derived from cholangiocarcinoma (TK) [12], pancreatic carcinoma (SUIT-2) [13] and colon cancer (SW1116) [14].

Analysis by immunofluorescence staining using monoclonal antibodies to AE1 and AE3 showed positive staining for human cytokeratins, verifying the epithelial origin of the tumor. RMCCA-1 also stained positively with antibodies to cytokeratins 7 and 19, distinguishing the bile duct epithelial cells from the hepatocytes. Together, these data confirm that RMCCA-1 indeed derived from the epithelium of the bile duct.

Tumor metastasis involves a series of complex processes including dysregulation of cell adhesion, cell motility, and enzymatic proteolysis of basement membrane and extracellular matrix. The RMCCA-1 cells exhibit a high level of migration rate, in contrary to the relatively low level of invasiveness and MMP activity. Thus the large discrepancy between the migration and invasion rates of RMCCA-1 is likely accounted, at least in part, by the lack of or the very low level of MMP activity. This discrepancy is smaller in the other 3 CCA cell lines, in which the MMP activity correlated with cell invasiveness relatively well (data not shown).

Karyotype analysis by G-banding revealed a complex pattern of chromosomal abnormalities. Most of the chromosomes were triplicates, suggesting that the cell line was originated from a tripoid cell. The lost of Y chromosome was not unusual as similar finding has been shown in human sarcomatous cholangiocarcinoma (SCK) cells [16]. Other aberrations involving Y chromosome have also been reported, including the translocation between Y chromosome and chromosome 1 as shown in a human cholangiocarcinoma cell line, PCI-SG231 [18].

With recent development of MFISH technique, it has become possible to identify and characterize complex chromosomal aberrations, previously unrevealed by G-banding analysis. This technique has allowed us to characterize the karyotype of RMCCA-1 in much greater detail with a high level of accuracy.

We have detected aberrations of chromosomes 1, 5, 7 and 12 in the RMCCA-1 cells, which were consistent with those of the other human cholangiocarcinoma cell lines including SCK, JCK, Cho-CK, Choi-CK, CC-SW-I, CC-LP-I, PCI: SG231, and RPMI-7451, and a rat cholangiocarcinoma cell line, CC-62 [15, 16, 17, 18]. In contrast, we did not detect structural rearrangement of X chromosome and chromosome 6 in the RMCCA-1 cells, nor did we detect the lost of chromosome 18, as found in SCK, JCK, Cho-CK, and Choi-CK [16].

In conclusion, we successfully established a new cholangiocarcinoma cell line which we named RMCCA-1. We have also performed preliminary characterization of its growth characteristics, karyotype, secreted tumor markers and invasive properties. This

cell line will be further used in our research towards understanding and combating against cholangiocarcinoma.

REFERENCES

1. **Khan S.A.**, Davidson B.R., Goldin R., Pereira S.P., Rosenberg W.M.C., Taylor-Robinson S.D., Thillainayagam, A.V., Thomas, H.C., Thursz, M.R., Wasan, H. Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document. *Gut* 2005; **51**(supp. VI): vi1-vi9
2. **Sirica, A.E.** Cholangiocarcinoma: Molecular Targeting Strategies for Chemoprevention and Therapy. *Hepatology* 2005;**41**: 5-15.
3. **De Groen P.C.**, Gores G.J., La Russo N.F., Gunderson L.L., Nagorney D.M. Biliary Tract Cancers. *The New England Journal of Medicine* 1999; **341**: 1368-1378.
4. **Vatanasapt V.**, Sriamporn S., Vatanasapt P. Cancer Control in Thailand. *Jpn. J. Clin. Oncol.* 2002; **32**: S82-S91.
5. **Thongprasert S.** The Role of Chemotherapy in Cholangiocarcinoma. *Annals of Oncology* 2005; **16** (suppl.2): ii93-ii96.
6. **Sripa B.**, Pairojkul C. Pathology of Cholangiocarcinoma. In *Liver Cancer in Thailand.* 2000; 84-88.
7. **Sirisinha S**, Tengchaisri T., Boonpucknavig S., Prempracha N., Ratanarapee S., Pausawasdi A. Establishment and Characterization of a Cholangiocarcinoma Cell Line from a Thai Patient with Intrahepatic Bile Duct Cancer. *Asian Pac J Allergy* 1991; **9**:153-157. [PMID: 1666951]
8. **Sripa B.**, Leungwattanawanit S., Nitta T., Wongkham C., Bhudhisawasdi V., Puapairoj A., Sripa C., Miwa M. Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100). *World J Gastroentero* 2005; **11**: 3392-3397. [PMID: 15948244]
9. **MacDougall JR**. The 92-kD gelatinase B is expressed by advanced stage melanoma cells. *Cancer Res.* 1995; **55**: 4174-81.

10. **Frandsen TL.** Asssays for the study of human cancer cell invasion and metastasis. *Fibrinolysis.* 1992; **6**: 71-76.
11. **Ozkan H**, Kaya M, Cengiz A. Comparison of tumor marker CA 242 with CA 19-9 and carcinoembryonic antigen (CEA) in pancreatic cancer. *Hepatogastroenterology.* 2003; **50**:1669-74. [PMID: 14571813]
12. **Watanabe M**, Chigusa M, Takahashi H, Nakamura J, Tanaka H, Ohno T. High level of CA19-9, CA50, and CEA-producible human cholangiocarcinoma cell line changes in the secretion ratios in vitro or in vivo. *In Vitro Cell Dev Biol Anim.* 2000; **36**:104-9. [PMID: 10718366]
13. **Taniguchi S**, Iwamura T, Kitamura N, Yamanari H, Kojima A, Hidaka K, Seguchi K, Setoguchi T. Protein-free culture of the human pancreatic cancer cell line, SUIT-2. *Hum Cell.* 1994; **7**: 207-14. [PMID: 7599109]
14. **Adachi M**, Sekine T, Umemoto A, Tsukikawa S, Imai K, Yachi A. Mechanism of clearance of circulating CA19-9 in rats. *Tumour Biol.* 1990; **11**: 51-8. [PMID: 2309066]
15. **Gil-Benso R**, Martinez-lorente A, Pellin-Perez A, Navarro-Fos S, Gregori-Romero MA, Carda C, Callaghan R, Peydro-Olava A,Llombart-bosch A. Characterization of a new rat cell line established from 2 AAF-induced combined hepatocellular cholangiocellular carcinoma. *In Vitro Cell Dev Biol Anim.* 2001; **37**; 17-25. [PMID: 11249201]
16. **Kim DG**, Park SY, You KR, Lee GB, Kim H, Moon WS, Chun YH, Park SH. Establishment and characterization of chromosomal aberrations in human cholangiocarcinoma cell lines by cross-species color banding. **Genes Chromosomes Cancer**. 2001; **30**: 48-56. [PMID: 11107175]
17. **Shimizu Y**, Demetris AJ, Gollin SM, Storto PD,Bedford HM, Altarac S, Iwatsuki S, herberman RB, Whiteside TL. Two new human cholangiocarcinoma cell lines and their cytogenetics and responses to growth factors, hormones, cytokines or immunologic effector cells. *Int J Cancer.* 1992: **9**; 252-60. [PMID: 1355757]

18. **Storto PD**, Saidman SL, Demetris AJ, Letessier E, Whiteside TL, Gollin SM. Chromosomal breakpoints in cholangiocarcinoma cell lines. *Genes Chromosome Cancer*.1990;**2**: 300-10. [PMID:2176543]

Figure and Tables

Figure 1 Hematoxylin and Eosin staining of the liver biopsy (10X magnification). The histopathological analysis of the biopsy indicates a well-differentiated peripheral cholangiocarcinoma with no vascular invasion.

Figure 2. The RMCCA-1 culture under a phase contrast microscope at 20X magnification. (A) at 16 th passage; (B) at 30 th passage. The RMCCA-1 cells exhibited circular to spindle shape with many processes and ornamental fringes. The nucleus and cytoplasm appeared granulated.

Figure 3. Growth kinetics of the RMCCA-1 cell line in vitro as analyzed by MTT assay. The tumor doubling time during the exponential phase of growth was 48 h. Each point represents the mean value from 3 experiments, each performed in triplicates.

Figure 4. Immunofluoresence staining of RMCCA-1 cells with antibodies against (A) Cytokeratin 7; (B) AE-1/AE-3 at 40X magnification.

Figure 5. Representative karyotypes of RMCCA-1 cell line as assessed by (A) G-banding ; (B) mFISH technique. The karyotype showed 54-61(3n)XX,-Y,der(1),t(1;2;7)(q31;q31;q22),t(2;17)(q33;q12),der(3)t(3;15)(q21;q?),t(4;17)(p14;q?),der(7)t(7;15)(p22;q?),der(8)t(8;7)(p12;?),der(10)t(10;13)(q21;q11),der(12)t(11;12)(q?,p11.2),der(13)t(13;9)(q11;q11),der(14)t(15;14)(q34;q32),der(16)t(13;16)(q11;p13.3),der(17)t(X;10;17)(p?,p?,p11.2),der(19)t(10;19)(q11.2;p13.3),der(19)t(17;19)(q?;q?),der(21)t(1;9;21)(?;?;q11),der(22)t(1;15;22)(?,?,?)

Figure 6. Invasion and migration rates of RMCCA-1 compared with KKU-213, KKU-100 and HuCCA-1 cell lines as determined by in vitro invasion and motility

assay. Approximately 1X105 cells were seeded into the upper chamber of a transwell, and incubated for 6 h before the filter was fixed, stained, and the invading/migrating cells were counted under microscope.

Table 1. Tumor markers secreted into the spent media after a 24 h starvation. The tumor marker level in the spent medium is significantly higher than the culture medium level

Table 1. Tumor markers secreted in the spent media after a 24 h starvation

	CEA (ng/ml)	AFP (U/ml)	CA 19-9 (U/ml)
RMCCA-1	<0.200	<0.500	72.92 *
[Normal serum]	[0.0 - 3.4]	[0.0-5.8]	[0.0-39]

* A Tumor marker in medium condition is higher than normal serum level.