



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

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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

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diagnosis of cholangiocarcinoma.

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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

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°C in a 5% (50 mL/L) CO₂ 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°C 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 H₂O and 2 µL of standard RNA template or RNA from

Table 1 The patient demographic data and blood chemistry data

	Benign biliary tract disease	Cholangio-carcinoma	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) ^a	3.8 ± 2.21	12.8 ± 5.24	1.05 ± 0.25
Alkaline Phosphatase (U/dL) ^a	309 ± 67.53	550 ± 24.44	98 ± 10.60

^a*P* < 0.005.

serum samples was prepared. The reaction conditions were reverse transcription at 60°C for 10 min, followed by initial denaturation at 95°C for 30 s and 40 cycles of denaturation at 95°C for 0.5 s, annealing at 60°C for 10 s, and extension at 72°C 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.

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-

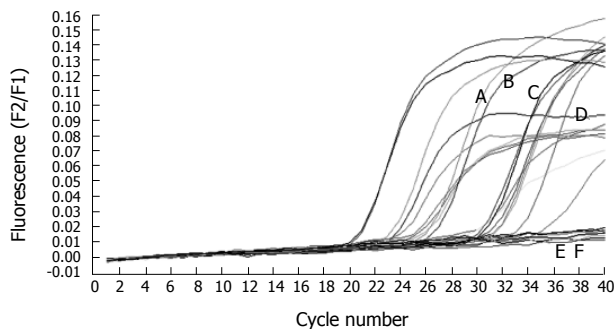


Figure 1 Continuously monitoring the development of signals in parallel standards and samples results in a series of amplification curves. The amplification of a 198 bp fragment of the generated hTERT cDNA was identified. (A: Signal from RMCCA1; B: Signal from HuCCA1; C, D: Signals from serum of cholangiocarcinoma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

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^[4]. 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

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 positive	mRNA negative
Stage I	2	1
Stage II	17	3
Stage III	9	1

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^[8]. 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.

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