RESEARCH COMMUNICATION

Ubiquitin-specific Protease 14 Expression Associated with Intrahepatic Cholangiocarcinoma Cell Differentiation

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Abstract

The purpose of this study was to identify the gene alterations amplified from AO16 primer and examine whether the expression patterns of USP14 in clinical specimens from patients with intrahepatic cholangiocarcinoma (ICC) is associated with cancer cells. DNA from tumor and corresponding normal tissues of 52 patients was amplified with 33 arbitrary primers. The DNA fragment that altered most frequently in ICC was cloned, sequenced, and identified by comparison with known nucleotide sequences in the genome database. The DNA copy numbers of the allelic alterations in cholangiocarcinoma were determined by quantitative real-time PCR and interpreted as allelic loss or DNA amplification by comparison with the reference gene. Associations between allelic imbalance and clinicopathological parameters of ICC patients were evaluated by $\chi 2$-tests. The Kaplan-Meier method was used to analyze survival rates. Immunohistochemically, USP14 showed weak cytoplasmic staining in normal bile duct epithelial cells. It was strongly detected in 21 cancer patients (43.8%). There were correlations between USP14 expression level and the clinicopathological features of ICC, histological grade ($P < 0.05$). However, there were no significant differences in age, gender, tumor size, metastasis, lymph node metastasis, and staging. USP14 expression was related to cholangiocarcinoma cell differentiation. Due to their emerging role in control of multiple signaling pathways and oncoproteins, USP14 inhibitors may be useful for anticancer agents.

Keywords: Intrahepatic cholangiocarcinoma - ubiquitin-specific protease 14 - cell differentiation

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Introduction

Intrahepatic cholangiocarcinoma (ICC) was predominantly found in Thai patients with bile duct cancer (Haswell-Elkins et al., 1992; Parkin et al., 1993) and its incidence is much higher in the northeastern region of Thailand, where it is closely associated with liver-fluke infestation (Srivatanakul et al., 1991; Watanapa et al., 1996; Shimonishi et al., 2000; Watanapa et al., 2002; Shaib et al., 2004) and the consumption of carcinogen-contaminated daily foods (IARC, 1994). With regards to genome scanning method, arbitrarily primed polymerase chain reaction (AP-PCR) (Williams et al., 1990), also called random amplified polymorphic DNA (RAPD) (Welsh et al., 1990), has proven a promising technique for identifying novel gene alterations in many human cancers (Navarro et al., 1999) including Wilms tumours (Singh et al., 2006), ovarian cancer (Pongstaporn et al., 2006), and cholangiocarcinoma (Chariyalertsak et al., 2005).

Ubiquitin-specific protease 14 (USP14) encodes Ubiquitin carboxyl-terminal hydrolase 14 enzyme, a member of the ubiquitin-specific processing (UBP) family of proteases that is a deubiquitinating enzyme (DUB) with His and Cys domains. This protein is located in the cytoplasm and cleaves the ubiquitin moiety from ubiquitin-fused precursors and ubiquitinylated proteins (Puente et al., 2003). It was strongly detected in the cytoplasm of colorectal cancer cells and may control the fate of proteins that regulate tumor invasion and metastasis (Shinji et al., 2006).

In this study, we evaluated USP14 expression level in ICC patients' tissue using immunohistochemistry and correlated to the clinicopathological features of the disease. Furthermore, the finding of genetic alteration, correlating with clinicopathological parameters of patients with ICC, was also demonstrated.

Materials and Methods

Subjects and tissue samples

Samples from 52 cases with primary ICC (42 males and 10 females) and corresponding normal tissues were obtained from Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand, during surgical resection. The median age at diagnosis was 56, range 26-75 years. This work was approved by the

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Ethics Committee of the Faculty of Medicine, Khon Kaen University, Thailand (HE471214). Samples were frozen after resection and stored at -80°C until DNA and RNA extraction. None of the patients received radiation or chemotherapy before surgery. All patients were residents of northeast Thailand, where liver-fluke infections are highly endemic. Hematoxylin-eosin stained sample sections from each tumor block were examined microscopically to confirm the presence of >80% cancer cells. Paired normal tissues from the same patient were used as controls and showed normal histological features. Genomic DNA was isolated from fresh cancerous and normal counterpart tissues by proteinase K digestion and salting-out method, with some modifications, as described previously (Chuensumran et al., 2007).

Cloning and sequencing of aberrant DNA fragment

The altered band employing the primer AO16 was excised from the 0.8% agarose gels and purified by DNA purifying kit (Nucleospin, Machery-Nagel GmbH & Co. KG, Germany). The eluted DNA was confirmed for purity and quantity on 0.8% agarose gel. The 2.8kb fragment was cloned using a TA cloning kit (Invitrogen, USA) following the manufacturer's instructions. Plasmid DNA was isolated from each bacterial colony using a QIAGEN plasmid kit (QIAGEN, USA). Restriction analysis of the recombinant plasmid DNA was carried out by EcoRI digestion. The plasmid DNA containing DNA fragment insertion was further nucleotide-sequenced with either forward or reverse M13 primer as the sequencing primers (customized by Macrogen Inc., Korea). The nucleotide sequences obtained from each clone were identified by comparison with known nucleotide sequences in the human genome database (http://www.ncbi.nlm.nih.gov/blastn) via BLASTn program.

Real-time quantitative PCR

Specific primers were designed according to the nucleotide sequences using the GeneFisher program (http://www.genefisher.com). They were 5´-GAGTTGGACCTTTCCAGA-3´ (forward primer) and 5´-TGCTTGCACAGATGTGA-3´ (reverse primer). Amplification of the altered DNA fragment was detected by real-time PCR with the designed specific primers (BioService Unit, Thailand) using β-globin as a reference gene. The PCR was performed in a total volume of 20 μl in each LightCycler glass capillary, containing 18 μl of LightCycler Mastermix; 8.8 μl water; 3.2 μl MgCl2 (4 mM); 2 μl forward primer (0.5 μM); 2 μl reverse primer (0.5 μM); and 2 μl LightCycler FastStart Enzyme (LightCycler FastStart DNA Master SYBR Green I, Roche Diagnostic, Germany) and 2 μl genomic DNA (40 ng). The PCR condition consisted of an initial denaturation step at 95°C for 15 s, 67-57°C (step delay 15 cycles, touchdown PCR) for 5 s, and 72°C for 20 s. Thermal cycling and fluorescent monitoring were performed using a LightCycler (Roche Applied Science, Germany). The point at which the PCR product is first detected above the fixed threshold, termed the cycle threshold (Ct), was determined for each sample. The DNA content of tumor and normal tissue preparations was normalized using β-globin within the PCR reaction. The relative concentrations were determined employing Ct values, which are equivalent to the cycle number at which the PCR product is first detected above a fixed threshold. The Ct values obtained from the analyzer were then calculated for the DNA copy number utilizing the delta-delta-Ct method[18]. Samples were run in duplicate. In this study, a sample with an amplification fold ≥1.5 was interpreted as having amplification, otherwise ≤0.5 was interpreted as having allelic loss, and the rest was no aberration[19].

Immunohistochemistry

Paraffin-embedded sections (3.5 μm) were subjected to immunostaining for anti-USP14 antibody produced in rabbit (Sigma Prestige Antibodies, USA). After deparaffinization, antigen retrieval was performed using an automated immunostainer (Ventana Medical Systems, USA) with standard CC1 reagent. The tissue sections were incubated with rabbit anti-USP14 (1:750 dilution) in DAKO diluent (S0809) for 32 min at 37°C followed by washing and detection with the Ultra-View DAB kit (Ventana Medical Systems, USA). Negative controls were performed by omitting the primary antibodies.

Estimation of USP14 protein expression level

The immunoreactivity of the USP14 protein in ICC cells was scored based on the intensity of cytoplasmic staining using the following classification system: -, no stainging; ±, weakly and equally stained with normal mucosa; and +, more strongly stained than normal mucosa. Patients positive for the USP14 protein were classified into the high expression cancer group, while the remaining patients were classified into the low expression cancer group. All specimens were evaluated by two investigators (P.P. and C.P.) who were blinded to the clinical information of patients.

Statistical analysis

Clinicopathological features of patients with ICC, including patient's age at initial diagnosis, gender, histological type, tumor size, lymph node and/or intrahepatic metastasis, were correlated with the alterations of the distinct region. Correlations between USP14 expression and the clinicopathological features of ICC were evaluated by χ2-test. Survival analysis was carried out with patients who were followed up for at least 200 weeks, or until death, after surgery. Three patients who died in the post-operative period were excluded and 4 cases were lost to follow-up. Thus, only 45 patients were available for the survival study. Overall survival distributions were calculated by Kaplan-Meier method and analyzed by log-rank test. P values < 0.05 were considered statistically significant.

Results

Cloning and sequencing results

According to AP-PCR analysis, the highest frequency of 2.8kb amplification (gain or increase of gene copy number) band was occurred from AO16 primer, the specific region from 18p11. The frequencies of distinguish

Table 1. Clinicopathological Parameters and Genetic Alterations of the DNA Sequence on Chromosome 18p11 in ICC

Parameters			18p11 alterations		P value
		N	+	-	
Age	≤ 50	16	9 (56.3)	7 (43.8)	0.455
	> 50	36	18 (50.0)	18 (50.0)	
Gender	Male	42	21 (50.0)	21 (50.0)	0.416
	Female	10	6 (60.0)	4 (40.0)	
Histological type	Well-diff	25	12 (48.0)	13 (52.0)	0.337
	Mod diff	14	6 (42.9)	8 (57.1)	
	Poor diff	13	9 (69.2)	4 (30.8)	
Tumor size (cm)	≤ 5	14	7 (50.0)	7 (50.0)	0.285
	6-10	27	17 (63.0)	10 (37.0)	
	> 10	9	3 (33.3)	6 (66.7)	
Metastasis	Positive	35	20 (57.1)	15 (42.9)	0.354
	Negative	15	7 (46.7)	8 (53.3)	
Lymph node metastasis	Positive	15	10 (66.7)	5 (33.3)	0.299
	Negative	30	16 (53.3)	14 (46.7)	
Staging	2 + 3	8	3 (37.5)	5 (62.5)	0.056
	4A	13	11 (84.6)	2 (15.4)	
	4B	24	12 (50.0)	12 (50.0)	

bands were 11 (37%) out of 30 cases.

Real-time PCR analysis

The specific region from 18p11 given high frequency was then analyzed by real-time PCR. The changes of DNA copy number at the definite region was analyzed by comparative Ct method. The real-time quantitative PCR results indicated that, of all 52 patients with ICC, the changes of DNA copy number at particular region on chromosome 18p11 were detected in 27 (52%) cases {2 (4%) were loss vs. 25 (48%) were amplification}. A housekeeping gene, β-globin, was amplified as a relatively external control. It was amplified in the same LightCycler run as the target, but not in the same capillary.

DNA alterations and clinicopathological data

Further, the aberrations of the unique region were investigated in relation to the clinicopathological data using chi-square test on SPSS program. The age, gender, and histological-type factors had 52 valid cases. Meanwhile, other factors including tumor size and metastasis had 50 (96%) informative cases. However, the DNA alterations on chromosome 18p11 did not correlate with the clinicopathological data (Table 1).

DNA alterations and survival analysis

The survival data were available for 45 patients. The observation period ranged from 1 to 200 weeks. At the most recent follow-up, only 1 patient was alive and 44 had died. For DNA alterations on chromosome 18p11, 27 of 44 (61.4%) cases showed DNA amplification, while 7 (15.9%) showed allelic loss. However, survival analysis demonstrated no relationship between amplification of the DNA sequences on the chromosome region (Figure 1).

USP14 expression in cholangiocarcinoma cells

Immunohistochemically, USP14 showed weak cytoplasmic staining in normal bile duct epithelial cells (Figure 2A). In 10 of 48 ICC patients, USP14 was not

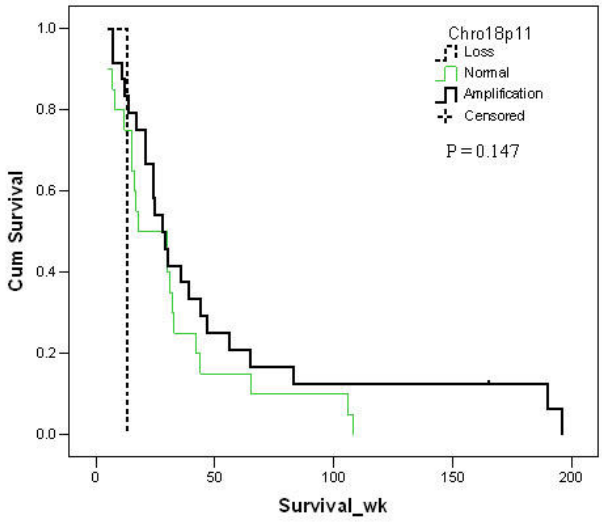

Figure 1. Overall Survival Curves for CCA Patients According to Genetic Alterations on Chromosome 18p11 (N = 44)

detected, and these patients were classified into the – group (Figure 2B). USP14 was weakly localized in the cytoplasm of 17 cancer patients, which were classified into the group (Figure 2C). On the other hand, USP14 was strongly detected in 21 cancer patients, which were classified into the ± group (Figure 2D). Thus, 21 (43.8%) + patients were classified into the high USP14 expression group and 27 (56.2%) – and patients into the low USP14 expression group.

Correlation of USP14 expression and clinicopathological factors

The correlations between USP14 expression and the clinicopathological features of ICC were summarized in Table 2. There were significant correlations between USP14 expression level and histological grade (P=0.048). However, there were no significant differences in age, gender, tumor size, metastasis, lymph node metastasis, and staging. The level of high USP14 expression increased with histological grading: 8% of well-differentiated type (8/21 patients), 88% of moderately-differentiated type (7/8 patients), and 40% of poorly-differentiated type (4/10

Table 2. Correlation between Clinicopathological features of Intrahepatic Cholangiocarcinoma and USP14 Expression

Parameters		USP14 expression High	Low	OR (95% CI)	P value
Age	≤ 50	7 (53.8)	6 (46.2)	1.36 (0.36-5.18)	0.651
	> 50	12 (46.2)	14 (53.8)	1.00 (referent)	
Gender	Male	15 (46.9)	17 (53.1)	0.66 (0.13-3.45)	0.622
	Female	4 (57.1)	3 (42.9)	1.00 (referent)	
Histological type					
	Well-diff	8 (38.1)	13 (61.9)	1.00 (referent)	0.048*
	Mod-diff	7 (87.5)	1 (12.5)	11.4 (1.17-110)	
	Poor-diff	4 (40.0)	6 (60.0)	1.08 (0.23-5.06)	
Tumor size				0.843	
(cm)	≤ 7	3 (20.0)	12 (80.0)	1.00 (referent)	
	> 7	3 (23.1)	10 (76.9)	1.20 (0.20-7.31)	
Metastasis					
	Positive	3 (25.0)	9 (75.0)	1.00 (referent)	0.690
	Negative	3 (18.8)	13 (81.3)	0.69 (0.11-4.24)	

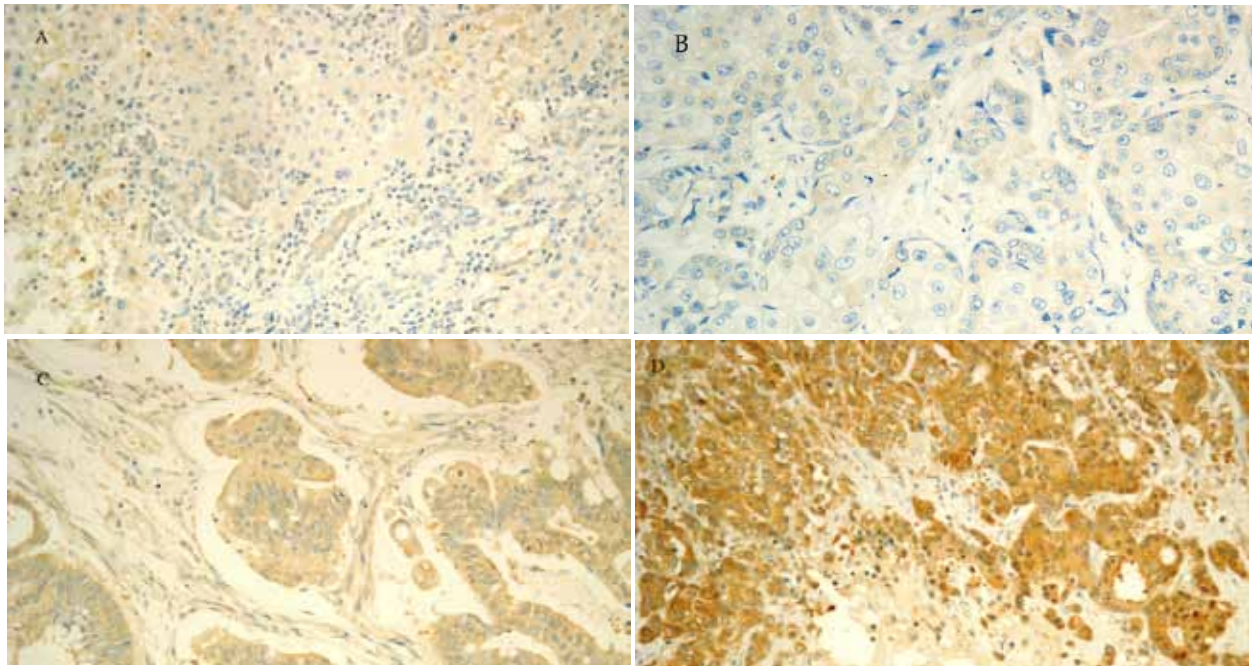

Figure 2. Representative Immunohistochemical Staining for USP14. The USP14 protein was weakly localized in normal bile duct epithelial cells (A). USP14 was not detected in 10 ICC patients classified into the – group (B). USP14 was detected more weakly than or equal to normal epithelial cells in 17 cancer patients classified into the ± group (C). USP14 was strongly detected compare with normal epithelial cells in 21 cancer patients classified into the + group (D). (Original magnification: A-D, x200)

patients).

Discussion

In this study, we used an AP-PCR technique to detect and characterize genomic instability in primary ICC. According to the delta-delta-Ct method (Livak et al., 2001), the DNA copy number was detected by real-time PCR and calculated compared with an internal control (Bodin et al., 2005; Prior et al., 2006). The result revealed allelic imbalance on chromosome 18p11 in 52% of ICC patients. BLAST analysis showed ubiquitin-specific protease 14 (usp14) as a candidate gene at this chromosomal region, conferred susceptibility to colorectal cancer (Shinji et al., 2006) and ovarian carcinogenesis (Wada et al., 2009).

Mammalian USP14 (Ubp6 in yeast) is unique among the ubiquitin-specific processing proteases (UBP) family of the deubiquitinating enzymes (DUBs), and aberrations in this pathway are known to lead to a variety of clinical disorders (Schwartz et al., 1999; Chung et al., 2001). From previous report, this enzyme family concerned with DNA repair, cancer, and apoptosis. The degradation of the tumor suppressor protein, p53 is regulated by ubiquitin-mediated proteolysis in which the responsible E3 enzyme (through sequential action of three classes of enzymes known as ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3)), which forms a complex with p53 called Mdm2 (Honda et al., 1997).

USP14 has been shown to associated with the regulatory subunit of proteasome (19S) and edited polyubiquitin chains on proteasome subunit (Ventii et al., 2008). Enhancement of proteasome activity through inhibition of USP14 may offer a strategy to reduce the levels of aberrant proteins in cells under proteotoxic stress (Lee et al., 2010).

In summary, USP14 expression was related to cholangiocarcinoma cell differentiation. Due to their emerging role in control of multiple signaling pathways and oncoproteins, USP14 inhibitors may be useful anticancer agents.

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