RESEARCH COMMUNICATION

Histological Type of Intrahepatic Cholangiocarcinoma Differentiated by Genetic Alteration from AP-PCR Fingerprint

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Abstract

Cholangiocarcinoma (CCA), the malignant neoplasm of the biliary epithelium, is usually fatal due to difficulty in early diagnosis and lack of availability of effective therapy. The genetic mechanisms involved in the development of CCA are not well understood and only a few cytogenetic studies have been published. In this study, genomic instability in 30 Thai cases of intrahepatic cholangiocarcinoma (ICC) was assessed using an arbitrarily primed-polymerase chain reaction (AP-PCR) method. Genetic alterations were analyzed as banding pattern changes between tumors and corresponding normal DNA. The abnormal band present at the highest frequency (23/30 cases, 77%) appeared with the AO16 primer. Statistical analysis also showed that DNA alteration from this primer was significantly associated with the moderately to poorly differentiated histological type (P = 0.038). Kaplan-Meier survival curves showed borderline significance for this DNA aberration (P = 0.06 by the log-rank test). This DNA fragment may thus be of use to predict degree of malignancy of the disease.

Keywords: Intrahepatic cholangiocarcinoma - ICC - CCA - genetic alteration - AP-PCR - Thailand

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Introduction

Intrahepatic cholangiocarcinoma (ICC) was predominantly found in Thai patients with cholangiocarcinoma (CCA) (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 infestations (Shimonishi et al., 2000; Watanapa et al., 2002; Shaib et al., 2004; Boonla et al., 2005; Jinawath et al., 2006; Dachrut et al., 2009) and the consumption of carcinogen-contaminated daily food (IARC, 1994). Data from the World Health Organization (WHO) database, and from national registries worldwide, showed an increase in ICC-related mortality (Patel, 2001; Khan et al., 2002).

Recent investigations into the underlying molecular mechanisms involved in cholangiocarcinogenesis and tumor growth have contributed greatly to understand the disease. Significant progress has been made over the past decade in defining molecular alterations associated with CCA. Alterations in p53 (Petmitr et al., 1998; Gores, 2003; Berthiaume and Wands, 2004) and p16INK4a (Taniai et al., 2002) are frequently detected in CCA and are likely contributing to oncogenesis in the biliary tract.

Other alterations that seem to occur early in cholangiocarcinogenesis include overexpression of the receptor tyrosine kinases (RTKs), ErbB-2, and Met (Endo et al., 2002) as well as the upregulation of COX-2 (Nakanuma et al., 2003). Likewise, WISP1v expression was found to be associated with lymphatic and perineural spread of CCA and a poor clinical outcome (Tanaka et al., 2003). Human telomerase reverse transcriptase (hTERT) has also been detected in a high percentage of analyzed cases of ICC, irrespective of tumor grade and subtype, as well as heterogeneously in dysplastic lesions, suggesting that acquired hTERT activity may reflect an early stage leading to CCA development (Ozaki et al., 1999). However, the clinical value of established survival predictors seems to be unfortuantely very limited since they fail to predict reliably survival of patients after resection.

With regards to genome scanning method, arbitrarily primed polymerase chain reaction (AP-PCR), also called random amplified polymorphic DNA (RAPD) (Williams et al., 1990; Welsh et al., 1990), has enormously wide application since it can be applied to the study of virtually any nucleic acid entity, whether previously characterized or not. In this study, it was used to evaluate genetic

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alterations as a prognostic factor of ICC patients after resection.

Materials and Methods

Sample collection

Tumor and corresponding normal tissues of 30 patients with ICC were provided from Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. All patients had given informed consent for genotyping. The study was also reviewed and approved by Ethical Committees at Khon Kaen University (HE471214).

DNA extraction

Genomic DNA was isolated from fresh cancerous, normal counterpart tissue cases by Proteinase K digestion and salting out methods (Miller et al, 1988), with some modification. The cancerous and normal tissues were embedded in Optimum Cutting Temperature (OCT) and cryostat tissue sections were performed (10 μm). The tissue sections were washed out from OCT by normal saline 3 times, then incubated in lysis buffer (10mM Tris-HCl, pH 8.0, 400 mM NaCl, 2mM EDTA), 200 μl of 10% Sodium Dodecyl Sulfate (SDS) and 10 mg/ml Proteinase K at 60°C for 3 hr. The solution mixture was mixed with 6 M NaCl, shaked and centrifuged at 10,000×g, 4 °C for 10 min. DNA was precipitated by absolute ethanol and washed 3 times with 70% ethanol. DNA pellet was then dissolved in TE buffer.

DNA quantification

The extracted DNA was quantitated using spectrophotometer to measure the absorbance at 260 nm. For further analysis, the concentration of DNA was adjusted to 20 ng/µl with steriled distilled water. Such a concentration of the DNA was confirmed by running on 0.8% agarose gel electrophoresis and comparing with EZ load Precision Molecular Mass Standard (Biorad laboratories, California, USA).

AP-PCR analysis

AP-PCR DNA fingerprint was performed using 30 chosen arbitrary primers (Pongstaporn et al, 2006). The template DNA was extracted from ICC and corresponding normal tissues of the same patients. PCR was performed based on the method of Williams et al (1990) with modifications (Singh and Roy, 2001). Amplification was carried out in 25 µl reaction mixture containing 2.5 µl of 10x PCR buffer, 200 µM each of dATP, dCTP, dGTP, dTTP, 2.5 mM MgCl2, 1 unit of taq DNA polymerase (Pharmacia Biotech, USA), 20 pmol/µl of random primers and 100 ng of genomic DNA. DNA amplification was performed in Gene Amp PCR System 9700 (Perkin Elmer, USA) programmed for 45 cycles. Each cycle consists of denaturation at 95°C for 2 minutes.

Electrophoresis and detection of AP-PCR products

AP-PCR products were resolved on 1.4% agarose gel and visualized by ethidium bromide staining. The band patterns of the AP-PCR fingerprints were compared between normal and tumor DNA in each case. Differences in the intensity of amplifies DNA fragments in the tumor DNA compared to corresponding normal tissue DNA were observed. The reduced and heightened intensity of the amplified DNA fragments represent respectively, allelic losses and gains of the corresponding genomic fragments in cancer cells (Singh and Roy, 2001).

Statistical analysis

Clinicopathological features of patients with ICC were correlated with the alterations of the bands. Results were evaluated by chi-square 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 23 patients were available for the survival study. Overall survival distributions were calculated by Kaplan-Meier method and analyzed using the log-rank test. P values < 0.05 were considered statistically significant.

Results

According to AP-PCR analysis of 30 ICC cases, DNA fingerprinting obtained from 21 out of 30 decamer primers have revealed this genetic aberration that are AO16, BC17, BB13, AB19, AO19, U8, S3, AA14, AU1, BF12, Q7, Y7, M7, AO10, AO5, BB3, BG4, H8, N20, 015, and S13 primers, ranked by frequency. The frequency of genetic instability amplified from 11 random primers, which show the prevalent between 9 and 77% were presented in Figure 1. Band characteristics captured from AP-PCR were shown in Figure 2. From all 30 cases, the highest frequency of genetic aberration occurred from primer

Table 1. Histological Parameters and Genetic Alterations in ICC Patients

Parameters	Genetic alterations +n (%)	-n (%)	P value	OR (95% CI)
AO16			0.038*	
Well-diff	5 (27.8)	13 (72.2)		1.00 (referent)
Mod diff	5 (71.4)	2 (28.6)		6.50 (0.71-73.74)
Poor diff	4 (80.0)	1 (20.0)		10.4 (0.72-318.8)
BC17			0.866	
Well-diff	5 (27.8)	13 (72.2)		1.00 (referent)
Mod diff	2 (28.6)	5 (71.4)		1.04 (0.10-10.04)
Poor diff	2 (40.0)	3 (60.0)		1.73 (0.14-20.58)
BB13			0.068	
Well-diff	2 (11.1)	16 (88.9)		1.00 (referent)
Mod diff	2 (28.6)	5 (71.4)		3.20 (0.23-47.14)
Poor diff	3 (60.0)	2 (40.0)		12.0 (0.82-279.9)
AB19			0.152	
Well-diff	2 (11.1)	16 (88.9)		1.00 (referent)
Mod diff	3 (42.9)	4 (57.1)		6.00 (0.52-83.95)
Poor diff	2 (40.0)	3 (60.0)		5.33 (0.34-101.8)
AB19			0.399	
Well-diff	4 (22.2)	14 (77.8)		1.00 (referent)
Mod diff	0	7 (100)		-
Poor diff	1 (20.0)	4 (80.0)		0.90 (0.13-6.35)
S3			0.186	
Well-diff	3 (16.7)	15 (83.3)		1.00 (referent)
Mod diff	0	7 (100)		-
Poor diff	2 (40.0)	3 (60.0)		0.30 (0.02-4.12)

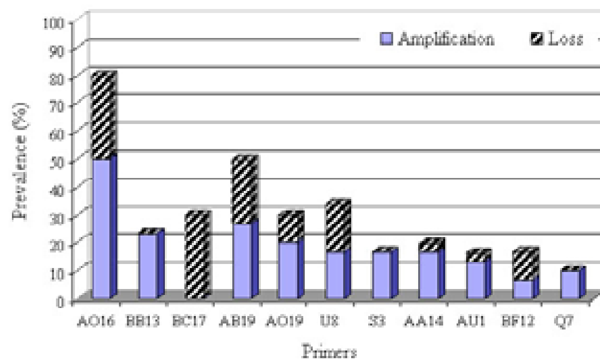

Figure 1. Genetic Instability in the 30 ICC Patients

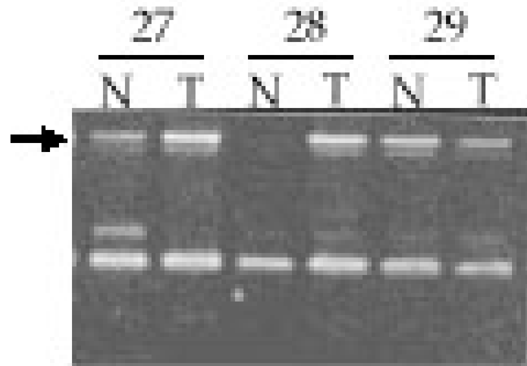

Figure 2. Band-pattern Changes Compared between Normal (N) and Tumor (T) Counterparts - Arrow Indicates Genetic Instability

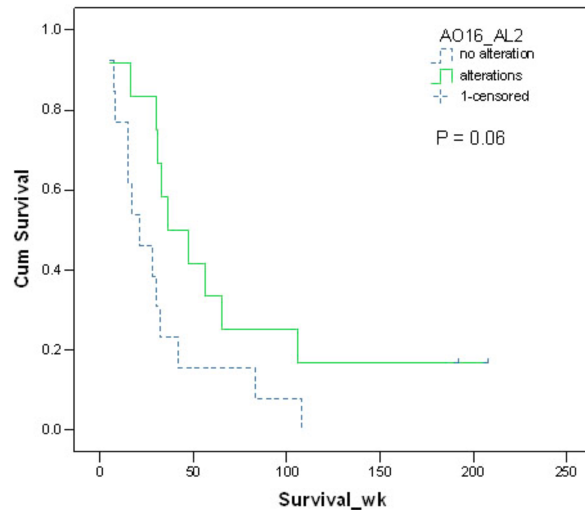

Figure 3. Overall Survival Curves for ICC Patients according to the Genetic Alteration from Primer AO16

AO16 (23 cases, 77%) as demonstrated in Figure 1. Moreover, only one pure loss band (decrease of gene copy number) appeared from primer BC17 (9 cases, 30%).

Statistical analysis showed that DNA alteration from AO16 primer associated significantly with histological type—moderately to poorly differentiated cells (P = 0.038) as shown in Table 1. However, Kaplan-Meier survival curves did not show significant correlation with the DNA aberration (P = 0.06) as shown in Figure 3.

Discussion

The modified AP-PCR technique is still a useful and feasible method for screening the genetic alterations in various cancers (Sood et al, 1996; Dil-Afroze et al, 1998; Ong et al, 1998; De Juan et al, 1999; Maeda et al, 1999; Sirivatanauksorn et al, 1999; Singh and Roy, 2001; Luo et al, 2003; Zhang et al, 2004; Chariyalertsak et al, 2005). This approach is much easier to perform and may detect DNA changes in the whole genome. Additionally, DNA fragments from unidentified amplified chromosomal regions can be easily and directly cloned. Moreover, the quantitative changes can be resolved using real-time PCR technique, which could offer much more precise quantification of DNA (Chariyalertsak et al, 2005). Based on the results reported here, this manner was applied to screen the genomic aberration of Thai patients with ICC.

The high occurrence of genomic sequence alterations in ICC demonstrated a crucial role for gene copy number in this cancer. As shown in Figure 1, the highest frequency of genetic alterations was revealed from primer AO16; ~80% of all genomic changes. A high incidence of gene amplification (>50%) has been previously reported in cholangiocarcinoma (Chariyalertsak et al, 2005). These fragments may concern the carcinogenesis of ICC. We thus investigated whether there was a correlation between AO16 amplification and survival rate. Interestingly, a curve came near to significant longer disease-free interval was seen when patients bearing the variation sequence were compared with those without this instability (P = 0.06 for all by log-rank test) as shown in Figure 3. The increased risk of recurrence in this group of patients suggests a good prognosis phenotype.

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