Prognostic significance of microsatellite alterations at 1p36 in cholangiocarcinoma

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AIM: To investigate loss of heterozygosity (LOH) and microsatellite instability (MSI) on the chromosomal region 1p36-pter in cholangiocarcinoma (CCA) patients and determine the association between microsatellite alterations and clinicopathological parameters.

METHODS: Ten polymorphic microsatellite markers were determined for LOH and MSI using GS-3000 gel scan fragment autoanalyzer.

RESULTS: Sixty-eight out of 90 cases (75.6%) showed LOH in one or more loci. LOH was found most frequently at D1S199 (40.0%), D1S507 (34.6%), D1S2845 (30.5%), and D1S2734 (30.1%). MSI was found in 34 of 90 cases (37.8%) at one or more loci. Fine mapping at 1p36 showed two distinctive regions of common loss, which were D1S2845 and the 25.5-cM region between D1S507 and D1S2734, indicating the existence of putative tumor suppressor genes that is likely to play important roles in the development of CCA. Patients with LOH at D1S234 showed less lymphatic invasion (P = 0.017), whereas patients with LOH at D1S2676 exhibited more lymphatic invasion than those without (P = 0.031). LOH at D1S2845 showed a significant correlation with nerve invasion (P = 0.029). Moreover, patients who demonstrated MSI at D1S228 showed a poor prognosis (P = 0.0026).

CONCLUSION: Allelic loss plays a major role in microsatellite alterations at chromosome 1p36, which may contribute to carcinogenesis and pathogenesis of liver fluke related CCA and these alterations can be used as molecular prognostic indicators for CCA patients.

Key words: Cholangiocarcinoma; Liver fluke; Chromosome 1p36; Loss of heterozygosity; Microsatellite instability

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INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor arising from bile duct epithelium and commonly found in the northeastern region of Thailand with the highest incidence in Khon Kaen[3]. In Thailand, chronic infection with the liver fluke, Opisthorchis viverrini (OV), is the major risk factor for the development of CCA[3]. Prolonged inflammation induced by parasitic infection causes continuous production of free radicals that not only combat the infection, but also act as carcinogens causing mutations and chromosomal breakage and aberrations within the body[8]. Patients with CCA are often diagnosed clinically and cholangiographically but mostly are in advanced stage and difficult to cure successfully. The prognosis of CCA is extremely poor[8]. Moreover, the molecular events involved in the development of CCA are not well understood.

Microsatellites are short tandem repeat sequences of unknown function scattered throughout the human genome. Loss of heterozygosity (LOH) and microsatellite instability (MSI) are the phenotypes of genetic instability caused by the abnormalities of tumor suppressor and DNA mismatch repair (MMR) genes, respectively. MSI...
DNA was extracted from blood and liver resection samples obtained from the patients who had undergone surgery at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

Patients and DNA preparation
Blood and liver resection samples were obtained from 90 patients with intrahepatic cholangiocarcinoma who underwent surgery at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Informed consent was obtained from each patient according to the guidelines of the Ethical Committee of Khon Kaen University. DNA was extracted from microdissected tissues using DNA isolation kit according to manufacturer’s instructions (Puregene, Gentra systems, USA). Matched peripheral blood leucocytes were prepared for DNA by the method described previously.

Microsatellite markers
Ten microsatellite markers specific for the chromosomal region 1p36-pter were used. The forward primer of each marker was labeled at 5’ end with 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein (HEX). Primer sequences were obtained from the Human Genome Database as shown in Table 1. D1S468 is located at telomeric end while D1S2676 is located toward centromeric end.

PCR amplification
PCR was performed in a 25 μL reaction containing 50 ng of DNA, 100 μmol/L of each deoxynucleoside triphosphate (dNTP), 5 pmol of each primer, 3 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris HCl pH 8.3, and 1.5 units of Taq DNA polymerase. Additives such as 0.4 μL Triton X-100 or 0.4 μL/L Tween 20 were added in the amplification of tissue DNA. The PCR conditions were denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s (or at 56°C for D1S468 and D1S2845, at 59°C for D1S199, and at 54°C for D1S2734), extension at 72°C for 30 s, and final extension at 72°C for 10 min.

Analysis of LOH and MSI
PCR products were denatured by 950 mL/L formamide and electrophoresed on 6% or 8% polyacrylamide gels containing 7 mol/L urea using GS-3000 gel scan fragment autoanalyzer (Corbett Research, Australia). MSI was defined as changes of size bands observed in tumor DNA but not visible in the corresponding normal DNA. For informative cases, allelic loss (LOH) was assessed if the signal of one allele was at least 50% reduced in the tumor DNA as compared with the corresponding normal allele. Our previous study showed the same pattern between non-tumorous tissue and leucocyte DNA, therefore in this study only leucocyte DNA was used as the corresponding normal DNA. Each assay was performed twice in order to ensure experimental reproducibility.

Statistical analysis
LOH and MSI frequencies were correlated with several clinical parameters using $\chi^2$ test. Survival curves were calculated using Kaplan-Meier and log rank test. The result was considered statistically significant when $P < 0.05$. 

RESULTS

LOH and MSI analysis
The percentage of LOH was analyzed based on informative cases while that of MSI could be analyzed within 90 cases. Representative examples of LOH and MSI at various loci are shown in Figure 1. LOH at one or more loci was observed in 68 out of 90 cases (75.6%). If a percentage of LOH at a locus was more than 30%, we determined that LOH frequency of the locus was significantly high. The percentage of LOH at each microsatellite marker varied from 17.3% to 40.0%. Among 10 loci, we observed...
4 loci with significantly high LOH frequency, which were D1S199 (40.0%), D1S507 (34.6%), D1S2845 (30.5%), and D1S2734 (30.1%). MSI was found in 34 (37.8%) of 90 cases at one or more loci. Among 10 loci, 3 loci showed percentage of MSI more than 10%, which were D1S234 (12.2%), D1S228 (11.2%), and D1S450 (10.1%). The percentages of LOH and MSI at each locus are shown in Figure 2. Interestingly, the percentage of MSI at each locus was much less than that of LOH, suggesting that LOH is a preferable pathway to MSI for the alterations of microsatellite markers on the chromosome 1p36 in liver fluke associated CCA.

**Fine mapping of chromosomal region 1p36-pter**

Fine mapping of the chromosomal region 1p36-pter in 90 CCA cases is shown in Figure 3. Four distinctive loci with high LOH frequencies were denoted giving rise to two common loss regions; D1S2845 and the 25.5-cM region between D1S507 and D1S2734.

**Correlation between microsatellite alterations and clinicopathological data**

The associations between LOH and MSI at each locus and clinicopathological parameters of CCA patients were analyzed. Patients with LOH at D1S2845 showed significantly more nerve invasion than those without ($P = 0.029$). Samples with moderately differentiated adenocarcinoma rarely found LOH at D1S234 ($P = 0.035$). Patients who had LOH at D1S234 exhibited significantly less lymphatic invasion ($P = 0.017$), whereas patients with LOH at D1S2676 showed significantly more lymphatic invasion ($P = 0.031$). Clinical features of patients with and without LOH at loci D1S2845, D1S234, and D1S2676 are summarized in Table 2. A survival curve by the Kaplan-Meier method showed a poor prognosis for patients who demonstrated MSI at D1S228 (Figure 4). The log-rank test confirmed a significant association between poor prognosis and MSI at D1S228 ($P = 0.0026$).

**DISCUSSION**

Microsatellite polymorphisms provide a large number of highly informative loci, which may allow simultaneous, rapid screening of LOH and MSI using very small amounts of DNA samples. Our finding of LOH at one or more loci was observed in 68 (75.6%) out of 90 cases. This finding is different from that of Momoi et al[11]. They determined fine mapping of chromosome 1p36 using 13 markers in 22 CCA patients and showed LOH at one or more loci in 13 (59.1%) cases. The differences...
Markers may lead to different percentages. However, by comparing 5 similar markers used in Momoi et al and our studies, the LOH frequencies at D1S450 (5%), D1S507 (18.7%), and D1S199 (22.2%) detected by Momoi et al were lower than those of our study, whereas the LOH frequencies at TP73 (54.5%) and D1S228 (29.4%) were higher than those of our study. This suggests differences in tumor etiology. In our study, all tumors were obtained from patients who were residents of northeastern region of Thailand where liver fluke infection remains highly endemic. Genetic alterations in CCA caused by liver fluke infection may be different from those caused by others such as hepatolithiasis and primary sclerosing cholangitis. The different percentages also suggested the specificity of genetic alterations in each tumor type.

The p73 gene is mapped to 1p36 at D1S468 and p73 protein is structurally similar to the p53 protein within its DNA-binding, transactivating, and oligomerization domains. The p73 protein also shares some functional characteristics with p53, including the ability to promote apoptosis when overexpressed in vitro and up-regulate p53 responsive genes involved in cell-cycle control such as p21. In neuroblastomas, the incidence of p73 LOH was found to be significantly higher in advanced tumors than in earlier stage. In breast carcinomas, LOH in the p73 region could be pathogenically related to breast cancer and possibly to a poor tumor prognosis.

A relatively high frequency of LOH at D1S468 (19.4%) in our study suggests that inactivation of p73 may play a role in CCA development. Fine mapping of chromosome 1p36 showed two regions of common allelic loss. The first region was at D1S2845 and the second region was from D1S507 to D1S2734 loci. A relative large area of deletion involving markers D1S507, D1S199, and D1S199 was frozen tissues which may have dilution effects from normal tissues, whereas the samples from patients who were residents of northeastern region of Thailand where liver fluke infection remains highly endemic. Genetic alterations in CCA caused by liver fluke infection may be different from those caused by others such as hepatolithiasis and primary sclerosing cholangitis. The different percentages also suggested the specificity of genetic alterations in each tumor type.
D1S2734 encompasses a 25.5-cM region. Candidate tumor suppressor genes located at D1S2845, D1S507, D1S199, and D1S2734 are DNA fragmentation factor, 40 kU, beta poly(ADP-ribose) polymerase (DFFB); caspase 9, apoptosis-related cysteine protease (CASP9); paired box homeotic gene (PAX7); and inhibitor of DNA binding 3 (dominant negative helix-loop-helix protein, ID3), respectively.

Statistical analyses were performed to determine whether the frequency of individual microsatellite marker showing alterations was correlated with clinical parameters. We found that MSI at marker D1S228 showed a significant correlation with poor survival ($P = 0.0026$). On the physical map, the retinoblastoma interacting zinc finger gene (RIZ) is adjacent to the polymorphic marker D1S228. In addition, RIZ harbors intragenic microsatellites and polyadenosine tracts within its coding region, it is a candidate for an inactivating mutation in MSI mediated carcinogenesis. MMR defect may cause not only MSI at D1S228 but also at the microsatellite coding sequences of RIZ, resulting in frame shift mutation of this gene and contributing to poor survival. However, our study showed that LOH at D1S228 did not affect patient survival, suggesting that LOH was not a major pathway of RIZ inactivation. An alternative pathway such as mutation and promoter hypermethylation may be a major pathway for RIZ inactivation in CCA, on which further study is needed. The high frequency of MSI found in this study (37.8%) suggests the involvement of MMR defect in liver fluke related CCA. However, the frequency of hMLH1 alteration found in MSI (+) tumors showed no significant difference from that of MSS tumors (data not shown), suggesting that other MMR members may be defective. In contrast, infrequent MSI in CCA was reported by Liengswangwong et al. This may be due to the differences in types of samples and markers.

D1S2845 located at 1p36.3 showed a significant correlation with nerve invasion ($P = 0.0029$). The putative tumor suppressor gene located on this region is DFFB, which function is involved in apoptosis. Deletion at this region may lead to tumor progression. Marker D1S234 is located at 1p36.1 in the same region of cell division cycle 42, Cdc42. Cdc42, a member of the Rho subfamily, is plasma membrane-associated small GTPase, which cycles between an active GTP-bound and an inactive GDP-bound state. In active state, it binds to a variety of effector proteins to regulate cellular responses involved in epithelial cell polarization processes. Cdc42, Rac and RhoA influences cell shape and structure by initiating actin cytoskeleton remodeling. RhoA and Rac1 generate actin stress fibers and lamellipodia/membrane ruffles, respectively, whereas Cdc42 stimulates the formation of microspikes and filopodia. Loss of D1S234 showed less lymphatic invasion, suggesting that lack of Cdc42 may cause tumor cells to be less invasive ($P = 0.017$). Our previous study on microsatellite alterations at loci D2S119, D3S1611 and TP53 showed the association with poor prognosis. The previous and present studies indicate the value of microsatellite alterations as prognostic indicators for liver fluke related CCA. With regard to the significant correlations of microsatellite alterations with clinicopathological parameters such as survival, lymphatic invasion and nerve invasion in this study, we propose the putative tumor suppressor genes; RIZ, Cdc42, and DFFB, which may play important roles in the development and progression of liver fluke related CCA.

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