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Cancer Letters 217 (2005) 213–219
CANCER Letters
www.elsevier.com/locate/canlet

Promoter hypermethylation is a major event of *hMLH1* gene inactivation in liver fluke related cholangiocarcinoma

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Received 20 April 2004; received in revised form 14 June 2004; accepted 15 June 2004

Abstract

Cholangiocarcinoma is a crucial health problem in Northeast Thailand where liver fluke infection is endemic. However, molecular genetic and epigenetic mechanisms involved in carcinogenesis of this cancer remain unclear. We attempted to study genetic and epigenetic alterations of *hMLH1* gene in 65 intrahepatic cholangiocarcinoma using polymerase chain reaction (PCR) based microsatellite marker D3S1611 and methylation-specific PCR, respectively. Of 65 cases, 29 (44.6%) showed hypermethylation of *hMLH1* promoter. Loss of heterozygosity (LOH) of *hMLH1* was detected in 12 of 51 informative cases (23.5%). Five out of 29 (17.2%) methylated cases demonstrated LOH. Aberrant methylation of *hMLH1* promoter was significantly associated with poorly differentiated type ($P=0.013$). Our study suggests that both genetic and epigenetic mechanisms cause the inactivation of *hMLH1* where epigenetic is a major event resulting in mismatch repair deficiency and contributing to carcinogenesis of liver fluke related cholangiocarcinoma. Since, gene silencing by methylation is an early event in carcinogenesis, promoter hypermethylation of *hMLH1* may be a molecular targeted therapy and prevention of liver fluke related cholangiocarcinoma.

Keywords: Epigenetics; Cholangiocarcinoma; Liver fluke; Hypermethylation; *hMLH1*

1. Introduction

Cholangiocarcinoma is a common hepatobiliary malignancy arises from the epithelium of the biliary

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doi:10.1016/j.canlet.2004.06.020

tree. Liver fluke (*Opisthorchis viverrini*) related cholangiocarcinoma accounts for about 89% of all liver cancer in Khon Kaen with the highest incidence in the world ($97.4/10^5$ in males and $39.0/10^5$ in females) [1,2]. The 3-year survival rates were 33, 30, and 12% for stages III, IVa, and IVb, respectively, whereas 5-year survival rates were 0% for all three late stages [3]. Cholangiocarcinoma like other common epithelial cancers is believed to develop through a multistep process driven by carcinogens inducing genetic and epigenetic alterations of bile duct epithelium. However, molecular mechanisms leading to carcinogenesis and pathogenesis of cholangiocarcinoma remain unclear.

Mismatch repair (MMR) is an important mechanism by which cells correct errors in DNA replication during proliferation to maintain the fidelity of the genome. Cells with MMR defects show mutation rates up to 1000-fold greater than those observed in normal cells. In human, two major MMR genes, *hMLH1* and *hMSH2*, as well as *hMSH3*, *hMSH6*, *hPMS1*, and *hPMS2* have participated in DNA MMR [4,5]. The abnormalities of MMR genes leading to intrinsic genomic instability and mutations of genes important for growth regulation have been proposed as an early step event in carcinogenesis [6,7]. Genetic and epigenetic alterations of *hMLH1* have been reported in various human cancers particularly, in colorectal and gastric cancers [8–12]. Aberrant methylation of promoter CpG islands of human genes has been known as an alternative mechanism of gene inactivation suggesting that epigenetic plays an important role in carcinogenesis [13–15].

Recently, genetic alterations of *hMLH1* in liver fluke related cholangiocarcinoma have been reported by Limpaiboon et al. [16] suggesting genetic mechanism involved in cholangiocarcinogenesis. To evaluate the possible involvement of *hMLH1* promoter hypermethylation in carcinogenesis of liver fluke related cholangiocarcinoma, we investigated the methylation status in 65 patients with intrahepatic cholangiocarcionoma using methylation-specific polymerase chain reaction (MSP) and also analyzed the association between the methylation status and clinicopathological parameters.

2. Patients and methods

2.1. Samples and DNA preparation

Samples of liver resection were obtained from 65 patients with intrahepatic cholangiocarcinoma who were admitted to Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. Informed consents were obtained from all patients who participated in the project approved by the ethical committee of Khon Kaen University. These patients were residents of the Northeast region of Thailand where liver fluke is highly endemic. Genomic DNA was prepared from frozen tumor and normal tissues according to standard procedure.

2.2. Methylation-specific PCR (MSP)

DNA methylation patterns in the *hMLH1* promoter were determined by bisulfite modification and followed by MSP as described previously [17]. Primer sequences of *hMLH1* for unmethylated reaction were 5′-TTT TGA TGT AGA TGT TTT ATT AGG GTT GT-3′ (sense) and 5′-ACC ACC TCA TCA TAA CTA CCC ACA-3′ (antisense), and for the methylated reaction were 5′-ACG TAG ACG TTT TAT TAG GGT CGC-3′ (sense) and 5′-CCT CAT CGT AAC TAC CCG CG-3′ (antisense) [11]. Polymerase chain reaction (PCR) was performed using the primer pairs under the following conditions: the PCR mixture (25 μl) contained 1× PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris pH 8.8, 10 mM 2-mercaptoethanol), 6 mM $MgCl_2$ (methylated reaction) or 7 mM $MgCl_2$ (unmethylated reaction), 200 μM of each deoxynucleoside triphosphate, 2.5 pmol of each primer, 100 ng bisulfite-modified DNA, and 1 unit of *Taq* DNA polymerase. Amplification was carried out in a PTC 200 Peltier Thermal Cycler (MJ Research, Inc) as follows: 5 min at 95 °C, followed by 30 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C for 35 cycles, and followed by a final 7-min extension at 72 °C. Human placental DNA treated in vitro with SssI methyltransferase (New England Biolabs) served as a positive control for the methylated reaction. Human leucocyte DNA was used as a control for unmethylated reaction. Ten microliter of each PCR product were directly loaded onto nondenaturing 10% polyacrylamide gels (29:1),