The Korean Journal of Pathology
2005; 39: 9-14

Microsatellite Instability and Mismatch Repair Protein (hMLH1, hMSH2) Expression in Intrahepatic Cholangiocarcinoma

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Received : October 6, 2004
Accepted : October 27, 2004

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Background : To clarify the role of the mismatch repair (MMR) system in the carcinogenesis of intrahepatic cholangiocarcinoma (ICC), we investigated the microsatellite instability (MSI) status and MMR protein expression in ICC. **Methods :** Thirty-six cases of ICCs were examined by microsatellite analysis for 55 markers that encompassed all of the chromosomal arms and BAT26. An immunohistochemical study for hMLH1 and hMSH2 was also performed. **Results :** Widespread MSI (MSI-H) accompanied with a loss of hMLH1 expression was found in one case (2.8%). This MSI-H case was an adenosquamous carcinoma showing intraductal tubulopapillary adenocarcinoma and invasive adenosquamous carcinoma component. Loss of hMLH1 was noted in both components while the frequency and shifted band patterns of MSI were not identical between the components. Another 10 ICCs (27.8%) revealed low level MSI with preserved MMR gene expression. **Conclusions :** Our data suggested that a genetic defect in the MMR system and MSI is not a major pathway in the carcinogenesis of ICC.

Key Words : Cholangiocarcinoma; Microsatellite repeats; hMLH1 protein; hMSH2 protein; Immunohistochemistry

Intrahepatic cholangiocarcinoma (ICC) is a malignant tumor arising from intrahepatic bile duct epithelial cells. It is the second most common tumor in the liver, and it has a high prevalence in parts of southeast and eastern Asia, including Korea.[1,2] Recent reports have suggested a remarkable increase in the incidence and mortality from ICC in the western countries.[3,4] Despite the recent advances in the diagnostic and therapeutic procedures for liver cancers, the prognosis of ICC remains dismal because of the lack of an early diagnostic method and the low rate of resectability.[5]

It is commonly held that cancer arises as the result of an accumulation of damage in the critical regulatory genes, and such genetic instability is an integral component of human neoplasia.[6] Genetic instability occurs in two different forms, chromosomal instability (CIN) and microsatellite instability (MSI). The CIN pathway usually begins with a high frequency of allelic losses and cytogenetic abnormalities. The MSI pathway occurs with the inactivation of genes that are responsible for DNA nucleotide mismatch repair (MMR), and this leads to mutations in DNA sequences at the nucleotide level.[6] High frequency of MSI (MSI-H) is a marker of an underlying MMR defect that fails to recognize replication errors. A group of genes including hMLH1, hMSH2, PMS1, PMS2, hMSH6/GTBP, and hMSH3 have been identified and known to be responsible for MSI.[7,8]

In the previous study, we provided the data for genome-wide allelotyping of ICC that showed high fractional allelic loss (FAL) value.[9] Several previous reports have evaluated MSI in biliary tract cancer; however, little is known about the possible role of MMR genes in the carcinogenesis of ICC.[10-18]

In the present study, we investigated the MSI status in ICC by using polymerase chain reaction (PCR) based methods, and evaluated the mismatch repair protein expression by immunohistochemical staining to clarify the putative role of the MMR system for this particular neoplasm.

MATERIALS AND METHODS

Tissue samples

The hepatic resection samples from 36 patients with pathologically defined ICC at Seoul National University Hospital (29 cases) and Inje University Seoul Paik Hospital (7 cases) were ana-

lyzed. All the samples were formalin fixed and paraffin embedded by a routine procedure. Microdissection and DNA extraction from the tumor and nonneoplastic liver tissue were performed as previously described.[9]

Microsatellite instability analysis

The MSI status was assessed by using fifty-five dinucleotide microsatellite markers that covered all of the non-acrocentric chromosome arms[9] and BAT26 mononucleotide marker. The PCR amplification was carried out in a 20 μL reaction mixture containing 50 ng DNA, 1×*Taq* polymerase buffer including 1.5 mM $MgCl_2$ (Promega, Madison, WI), 0.4 pmol of each primer, 0.2 mM dNTP, 1.5 μCi of [α-32P]dCTP, and 1 unit *Taq* polymerase. PCR was performed with the use of a thermal cycler (version 2.0; Perkin Elmer Cetus, Norwalk, CT). The one cycle of denaturation was carried out at 94℃ for 4 min; this was followed by 29 to 32 cycles of denaturation at 95℃ for 30 sec, annealing at 55-60℃ for 30 sec, an extension step at 72℃ for 30 sec and a final extension at 72℃ for 10 min. The PCR products were resolved on a denaturing 6% polyacrylamide gel and visualized by autoradiography. MSI was determined to be present when an additional, abnormal-sized band was detected in the tumor DNA in comparison with the normal DNA at a given locus.

Immunohistochemistry

Immunohistochemical staining was performed using mouse monoclonal antibodies for hMLH1 (clone G168-15; BD biosciences, San Jose, CA) and hMSH2 (clone FE11; Oncogene science, Cambridge, MA) at 1:50 dilutions as described previously.[12] The normal staining pattern for both hMLH1 and hMSH2 was nuclear, and discrete nuclear staining in 5% or more of the tumor cells was considered as positive. The absence of nuclear staining in the presence of an internal positive control, which was represented by nonneoplastic epithelial cells or stromal cells, was considered negative.

RESULTS

MSI status and hMLH1, hMSH2 immunohistochemical expression

Eleven of the 36 ICC cases (30.6%) demonstrated MSI for at least one of the 55 markers analyzed, and the remaining 25 cases were stable for all the microsatellite markers (MSS). Ten of the 11 tumors showed MSI at one (7 cases) or two markers (3 cases) (low frequency MSI, MSI-L). Only one of the 11 tumors (2.8%) showed widespread MSI at 37 markers (MSI-H) (Fig. 1). The MSIs were evenly dispersed throughout the markers. All the ICCs exhibited a single normal allele at BAT 26 and none of the ICC cases revealed any allelic shift (Fig. 1). No MSI was noted in the nonneoplastic liver tissue.

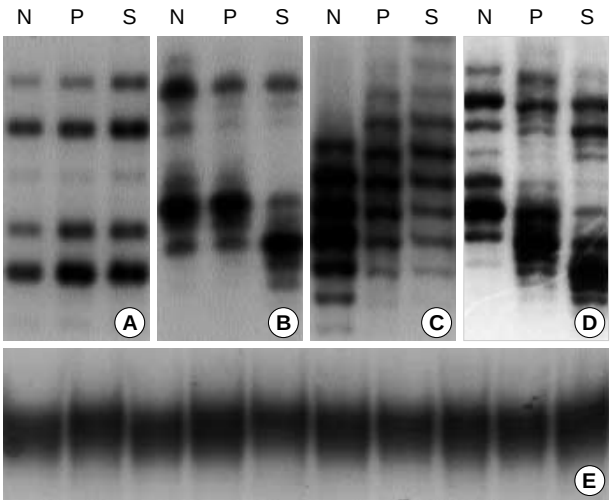

Fig. 1. (A-D) Examples of widespread MSI in one of the ICC cases. No shifted band in D9S103 (A), MSI in S only in D8S254 (B), same MSI pattern in both P and S in D6S271 (C), and MSI in both P and S with further shifted band in S in D13S118 (D). (N: nonneoplastic liver, P: intraductal papillotubular adenocarcinoma, S: invasive adenosquamous carcinoma) (E) No alteration of the BAT26 microsatellite marker in ICC cases.

The ICC case with MSI-H showed a loss of immunoreactivity for hMLH1 protein. All of the MSI-L and MSS cases revealed the nuclear hMLH1 expression. For the hMSH2 protein, none of the ICC cases demonstrated a loss of expression (Fig. 2).

ICCs with microsatellite instability

The ICC case with the simultaneous MSI-H and loss of hMLH1 expression was an adenosquamous carcinoma showing an intraductal, well differentiated, tubulaopapillary adenocarcinoma that was combined with an invasive adenosquamous carcinoma component (Fig. 2). Loss of hMLH1 expression and the preserved nuclear hMSH2 expression were equally identified in both components. It was interesting that the amplicon lengths for markers with MSI were different in the intraductal and invasive components of the carcinoma for 27 out of 37 microsatellite markers: In the invasive component, MSI was noted only for 9 markers, and additional or further shifted bands was noted for anoth-

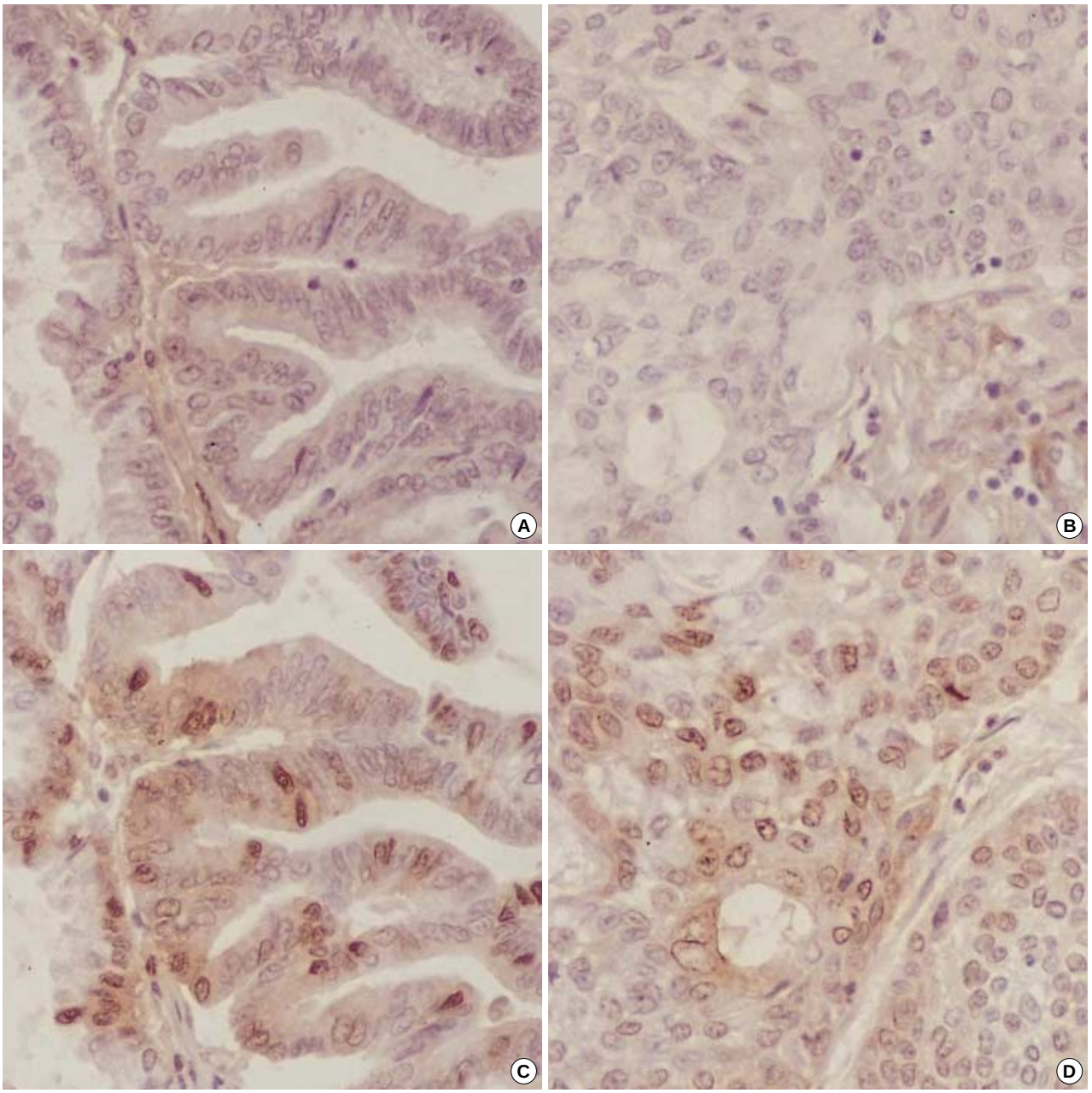

Fig. 2. (A-D) Photomicrographs of the expression of mismatch repair proteins in ICC with widespread MSI showing loss of hMLH1 in intraductal (A) and invasive (B) carcinoma, and preserved hMSH2 in intraductal (C) and invasive (D) carcinoma component. *(Fig. 2 continued next)*

er 18 markers. For the remaining 10 markers, the intraductal and invasive components of ICC showed the same MSI pattern (Fig. 1)

One of the MSI-L ICCs showed no LOH at any of the markers; however there was no difference for the FAL values between the MSI-L and MSS ICCs. Other clinicopathological parameters including age, gender, tumor size, histological type, degree of differentiation, tumor location and the gross type did not show any difference between the MSI (low or high) and MSS ICCs (Table 1).

DISCUSSION

Little is known about the incidence of MSI and the underlying MMR defect in biliary tract cancer including ICC.[10-18] Various frequencies of MSI have been found in ICC by several authors[13-17] and the highest reported MSI frequency was 62.5% in thorotrast-induced human intrahepatic cholangiocarcinoma.[17] However, several of the previous studies did not distinguish between MSI-H and MSI-L, and the authors regarded MSI-L as MSI positive

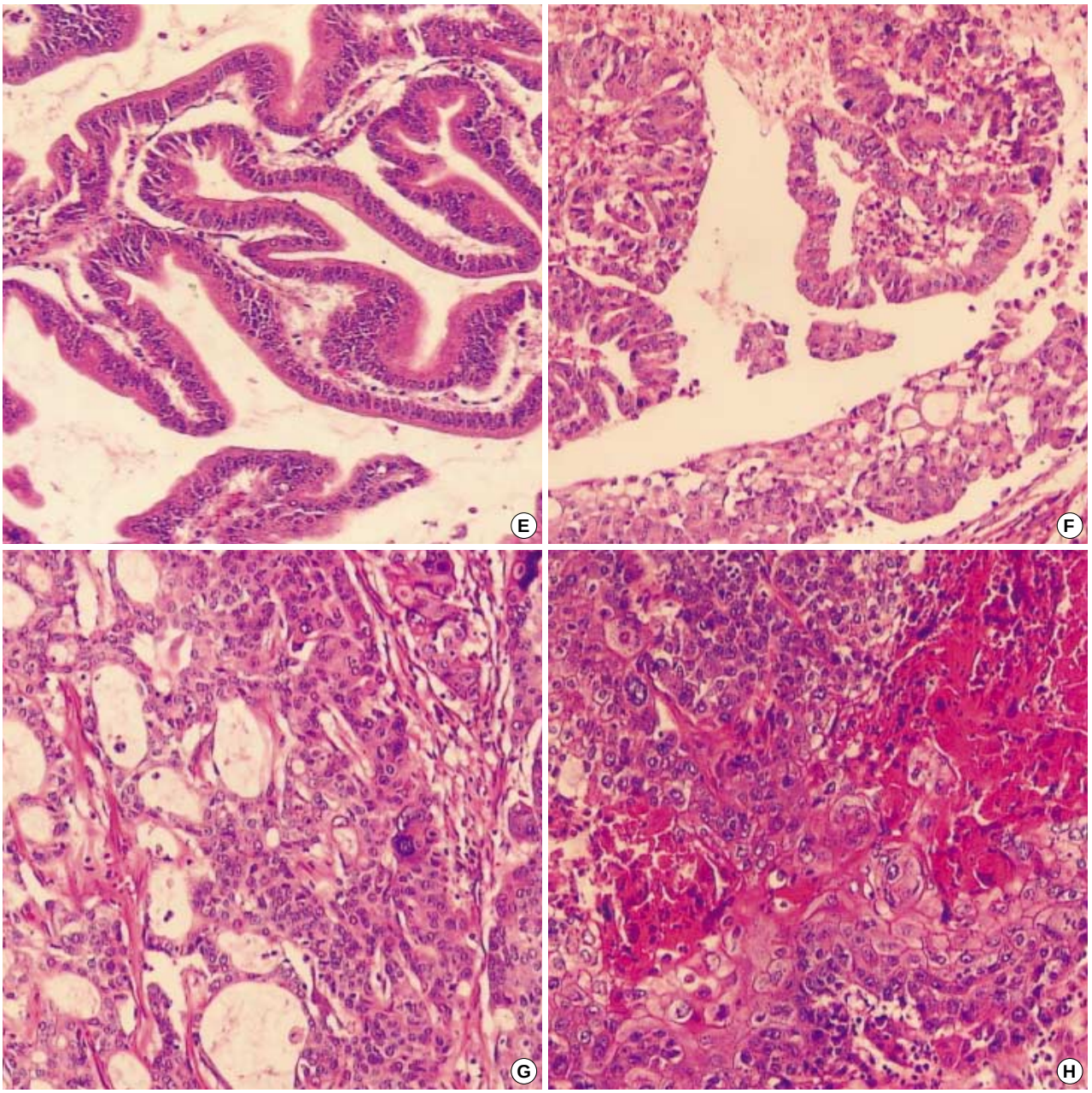

Fig. 2. *(Continued from the previous page)* (E-H) Histologically, the MSI-H case was an adenosquamous carcinoma consisted of intraductal papillotubular adenocarcinoma (E) and invasive adenosquamous carcinoma component (G, H) with area of gradual transition (F).

tumors.[14,17] Others have used selected microsatellite markers that are not recommended for MSI analysis.[13,16] Momoi *et al*.[15] studied MSI in 22 ICCs with eight microsatellite markers and they reported 4 MSI-H tumors (18.2%); however, they did not evaluate the status of the MMR system. Our MSI data was obtained from 36 ICCs by using 55 microsatellite markers that encompassed all the chromosomal arms as well as the important mononucleotide marker, BAT 26, and we showed a lower frequency of widespread MSI (2.8%) than those values obtained from the previous studies. This low frequency of MSI was comparable to the data that was obtained from biliary tract cancer including gallbladder carcinoma[10,12] and other non-hereditary nonpolyposis colon cancer (HNPCC) type cancers.[8]

We also performed an immunohistochemical analysis for hMLH1 and hMSH2 protein, and showed a concurrent loss of expression of hMLH1 protein in the MSI-H ICC. To the best of our knowledge, this is the first study for the immunohistochemical evaluation of hMLH1 and hMSH2 in ICC that provides reli-

Table 1. Correlation of MSI status of intrahepatic cholangiocarcinoma with FAL value and clinicopathological parameters

	MSI status		*p* value
	MSI*	MSS	
FAL value†	0.340±0.210	0.316±0.193	NS
Sex Male	9	21	NS
Female	2	4	
Age >50	8	15	NS
≤50	3	10	
Size >5 cm	6	12	NS
≤5 cm	5	13	
Histology			
Tubular	9	21	NS
Papillary	0	2	
Mucinous	1	2	
Adenosquamous	1	0	
Differentiation			
Well	2	6	NS
Moderate	7	16	
Poor	2	3	
Location			
Hilar	4	6	NS
Peripheral	7	19	
Gross type			
Mass forming	1	8	NS
Spicula forming	7	15	
Periductal	3	2	

*Cases include both MSI-H and MSI-L. †Mean±standard deviation and values are analyzed by one way ANOVA test. NS, not significant.

able data for MMR defects in MSI-H ICC. There are two previous studies that examined the loss of heterozygosity (LOH) or promoter hypermethylation of hMLH1,[16,17] providing an association between alterations of hMLH1 and the MSI positive phenotype.

In this work, the ICC with a MMR alteration was an adenosquamous carcinoma showing an intraductal, well differentiated, tubulopapillary adenocarcinoma and an invasive component of adenosquamous carcinoma. The MSI and loss of hMLH1 expression identified in both components provided evidence that they arose from the same progenitor cell and the MMR deficiency is an early event in the carcinogenesis of ICC. However, the higher rate and additional band-pattern MSI found in the invasive adenosquamous carcinoma than the intraductal adenocarcinoma component suggests that there were subsequent alterations leading to genetic heterogeneity in the invasive component. Different amplicon sizes and additional shifting loci were previously reported in the invasive carcinoma associated with biliary intraductal papillary neoplasm[18] and also in the squamous cell carcinoma component of gastric adenosquamous carcinoma.[19]

It is now generally accepted that MSI tumors exhibit different clinicopathological behaviors and MSI-H has been reported as a marker for a better prognosis in HNPCC type cancers.[7,8] Several studies have reported an association of MSI-H with the mucinous histologic type, an early age of onset of cancer[10] and a better cumulative survival rate[11] in biliary tract cancer. In ICC, the association of MSI-H in the carcinogenic steps with certain gross subtypes of cancer (mass with periductal infiltrating subtype) and higher clinical stage has been presented.[15] Limpiboon et al.[16] have reported a significant association of MSI at the hMSH2 related marker (D2S119) with poor survival in the liver fluke related cholangiocarcinoma. In the present study, we analyzed the clinicopathological parameters between MSI (low or high) and MSS ICCs, however, no distinguishing finding was noted because only one of MSI-H ICC was identified.

Our data suggested that genetic defect in the MMR system and MSI is not a major pathway in the carcinogenesis of ICC.

ACKNOWLEDGEMENT

This study was supported by the 2002 Inje University Research Fund.

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