Acta Medica Okayama

Reduced expression of hMLH1 and hMSH2 gene products in high-grade hepatocellular carcinoma.

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Abstract

We performed an immunohistochemical analysis of 2 major DNA mismatch repair proteins, human Mut L homologue-1 (hMLH1) and human Mut S homologue-2 (hMSH2), in hepatocellular carcinoma (HCC) using 33 biopsied and 58 surgically resected specimens, as well as 30 samples from non-cancerous livers. In well-differentiated HCCs, the immunoreactivity for these antigens was well preserved, and the staining intensity was stronger compared to the surrounding liver tissues. However, among 41 moderately-differentiated and 9 poorly-differentiated HCCs of the resected cases, hMLH1- and hMSH2-positive cells were significantly reduced in 19 (38%) and 9 (18%) cases, respectively. In 9 resected tumors, the expression of both of these antigens was reduced. Moreover, in 41 tumors of differing histological grades, 10 and 5 tumors for hMLH1 and hMSH2, respectively, contained a less-differentiated area with a reduced number of immunoreactive cells. The samples from non-cancerous biopsied liver and fetal autopsy tissue were well immunostained for both hMLH1 and hMSH2. We confirmed in this series that the hMLH1 and hMSH2 defect did commonly occur in high-grade HCCs, and that it might play a role in tumor progression.

KEYWORDS: hepatocellular carcinoma, human Mut L homologue-1(hMLH1), human Mut S homologue-2(hMS2), mismatch repair proteins, immunohistochemistry

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Reduced Expression of hMLH1 and hMSH2 Gene Products in High-Grade Hepatocellular Carcinoma

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We performed an immunohistochemical analysis of 2 major DNA mismatch repair proteins, human Mut L homologue-1 (hMLH1) and human Mut S homologue-2 (hMSH2), in hepatocellular carcinoma (HCC) using 33 biopsied and 58 surgically resected specimens, as well as 30 samples from non-cancerous livers. In well-differentiated HCCs, the immunoreactivity for these antigens was well preserved, and the staining intensity was stronger compared to the surrounding liver tissues. However, among 41 moderately-differentiated and 9 poorly-differentiated HCCs of the resected cases, hMLH1- and hMSH2-positive cells were significantly reduced in 19 (38%) and 9 (18%) cases, respectively. In 9 resected tumors, the expression of both of these antigens was reduced. Moreover, in 41 tumors of differing histological grades, 10 and 5 tumors for hMLH1 and hMSH2, respectively, contained a less-differentiated area with a reduced number of immunoreactive cells. The samples from non-cancerous biopsied liver and fetal autopsy tissue were well immunostained for both hMLH1 and hMSH2. We confirmed in this series that the hMLH1 and hMSH2 defect did commonly occur in high-grade HCCs, and that it might play a role in tumor progression.

Key words: hepatocellular carcinoma, human Mut L homologue-1 (hMLH1), human Mut S homologue-2 (hMSH2), mismatch repair proteins, immunohistochemistry

M icrosatellite instability (MSI) is a marker of defective mismatch repair (MMR). MSI occurs in hereditary non-polyposis colorectal carcinoma (HNPCC)-associated tumors [1], in sporadic cases of gastrointestinal carcinoma [2] and in other tumors [3, 4]. It is currently considered to represent an alternative pathway of carcinogenesis (so-called mutation pathway) driven by the defects of MMR genes, as opposed to the tumor-suppressor pathway causing mutations of key proto-oncogenes and inactivation of tumor-suppressor genes [5]. The DNA MMR system is a group of proteins that repair short DNA duplex loops and nucleotide base mismatches in DNA synthesis. Of the MMR genes, human Mut L homologue-1 (hMLH1) and human Mut S homologue-2 (hMSH2) are most frequently affected by germline mutations in HNPCC, and their functional loss is believed to promote tumor development.

MSI analyses of hepatocellular carcinoma (HCC) have shown conflicting results among authors. Some authors have described infrequency of MSI [6-8], while others have noted that it occurred commonly, up to 63% in the cases examined [9-11]. One of the latter series [10] further pointed out that loss of heterozygosity at the microsatellites linked to hMLH1 and/or hMSH2 was important in hepatocarcinogenesis. Yano et al. [12] also demonstrated in a sequential analysis that point mutations of the hMSH2 gene were identified in 7 of 38 HCCs. These studies indicate that MMR genes, as well as MSI,

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may play a role in hepatocarcinogenesis.

Immunohistochemical defects of hMLH1 or hMSH2 are now recognized as a sign of MSI-high, having confirmed by the comparison of immunohistochemical and cytogenetic studies in colon carcinoma [13, 14], endometrial carcinoma [15], and medullary carcinoma of the pancreas [16]. Compared to cytogenetic study for MSI, immunohistochemical study could reveal the foci of negative staining, where the results could be easily correlated with histomorphological features. Conversely, such focal events might be missed in cytogenetic study for MSI. Moreover, a large number of tumors could be easily estimated immunohistochemically with the antisera against hMLH1˙ and hMSH2 that work on formalin-fixed, paraffin-embedded sections.

In the present study, we performed an immunohistochemical analysis for hMLH1 and hMSH2 proteins in HCC, which, to our knowledge, has never been performed so far.

Materials and Methods

Tissue specimens.   Seventy-one surgically resected and 38 biopsied HCCs were retrieved from the files of the Department of Pathology, Kurashiki Central Hospital, Kurashiki, and the Department of Clinical Pathology, Kurashiki Medical Center, Kurashiki. All tumors were fixed in formalin, and embedded in paraffin. For each case, hematoxylin-eosin-stained sections were available, and reviewed by 2 of the authors (W.Y., N.K.). Tumors were histologically classified as well-differentiated HCC (wHCC), moderately-differentiated HCC (mHCC) and poorly-differentiated HCC (pHCC), according to the classification of Liver Cancer Study Group of Japan [17]. Representative tumor grade was defined as the most prevalent component in the tumor, as recommended in the classification system. However, mosaic patterns with multiple histological grades were often noted in these specimens, and each HCC nodule was further divided into 2 categories as follows: simple tumor, which contained only one histological grade, and complex tumor, which was composed of more than one histological grade. Clinical data were available from the patient records.

Five biopsy cases and 13 resected tumors were deleted from the study, according to the criteria for suboptimal staining of the internal positive controls mentioned in detail below. Thus 33 biopsied tissues and 58 resected tumors remained for analysis. The patients in the biopsy series consisted of 21 males and 12 females, and their ages ranged from 40 to 77 years (mean 65.3). These tumors consisted of 28 wHCCs and 5 mHCCs. Three cases exhibited complex tumors (2 wHCC with mHCC and 1 mHCC with pHCC). HBV and HCV infections were found in 3 and 24 patients, respectively, and one patient had been infected with both viruses. Two had alcoholic hepatitis.

The patients in the surgically resected series consisted of 45 males and 13 females, and their ages were distributed from 43 to 81 years (mean: 62.7). This group was comprised of 8 wHCCs, 41 mHCCs and 9 pHCCs. HBV and HCV infections were found in 15 and 38 cases, respectively. One patient had alcoholic hepatitis.

Twenty-four samples of adult non-cancerous liver tissues and 6 fetal liver tissues were also retrieved for comparison. The former included 9 examples of acute hepatitis, 5 of HCV-related hepatitis, 4 of alcoholic hepatitis, and one each of autoimmune hepatitis, fatty liver, sarcoidosis, systemic lupus erythematosus, idiopathic portal hypertension, and a hyperplastic nodule. The patients consisted of 11 males and 13 females between 34 and 71 years of age (mean: 51). The latter group was comprised of autopsy cases at 17, 19, 21, 23, 28 and 30 weeks of gestation, except for one, which was resected due to ectopic tubal pregnancy at 8 weeks. None of 6 cases showed morphological abnormalities of the liver.

Immunohistochemistry.   Immunohistochemical staining was performed manually with formalin-fixed, paraffin-embedded tissue sections using the catalyzed signal amplification (CSA) system (DAKO Japan, Kyoto, Japan) with appropriate positive and negative controls. Tissues were sectioned at 4 μm. After deparaffinization and rehydration in xylene and gradient alcohols, slides were rinsed in 10 mM citrate buffer (pH, 6.0) and irradiated in a microwave oven (500 W) for 17 min for antigen retrieval. For hMLH1, sections were further sonicated for 2 min in an ultrasonic cleaner after cooling down at room temperature for 15 min. After cooling down at room temperature, endogeneous peroxidase activity was blocked using 3% of hydrogen peroxide in phosphate-buffered saline (pH 7.4). Sections were then incubated overnight with mouse monoclonal antibodies against hMLH1 protein (1:100; PharMingen, San Diego, CA, USA) [13-16] and hMSH2 protein (1:100; Oncogene Research product, Cambridge, MA, USA) [13, 14, 16, 18]. Diaminobenzidine was adopted as a chromogen, and the slides were counterstained with

hematoxylin.

Grading of immunostaining. Only cases with appropriate staining of the internal positive controls, such as lymphocytes and stromal cells, adjacent to or admixed in tumors, were considered suitable for evaluation.

The immunostaining was scored semiquantitatively by 2 of the authors (W.Y., N.K.) using − through + + scales as follows; − for negative stain, + for less than 25% of reactive cells; + + for more than 25% of positive cells. We described − and + as 'reduced' and + + as 'preserved' as previously described [19].

Statistical analysis. The χ-square test was used to determine whether MMR protein expression differed significantly between HCC grades.

Results

Only nuclear immunostaining for hMLH1 or hMSH2 was considered positive in this study. Intracytoplasmic staining was neglected because it was also observed in negative control slides. The latter is probably due to enhanced reaction against endogenous biotin, which could often be noted with the CSA system.

Tumor biopsy specimens. Reduction of both hMLH1- and hMSH2-positive cells was found in 3 of 5 mHCCs, while, in 28 wHCCs, most of the tumor cells were immunostained for both the antigens (Table 1, Fig. 1 A-C). Among 3 complex tumors, one wHCC with focal mHCC showed significant reduction of hMSH2-positive cells in the mHCC area. The staining intensity for these antigens was sometimes stronger in wHCC, compared to the surrounding non-cancerous tissues.

Surgically resected specimens. In 8 wHCCs, reduction of hMLH1- and hMSH2-positive cells

was noted only in 2 and 1 tumors, respectively (Table 2, Fig. 1 D-F). Among 50 tumors consisting of 41 mHCCs and 9 pHCCs, however, 19 (38%) and 9 (18%) tumors possessed reduced numbers of positive cells for hMLH1 and hMSH2, respectively. These rates were significantly higher compared with those of the wHCCs ($P < 0.01$). In 9 tumors, including 1 wHCC, 7 mHCCs and 1 pHCC, positive cells were reduced for both hMLH1 and hMSH2.

Table 3 shows the immunohistochemical features of the 41 complex tumors. Although, in most cases, the immunohistochemical status was consistent throughout the whole tumor, 10 and 5 tumors for hMLH1 and hMSH2, respectively, contained a less-differentiated area with a reduced number of immunoreactive cells (Fig. 2, A-D). The reverse immunoreactivity, with an increased number of positive cells in a less-differentiated area, was exceptional for both hMLH1 and hMSH2. The staining intensity for both hMLH1 and hMSH2 appeared stronger in the tumors, especially in wHCC, compared with non-cancerous tissues. This was confirmed through observing the areas where the 2 components apposed each other, and positive control tissues including lymphocytes and stromal cells, were properly stained. In some areas, however, the immunoreactivity of non-cancerous tissues, including internal positive controls, were too weakly immunostained to make the comparison unequivocal.

Non-cancerous specimens. Regardless of histological diagnoses, all of 24 cases were positive for both hMLH1 and hMSH2, although the ratio of positive cells and staining intensity were variable. HCV-related hepatitis tended to express more intense staining than did other lesions, but it was unrelated to the activity or chronicity of the inflammation. Both hMLH1 and hMSH2 were diffusely positive in every fetal liver. In cases at 28 and 30

Table 1 Immunohistochemical results of hepatocellular carcinoma in the biopsy materials

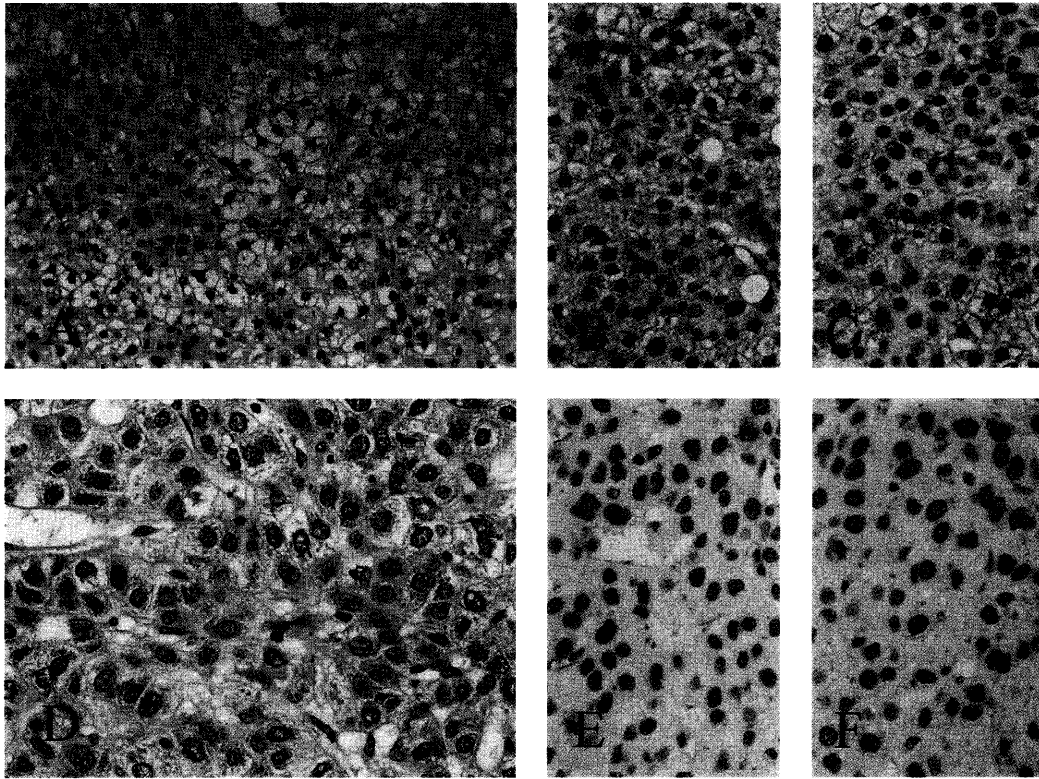| Histological grade	hMLH1			hMSH2		
	+ +	+	−	+ +	+	−
wHCC (n = 28)	28	0	0	28	0	0
mHCC (n = 5)	2	0	3	2	1	2

wHCC, well-differentiated hepatocellular carcinoma; mHCC, moderately-differentiated hepatocellular carcinoma.
+ +, more than 25% positive cells; +, less than 25% positive cells; −, negative.

Table 2 Immunohistochemical results of hepatocellular carcinoma in the surgically resected materials

	hMLH1			hMSH2		
	+ +	+	−	+ +	+	−
wHCC (n = 8)	6	1	1	7	1	0
mHCC (n = 41)	24	15	2	34	5	2
pHCC (n = 9)	7	2	0	7	2	0

pHCC, poorly differentiated hepatocellular carcinoma.

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Fig. 1   A-C, a representative example of well-differentiated hepatocellular carcinoma in biopsy specimen. Tumor with scarce cellular and structural atypia and focally increased cellularity (A, hematoxylin-eosin stain). Immunohistochemical staining for hMLH1 (B) and hMSH2 (C) exhibits diffuse and strong nuclear positivity. D-F, moderately-differentiated hepatocellular carcinoma in a surgically resected material. Tumor with obvious cellular atypia and thick trabecular pattern (D, hematoxylin-eosin stain). Both hMLH1 (E) and hMSH2 (F) are clearly stained in the nuclei of carcinoma cells.
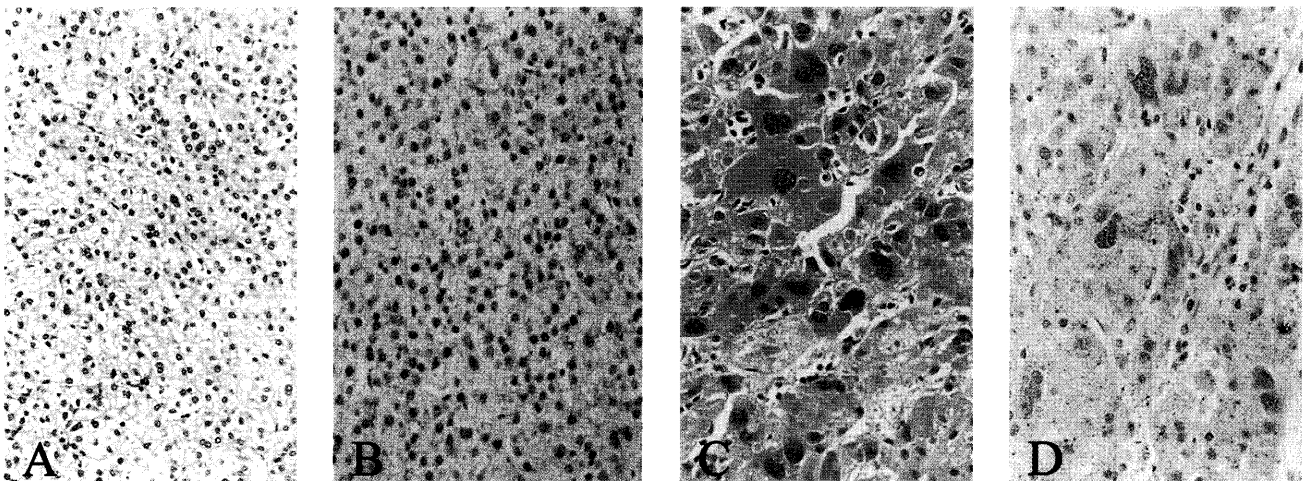
Fig. 2   A surgical example of complex tumor. A, B, well-differentiated area. Tumor with increased cellularity and minimal cytologic atypia (A, hematoxylin-eosin stain). Immunostaining for hMSH2, showing diffuse nuclear positivity (B). C, D, poorly-differentiated focus. Tumor cells with anaplasia (C, hematoxylin-eosin stain). Negative reaction for hMSH2 (D).

Table 3   Comparison of immunoreactivity among foci with different histological grades in the complex tumors

Histological grades found	hMLH1				hMSH2			
	wHCC	mHCC	pHCC	NOC	wHCC	mHCC	pHCC	NOC
wHCC + mHCC + pHCC (n = 8)	○	○	○	2	○	○	○	5
	○	○	●	1	○	●	○	2
	○	●	●	1	●	●	●	1
	●	○	●	1				
	●	●	●	3				
wHCC + mHCC (n = 19)	○	○		9	○	○		15
	○	●		3	○	●		3
	●	●		7	●	●		1
mHCC + pHCC (n = 14)		○	○	6		○	○	9
		○	●	5		○	●	2
		●	●	3		●	●	3

The open circle (○) and closed circle (●) represent more than 25% of positive cells (+ +) and less than 25% of positive cells (+ or −), respectively, in the given histological grade area.
NOC, number of cases.

Discussion

weeks, the number of positive cells was more reduced than in others.

To date, so far as we know, immunohistochemical studies of the MMR gene products have never been performed in HCC. In the present series, we clarified that the expression of hMLH1 and hMSH2 in wHCC was consistent and was often more intensified than in the adjacent liver tissues. We also identified that it was often reduced in mHCCs and pHCCs (38% and 18% for hMLH1 and hMSH2, respectively, in the resected samples), and that 9 (16%) of the 58 resected tumors had a reduced number of positive cells for both hMLH1 and hMSH2.

We considered that the enhanced expression of hMLH1 and hMSH2 in HCC is an upregulation caused by increased cell proliferation. Similar features had been observed in breast cancer, in which hMSH2 immunoreactivity was stronger in comparison with surrounding non-cancerous tissue [20]. Regarding liver cells, hMSH2 expression is consistent in fetal cell lines [21, 22], and we confirmed this immunohistochemically using autopsy tissues. However, this is not a feature of adult cell lines [21]. In the normal colonic epithelia, the immunoexpression of these MMR gene products are always found in the lower thirds of the crypts, where the cell proliferation is physically continuing [13]. Moreover,

Marra et al. directly demonstrated that hMSH2 expression obviously increased in proliferating cells [23]. These facts strongly suggest that the MMR gene products are upregulated in actively proliferating cells, whereas it might be absent or minimal in mitotically quiescent cells, such as adult hepatocytes.

We identified that MMR gene products were often reduced in mHCCs and pHCCs (38% and 18% for hMLH1 and hMSH2, respectively, in the resected samples) compared with those seen in wHCC. This is in concordance with the report of Kondo et al. [24], who described cytogenetically that 4 out of 38 HCCs had a replication error in 1 or 2 microsatellite markers, and that these were all pHCCs. We further demonstrated that this defect might play a role in the tumor progressions, because it sometimes appeared at high-grade foci in otherwise well-immunostained tumors. Our series also revealed that 9 (16%) of the 58 resected tumors had a reduced number of positive cells for both hMLH1 and hMSH2. This is difficult to explain solely in terms of coincidental mutations of both the hMLH1 and hMSH2 genes, and might be attributed to enhanced hypermethylation of gene promoters. Further cytogenetic studies are mandatory to prove this speculation.

The immunohistochemical defect of hMLH1 and/or hMSH2 has been described in advanced and poorly-differentiated tumors in various organs. In colon carcinoma, 33% and 9% of the grade III tumors were immunoreactive for hMLH1 and hMSH2, respectively,

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while in grades I and II cases, 67% and 91% of the tumors were positive [13]. In breast carcinoma, decreased immunoreactivity for hMSH2 was more common in invasive tumors, compared to *in situ* carcinomas [20], and negative staining in invasive tumors further correlated with higher histological grade, higher mitotic rate, and positive lymph node status [25]. In bladder carcinoma, reduced hMSH2 expression was more common in the cases with higher histological grade and tumor recurrence [19].

The status of MMR gene products is also a concern in non-cancerous tissues surrounding HCC, because HCC sometimes manifests multicentric tumors, and a part of such tumors might arise in genetically modified liver tissues. In fact, Salvucci *et al.* reported that MSI was frequent in cases of HBV-associated liver cirrhosis [26]. We did find negative parenchyma in resected specimens, but we cannot definitely assert its frequency. Although we deleted those cases with suboptimal staining that could cause serious problems for evaluating tumors, so far as non-cancerous tissue was concerned, the adopted cases also showed focal suboptimal staining, such as negative reactions in lymphocytes. This is probably due to the artifacts during the surgical procedures, tissue degeneration after resection, or inappropriate fixation. The low transcription level in quiescent liver cells further complicates the problem, because negative reaction in hepatocytes might not always indicate a pathological state. In many of these cases, consistently positive immunohistochemical staining in the non-cancerous biopsy samples might be attributable to the presence of an active inflammatory process.

In summary, we have demonstrated immunohistochemically that the intensity of HCC was more enhanced than non-cancerous tissues and that the expression of hMLH1 and hMSH2 was often reduced in mHCC and pHCC, compared with wHCC. We also suggested that this defect might play a role in tumor progression.

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