Diagnostic application of hMLH1 methylation in hereditary non-polyposis colorectal cancer

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Abstract. Colorectal cancer (CRC) due to mismatch repair (MMR) defect has distinct characteristics among unselected CRCs. These CRCs are biologically less aggressive and, thus, showing better prognosis but less sensitive to the 5FU-based chemotherapy. CRCs with MMR defect derive from both hereditary and sporadic reasons. Germline inactivation of MMR genes (hMLH1, hMSH2, hMSH6, and hPMS2) underlies the hereditary CRC with MMR defect (Lynch syndrome) and epigenetic silencing of hMLH1 gene causes the sporadic CRC with MMR defect. Hereditary and sporadic CRC with MMR defect can be detectable by microsatellite instability (MSI) test or immunohistochemical analysis among general CRCs. Lynch syndrome can be diagnosed by the clinical criteria or by genetic test to detect pathogenic germline mutations in MMR genes. However, both clinical criteria and genetic test are inadequate for the diagnosis of Lynch syndrome. Here we propose a possible application of methylation test combined with MSI or pathological analysis as an effective and a cost-saving new strategy for screening of Lynch syndrome.

Keywords: Hereditary non-polyposis colorectal cancer, hMLH1, methylation, immunohistochemistry, microsatellite instability.

Abbreviations: CRC, colorectal cancer; MMR, mismatch repair; MSI, microsatellite instability; MNR, mononucleotide repeat; CIMP, CpG island methylator phenotype; IHC, immunohistochemical.

1. Introduction

Colorectal cancer (CRC) causes a major public health problem in Western nations and in parts of the Orient including Japan. If one knows who is at high risk due either to certain environmental exposures and/or to a hereditary risk, CRC can be better controlled. Thus, the knowledge of a hereditary cancer syndrome is important for making effective screening programs, and ultimately the mortality rate due to CRC can be decreased.

Taking family history is of course the initial step for the diagnosis of Lynch syndrome (hereditary non-polyposis colorectal cancer (HNPCC)) because the CRC involved in this syndrome has less obvious clinical features compared to familiar adenomatous polyposis (FAP), another hereditary CRC syndrome that has observable numbers of polyps in colon and rectum. High-level microsatellite instability (MSI-H) is demonstrated in majority of the HNPCC and in 10 to 15% of sporadic CRC [1–4]. Accordingly, MSI test is a good way to recruit highly probable Lynch syndrome patients from general CRC patients. Deficiency in mismatch repair (MMR) machinery causes instability (length alteration by slippage) of microsatellite sequences either in hereditary or sporadic CRCs with MMR defect and mononucleotide repeat markers such as BAT25, BAT26,
products, such as hMSH2, hMLH1, hPMS2, and hMSH6, in tumor samples has been applied to help the diagnosis of Lynch syndrome [11] because this syndrome is caused by germline inactivation of MMR genes. Recently the CRCs with MMR deficiency can simply be identified by the histopathological examination without IHC, because observable histopathological features of these tumors have been elucidated and been generally recognized. If one can identify MSI-H CRC and distinguish the sporadic subset then most of the remainder should be HNPCC. Accordingly, elucidating of the distinguishable differences in methylation pattern of hMLH1 promoter between sporadic MSI-H and HNPCC may be important for such purposes.

2. Methylation pattern of hMLH1 in sporadic MSI-H CRC and HNPCC

Hypermethylation of the promoter region of the hMLH1 gene is considered to be associated with absent expression of hMLH1 protein in sporadic CRCs with MSI-H [8,12–14]. If the precise difference including incidence or pattern of the hMLH1 promoter hypermethylation in Lynch syndrome versus MSI-H sporadic CRC is identified, that could ultimately help to diagnose HNPCC. However, difficulty in promoter analysis of hMLH1 has been demonstrated in many studies because the methylation pattern in hMLH1 promoter is a little different from other genes also silenced by methylation.

Methylation-sensitive enzyme (e.g., HpaII) digestion and MSP have been applied to determine the methylation status of the hMLH1 promoter [8,12–14]. However, methylation-sensitive enzyme digestion can identify only those CpG sites in enzyme-recognition-sequences and, therefore, fails to recognize most CpG sites. Similarly, the methylation specific PCR (MSP) can analyze only two CpG sites at the 3′ ends of each two primers. One seemingly straightforward way of determining the precise methylation status of a gene promoter in surgically removed tumor samples would involve multiple subcloning of the relevant bisulfite-treated (and untreated) DNA fragments and sequencing them. Unfortunately most of the colorectal tumors are heterogeneous, and so we cannot be sure the reliability and reproducibility of the results that we obtain from bisulfite sequencing of the extracted DNA. Accordingly, precise methylation status of hMLH1 related to the transcriptional silencing and hence role of MMR deficiency was not well elucidated in surgically removed materials.

Another problem for the analysis of hMLH1 methylation is its unique methylation pattern for the gene silencing compared to other gene silenced by methylation. For example O6-methylguanine-DNA methyltransferase (MGMT) has dense CpG islands in its promoter, and methylation occurs simultaneously over the entire promoter except central ‘C’ region. Thus, MSP primer designed to hybridize anywhere but ‘C’ region could effectively detect the silencing of the MGMT gene. On the other hand, hMLH1 has less number of CpG islands in its promoter especially at 3′ side. Previous authors have studied different regions of hMLH1 promoter, such as its 5′-promoter part [12], 3′-promoter part [15], a small number of CpG sites, or combinations of above (Fig. 1). Methylation at the 5′-most CpG sites was initially reported to correlate with loss of expression of hMLH1 both RNA and protein levels [12]. However, most of the methylation dense area located upstream promoter has been shown to have no relation to the silencing of hMLH1, and even normal mucosa acquires hypermethylation at these upstream regions [16]. On the other hand, methylation at a 3′-small region closer to the transcription start site invariably correlated with the absence of the hMLH1 expression in vitro and colorectal tumors [17]. However, methylation status in 3′ region sometimes does not correlate with the gene silencing (unpublished data). Even the semi-quantitative COBRA analysis on that 3′ region [18] did not reveal stationary results (unpublished data). Another report demonstrated that the promoter methylation of the hMLH1 initiates from 5′ end and accumulate CpG island methylation over downward to the 3′ site to complete full-methylation [19] (Fig. 2). Age-related hypermethylation of the 5′ region of hMLH1 already starts in normal colonic mucosa and is considered to be associated with microsatellite-unstable colorectal cancer development. Full methylation from 5′ to 3′ region of the promoter only accounts for the silencing of the hMLH1. Accordingly, the conventional MSP method have failed to identify the silencing of the gene due to the difficulty in primer design, since the limited CpG site responsible for the hMLH1 silencing was not well elucidated (Fig. 1).

There were several reports with attempt to identify more precise transcriptional regulating regions in
**hMLH1** mostly by using cell lines. Methylation silencing of the **hMLH1** 3′ proximal promoter region is not uniform, but instead, appears to be confined to several targeted regions. Deng et al. identified a CCAAT box in this region, which specifically bound transcription factor CBF [20]. Methylation at CpG site two base pairs upstream of the CCAAT box inhibited the binding of CBF to CCAAT box, and is one of the causes of **hMLH1** gene silencing in colon cancer cells (Fig. 1). The second element is included between nucleotides –273 and –222, and it is recognized by a nuclear factor of about 120 kDa [21].

Thus, if one can identify the precise methylated CpGs to inhibit binding of these important elements for the silencing of the **hMLH1**, analysis of these specific CpG islands could be helpful to diagnosis HNPCC before germline mutation analysis of MMR genes.

### 3. Microsatellite markers

MSI in sporadic colorectal tumors was discovered independently in 1993 [3,4]. That same year MSI was linked to the majority of HNPCC [1]. In December 1997, the National Cancer Institute (NCI), Division of Cancer Prevention, held a workshop entitled “The International Workshop on Microsatellite Instability and Replication Error (RER) Phenotypes in Cancer Detection and Familial Predisposition” [22]. Recommendations from this workshop included five specific microsatellite markers (2 mono-nucleotide and 3 di-nucleotide markers) and reference panels for future research. This reference panel (called NCI panel for MSI) has broad utility in several experimental and diagnostic settings. At that time MSI panel was intended to elucidate the tumors caused by MMR deficiency. However, the latest findings in this very active area of
research required some modification on the definition because false positive and false negative cases by the usage of the NCI panel have been identified. In December 2002, NCI held a workshop entitled “The International Workshop on Diagnostic Guidelines for HNPCC and MSI”. At that workshop, over-diagnosis and under-diagnosis of MSI-H by the NCI panel was discussed. Jass et al. demonstrated that alteration in more than 60% instead of 30–40% of markers could be more reliable for the diagnosis of tumor with DNA MMR gene inactivation. Moreover, instability in mono-nucleotide markers (MNMs) are most specifically observed in such tumors, thus examination of MSI with multiple MNMs is important and sufficient for the diagnosis of tumor with MMR inactivation, either in hereditary or sporadic. Hemelin’s group proposed the mononucleotide repeats pentaplex (MRP) PCR that includes BAT25, BAT26, and 3 more mononucleotide markers they identified [6]. By using their panel, they can distinguish the subset of CRCs with distinctive clinicopathological features due to the true MMR defects either in hereditary or sporadic. The specificity and sensitivity for the diagnosis of MSI-H is both 100% in CRC. MSI-H CRC due to the germline mutation of hMLH6 infrequently shows MSI-H but more frequently shows MSI-L/S because such tumors have rare instability in dinucleotide markers. But by using MRP markers, hMLH6 mutants can be properly diagnosed as MSI-H. Thus, only mononucleotide repeat markers may be sufficient for the diagnosis of true MSI-H. Dinucleotide markers may only be useful for the diagnosis of MSI-L cancers, and 5 markers are not enough but nearly 10 markers are required to identify MSI-L to exclude the background low-instability, and proper specific markers for the detection of MSI-L should be selected for this purpose.

4. Pathological diagnosis of Lynch syndrome and MSI-H sporadic CRC

Limitation of immunohistochemistry (IHC) in the evaluation of MMR status in CRC was due to the frequent failure of antigenic retrieval, this being especially problematic in the case of hMLH1. These technical problems have proven to be surmountable, though false negative staining still occur in laboratories that have not fully worked up a reliable protocol.

On the other hand, histopathological features for MSI-H CRC have been discussed in the last NCI consensus meeting. The most important variables for MSI-H CRC were mucinous adenocarcinoma and ‘medullary’ or ‘undifferentiated’ carcinoma, poor differentiation, and the presence of intra-epithelial lymphocytes, mainly cytotoxic T lymphocytes described as tumor infiltrating lymphocytes (TILs). TILs can be identified in haematoxylin and eosin stained sections and more TILs are present in HNPCC. Given that more pathologists understand the features of CRC with MMR deficiency, MSI-H CRC can be now easily picked up in routine clinical setting after colorectal biopsy or surgery. If so, histopathological diagnosis plus methylation analysis of hMLH1 promoter could be most cost effective for the screening of the HNPCC.

5. Is it true that all sporadic MSI-H CRCs are caused by silencing of the hMLH1 gene and HNPCC has no methylation in hMLH1?

Germline mutation of MMR genes including hMLH1, hMSH2, hMSH6, and hPMS2 has been identified in HNPCC. On the other hand, promoter hypermethylation of hMLH1 is only known for sporadic MSI-H CRC. The question to be resolved is “Is there any HNPCC whose hMLH1 key promoter is methylated?” There was no consensus description regarding this question, and the frequency of HNPCC with promoter hypermethylation of hMLH1 were reported with a range from 0% to 30%, because method employed for the hMLH1 methylation may not be properly chosen to detect true silencing of the gene. Collaborative study to ask this question should be needed to identify the true incidence of hMLH1 methylation among HNPCC cases with pathological germline mutation.

6. CIMP

It has recently been discovered that some tumors contain several genes whose promoter regions have all been silenced by methylation; cells displaying this property are said to have an unusual phenotype known which has been described as CpG island methylator phenotype (CIMP) by Toyota et al. [18]. In the study of the epigenetic alterations in cancer causation, there was a high correlation between the methylation status of the hMLH1 gene and the methylation status of four typical CIMP genes (p16, MINT 1, 2, and 31) in the CRCc [18]. Accordingly, CIMP tumors were markedly more common in the proximal colon; frequencies of K-ras mutations were significantly higher; and mutation and/or LOH of p53 were significantly lower.
Since most of the colorectal tumors with promoter hypermethylation of hMLH1 fulfill CIMP criteria, it may be the case that MSI-H CRC with CIMP is more likely to be sporadic while those without CIMP are HN-
PCC. However, we do not have a consensus definition of CIMP criteria regarding which genes are involved in CIMP, and some of the HNPPC has promoter hypermethylation in several genes other than p16, MINT 1, 2, and 31 (unpublished data). In the aspect of co-
rolectal carcinogenesis, it may be possible that CIMP tumor without hMLH1 methylation and thus exhibit MSS/MSI-L (non-MSI-H) may become MSI-H after the acquisition of hMLH1 methylation in the course of progression.

7. Conclusions

As key methylation site in hMLH1 promoter where directly silence the expression of the gene has been elucidated, reliable methylation test to detect hMLH1 silencing becomes available, which could help cost ef-
fecive and time saving diagnosis of HNPPC before confirmed by germline analysis. Promoter hy-
permethylation may not cause hMLH1 defect in HNPPC, but large samples of HNPPC confirmed by germline in-
activation of hMLH1 should be evaluated for the true occasion of hMLH1 methylation on opposite allele as a second hit. This information should be rationale of the methylation test for HNPPC.

Since clinicopathological feature of MSI-H CRC has significant differences from others especially in the as-
pect of prognosis and sensitivity to the chemothera-
pic agent, diagnosis of the CRC with MMR defi-
ciency has become essential for the treatment of the CRC patient. Most cost effective and desirable diag-
nosis of CRC with MMR defect can be simply made by histopathology. However, IHC examination of MMR protein including hMLH1, hMSH2, hMSH6, and hPMS2, may be more important to diagnose CRC with MMR defect including sporadic MSI-H and Lynch syn-
drome because there are few sporadic CRC with alteration of hMSH2 gene. MSI test with (3 to) 5 mononu-
elotide microsatellite markers would be another al-
ternative for the diagnosis MMR defect cancer. This method can be done in a single PCR on tumor samples
without normal counterpart, which will make cost sav-
ing and can be done by tumor biopsy samples. Then, in
combination with either of the test (pathology or MSI) and the test for hMLH1 promoter hypermethylation,
identification of HNPPC can be made before the anal-
ysis of the germline mutation with cost effective and
time saving manner.

Acknowledgements

I appreciate Takeshi Nagasaka, Kenji Notohara,
Takeshi Kambara, Barbara Legget, and Jeremy R. Jass
for helpful advice and support.

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