Clinical Research

MLH1 and MSH2 mismatch repair protein profile using immunohistochemistry in Nepalese colorectal cancer patients

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ABSTRACT

BACKGROUND Hereditary nonpolyposis colorectal cancer, or Lynch syndrome, caused by germline mutations or genetic defects in mismatch repair (MMR) genes (*MLH1*, *MSH2*, *PMS2*, *MSH6*, and *epithelial cellular adhesion molecule*), is an autosomal dominant condition accounting for 2–5% of all colorectal carcinomas (CRCs). Reports on MMR loss in many populations are available; however, there are no reports on the frequency of MMR protein expression in Nepalese cohorts. Therefore, this study was aimed to assess the expression profiles of MLH1 and MSH2 protein by immunohistochemistry (IHC) in Nepalese CRC patients.

METHODS This retrospective study used archived formalin-fixed paraffin-embedded tissue blocks from 43 Nepalese CRC patients. IHC staining was performed using MLH1 and MSH2 antibodies. IHC scoring analysis was assessed using semiquantitative scoring.

RESULTS Of the 43 CRC patients, 8 (18.6%) showed loss of staining for MLH1 antibody, 5 (11.6%) showed loss of staining for MSH2 antibody, and 4 (9.3%) showed loss of staining for both MLH1 and MSH2 antibodies.

CONCLUSIONS IHC is a potential screening method of determining the MMR expression profile of Nepalese CRC patients. IHC can be performed in local clinical laboratories to find MMR protein defects in selected cases prior to expensive molecular tests.

KEYWORDS colorectal cancer, immunohistochemistry, mismatch repair, MutL homolog 1, MutS homolog 2

Colorectal cancer (CRC) is a common cancer in Nepal, and the majority of CRC patients are young adult Nepalese.[1] The rectum is the most common site, with incidents of right-sided colonic cancer increasing worldwide.[1] Sporadic disease has been commonly reported in CRC, but there is an underlying genetic component as well.[2] Hereditary nonpolyposis colorectal cancer (HNPCC), or Lynch syndrome, is a cancer syndrome that contributes to 5–10% of CRC cases. HNPCC is caused by a germline defect in at least one of the five mismatch repair (MMR) genes: *MutL homolog 1* (*MLH1*), *MutS homolog 2* (*MSH2*), *MSH6*, *PMS1 homolog 2* (*PMS2*), and *epithelial cellular adhesion molecule*. Inherited mutation carriers have an increased tendency to developing HNPCC at an early age.[3] DNA replication errors also cause mutation, which accumulates at simple repetitive sequences (microsatellites), or short tandem repeats, and leads to microsatellite instability (MSI).[4,5] MSI is the mechanism underlying carcinogenesis in sporadic CRC, sporadic endometrial cancer, and

numerous other cancers.[6] MSI is caused by a defect in any of the MMR genes that fail to recognize and repair DNA replication errors.[7] Tumors with high frequency of MSI have a higher probability to grow from polyp to cancer, they are most prevalent in the proximal colon, and tend to be diploid and mucinous, surrounded by lymphoid reactions.[2] Immunohistochemistry (IHC) for analyzing MMR protein expression and polymerase chain reaction (PCR) amplification are commonly used to determine the MSI status in CRC.[8]

MMR system defects lead to increased risk of HNPCC and sporadic tumors.[9] Aberrant hypermethylation of the *MLH1* promoter CpG islands or *MLH1*, *MSH2*, or *MSH6* mutations can inactivate the MMR system.[10,11] Germline *MLH1* and *MSH2* mutations are found in approximately equal proportions in HNPCC patients.[12] *MLH1* and *MSH2* mutations account for ~90% of all cancer patients,[3] while *MSH6* mutation accounts for 7% and other genes account for 3% of all cancer patients.[13,14] MMR gene defects lead to a loss of MMR protein expression in tumor tissue, while MMR proteins are abundantly expressed in proliferating tissue. The loss of immunoreactivity in IHC staining can be used to determine a hypermethylated MMR gene, as well as demonstrating the correlation between MSI and loss of MMR protein expression.[15–17]

Direct sequencing or Sanger sequencing is the gold standard to detect MMR gene mutation and diagnose HNPCC in the germline,[18] but it is expensive and laborious. IHC sensitivity and specificity in detecting *MLH1* and *MSH2* mutation are 92.3% and 100% respectively; therefore, IHC is a good alternative prior to mutational analysis to determine the loss of MMR expression.[15] Although MLH1 and MSH2 are the most common proteins involved in the MMR system, there are no reports on MLH1 and MSH2 expression profiles in Nepalese CRC patients. Therefore, this study was aimed to determine the expression profiles of MLH1 and MSH2 by IHC staining in Nepalese CRC patients, corresponding to their clinicopathological characteristics.

METHODS

Sample collection

In this retrospective study, we used all available archived formalin-fixed paraffin-embedded (FFPE) tissue blocks obtained from Nepalese CRC patients (n = 43) diagnosed with CRC from August to November 2012 at the B.P. Koirala Memorial Cancer Hospital (BPKMCH), Chitwan, Nepal. Each sample was reviewed by observing hematoxylin and eosin stained slides, and the clinical information of each sample was collected to further confirm cancer. The procedure was performed in the Pathology Laboratory, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia, as part of research collaboration. The study was approved by the ethical review board of the Nepal Health Research Council, Kathmandu, Nepal, and the BPKMCH (Reg. No. 145/2016). All patients provided written informed consent.

IHC

Each FFPE tissue block obtained from a biopsy or a resected bowel specimen of each patient was cut into 4-µm-thick sections, and the sections were mounted on positively charged or aminopropyltriethoxysilane-coated slides (DakoCytomation, Glostrup, Denmark). Next, the sections were dewaxed using xylene and rehydrated with graded alcohol concentrations in distilled water. The slides were subjected for antigen retrieval treatment for 3 min at 121°C (15 lb) in tris-ethylenediaminetetraacetic acid buffer prior to incubation with 200 µl of MLH1 or MSH2 primary antibody (Dako, Denmark) at 1:200 dilution (initial concentration: hMLH1 = 78.1 mg/l and hMSH2 = 23.7 mg/l) overnight at 4°C.

The blocking step for endogenous peroxidase activity was performed by 5 min incubation in peroxidase-blocking reagent. The slides were washed with Tris-buffered saline (TBS) and incubated in two drops of horseradish peroxidase-labeled polymer conjugated to a secondary antibody. Subsequently, the slides were washed with TBS, followed by 3 min incubation in 200 µl of 3,3'-diaminobenzidine chromogen. Next, hematoxylin counterstaining was performed, followed by subsequent dehydration and mounting. Normal appendix tissue served as an external positive control, while lymphocytes and benign colonocytes were used as internal positive controls. The presence of nuclear staining in normal appendix tissue and the absence of nuclear staining in adjacent malignant cells indicated loss of protein expression.[19]

Staining assessment and scoring

Semiquantitative scoring was performed on IHC specimens. The result validity was determined based

on the presence of internal controls (lymphocytes and benign colonocytes). A scale of 0 to 3 was used to measure the intensity of immunoreactivity of the nuclear compartment of malignant epithelial cells, in which comparison was based on the intensity of reactivity of tumor cells with positive control cells: 0 = no reactivity; 1 = mild intensity; 2 = moderate intensity; 3 = intensity equivalent to positive control cells.[19] The percentage scoring of tumor cell staining was measured on a scale of 0 to 4: 0 = no tumor cell immunoreactivity; 1 = 1–10% positive tumor cells; 2 = 11–50% positive tumor cells; 3 = 51–80% positive tumor cells; 4 = >80% positive tumor cells. Tumor cells were differentiated from tumor-infiltrating lymphocytes, which were also considered immunopositive.[20] The percentage and intensity scores were multiplied to obtain the total score. A total score of ≥4 was considered normal protein expression, while a total score of <4 was considered loss of protein expression.[17,19]

RESULTS

Nuclear staining in the normal colonic epithelium and neoplastic cells showed normal protein expression (Figure 1, a and c). Nuclear staining occurred in all 43 patients, although MLH1 and MSH2 antibody staining often showed a heterogeneous pattern, in which many tumor cell nuclei were positively stained. A semiquantitative score of <4 indicated tumors with loss of MLH1 and MSH2 expression, while a semiquantitative score of ≥4 indicated tumors with normal or intact MLH1 and MSH2 expression.

Table 1 shows the clinicopathological characteristics of CRC patients with MLH1 and MSH2 expression. Younger patients aged <40 years showed the highest percentage (75%) of loss of MLH1 and MSH2 expression as compared to CRC patients in other age groups. The most common site of cancer was the rectum (60%). Of all sites of tumors, loss of MLH1 and MSH2 expression was not observed in the cecum, descending colon, and ascending colon. Although higher percentage of patients had well-differentiated tumors (65%), loss of MLH1 and MSH2 expression were highly observed in patients with moderately differentiated tumors. Five patients with loss of MLH1 expression had a tumor diameter of ≤20 mm, but patients with a tumor diameter of ≤20 mm and 21–50 mm showed equal loss of MSH2 expression. In addition, tumors that had lost the MMR protein

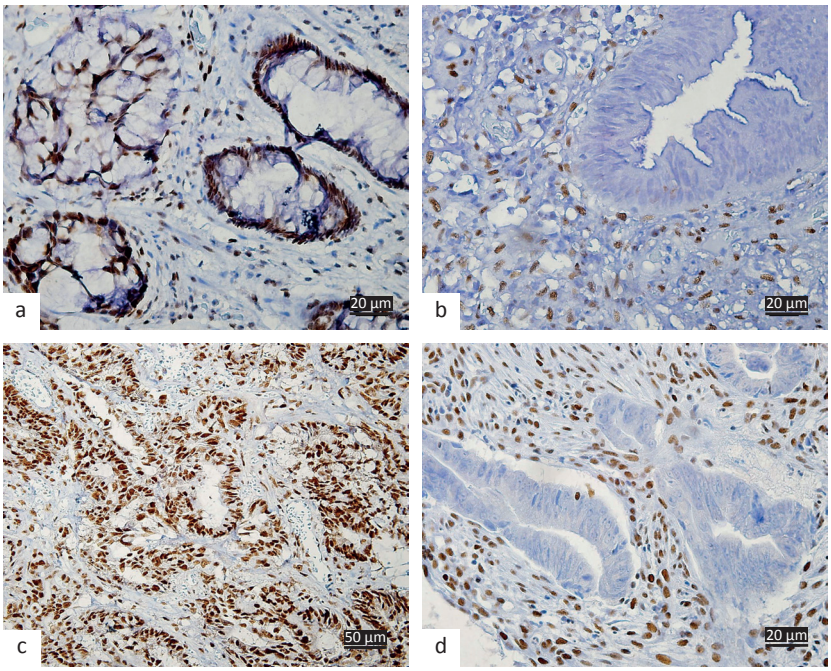

Figure 1. IHC staining for MLH1 and MSH2 expression. (a) Normal MLH1 expression in the presence of nuclear staining (400x magnification); (b) loss of MLH1 expression in the absence of nuclear staining in malignant CRC cells with adjacent nuclear staining of lymphocytes (internal control) (400x magnification); (c) normal MSH2 nuclear staining for normal protein expression (200x magnification); (d) loss of MSH2 expression with adjacent control staining of lymphocytes (400x magnification). IHC=immunohistochemistry; MLH1=MutL homolog 1; MSH2=MutS homolog 2; CRC=colorectal cancer

Table 1. Clinicopathological characteristics of Nepalese CRC patients with MLH1 and MSH2 expression

Characteristic	Total no. of patients, n (%) (N = 43)	MLH1 normal expression, n (%) (N = 35)	MLH1 loss expression, n (%) (N = 8)	*p*	MSH2 normal expression, n (%) (N = 38)	MSH2 loss expression, n (%) (N = 5)	*p*
Age (years)				**0.009***			**0.001***
<40	4 (10)	1 (3)	3 (38)		1 (2)	3 (60)	
40–49	10 (23)	7 (20)	3 (38)		10 (26)	0 (0)	
50–59	9 (21)	9 (26)	0 (0)		9 (24)	0 (0)	
60–69	10 (23)	8 (23)	2 (25)		9 (24)	1 (20)	
≥70	10 (23)	10 (28)	0 (0)		9 (24)	1 (20)	
Mean (SD)	57.49 (13.70)	60.06 (12.60)	46.25 (13.60)		58.53 (13.10)	49.60 (17.30)	
Median (min–max)	58 (30–85)	60 (30–85)	40.5 (35–69)		58 (30–85)	38 (35–70)	
Gender				0.223*			0.635*
Male	27 (63)	20 (57)	7 (87)		23 (60)	4 (80)	
Female	16 (37)	15 (43)	1 (13)		15 (40)	1 (20)	
Types of biopsy				0.629†			0.714†
Minor	42 (98)	34 (97)	8 (100)		37 (97)	5 (100)	
Major	1 (2)	1 (3)	0 (0)		1 (3)	0 (0)	
Tumor diameter (mm)				0.081†			0.302†
≤20	31 (72)	26 (74)	5 (63)		28 (74)	2 (40)	
21–50	9 (21)	8 (23)	1 (12)		7 (18)	2 (40)	
>50	3 (7)	1 (3)	2 (25)		3 (8)	1 (20)	
Diagnosis				0.341*			0.222*
Adenocarcinomas	41 (95)	34 (97)	7 (88)		37 (97)	4 (80)	
Squamous cell carcinomas	2 (5)	1 (3)	1 (12)		1 (3)	1 (20)	
Tumor grade				0.124†			0.106†
Well differentiated	28 (65)	25 (71)	3 (38)		26 (68)	2 (40)	
Moderately differentiated	10 (23)	6 (17)	4 (50)		7 (18)	3 (60)	
Poorly differentiated	5 (12)	4 (12)	1 (12)		5 (14)	0 (0)	
Site of tumor				0.065†			0.600†
Rectum	26 (60)	22 (63)	4 (50)		23 (60)	3 (60)	
Cecum	3 (7)	3 (8)	0 (0)		3 (8)	0 (0)	
Sigmoid colon	2 (5)	0 (0)	2 (25)		1 (3)	1 (20)	
Descending colon	1 (2)	1 (3)	0 (0)		1 (3)	0 (0)	
Ascending colon	2 (5)	2 (6)	0 (0)		2 (5)	0 (0)	
Anal canal	9 (21)	7 (20)	2 (25)		8 (21)	1 (20)	

CRC=colorectal cancer; MLH1=MutL homolog 1; MSH2=MutS homolog 2; SD=standard deviation
*Fisher's exact test; †chi-square test

were mostly adenocarcinomas, while two were squamous cell carcinomas.

Table 2 shows a comparison of the loss of MLH1 and MSH2 expression in all 43 patients according to different ethnic groups in Nepal whereby Brahmins and Chhetris contributed to the largest number of CRC patients as compared to other ethnic groups. Of total CRC patients in all ethnic groups, an overall percentage of 18.6% and 0.12% of MLH1 and MSH2 loss of expression were identified, respectively.

Table 2. A different ethnic group of Nepal related to the MLH1 and MSH2 protein expression

Ethnic group	Total no. of patients, n (%) (N = 43)	MLH1 normal expression, n (%) (N = 35)	MLH1 loss of expression, n (%) (N = 80)	MSH2 normal expression, n (%) (N = 38)	MSH2 loss of expression, n (%) (N = 5)
Brahmin/Chhetri	23 (53)	19 (54)	4 (50)	20 (53)	3 (60)
Kirati	3 (7)	3 (9)	0 (0)	3 (8)	0 (0)
Newar	1 (2)	1 (3)	0 (0)	1 (3)	0 (0)
Tamang	2 (5)	2 (6)	0 (0)	2 (5)	0 (0)
Magar	2 (5)	1 (3)	1 (13)	2 (5)	0 (0)
Chaudhari	6 (14)	5 (14)	1 (13)	5 (13)	1 (20)
Gurung	1 (2)	1 (3)	0 (0)	1 (3)	0 (0)
Tharu	1 (2)	1 (3)	0 (0)	1 (3)	0 (0)
Mandal	2 (5)	1 (3)	1 (13)	2 (5)	0 (0)
Damai	2 (5)	1 (3)	1 (13)	1 (3)	1 (20)

MLH1=MutL homolog 1; MSH2=MutS homolog 2

DISCUSSION

The loss of MLH1 expression identified in this retrospective study was higher than the loss of MSH2 protein expression. Previous studies have shown that loss of MLH1 expression, as a predominant MMR protein, constitutes the highest percentage of loss of protein expression, followed by MSH2.[21,22] MMR proteins form heterodimeric complexes in their functional state: MSH2 forms a heterodimeric complex with MSH6 to form MutSα,[23] while MLH1 forms a heterodimeric complex with PMS2 to form MutLα.[24] Therefore, loss of MLH1 and MSH2 expression might cause degradation of respective dimers and concurrent loss of obligatory and secondary partner proteins, resulting in functional loss of MMR proteins. In the present study, the expression profile of MSH6 and PMS2 was not performed as the mutations in the secondary proteins, MSH6 and PMS2 do not cause a concomitant loss of their respective obligatory proteins MLH1 and MSH2, respectively.[25] Hall et al[25] recommended IHC testing for PMS2 and MSH6 alone for MMR proteins, because other proteins (MSH3, MLH3, and PMS1) are available to compensate the function of secondary proteins MSH6 and PMS2. Therefore, the loss of a dimer formed by MLH1 and PMS2 or MSH2 and MSH6 is because of MLH1 or MSH2 mutations, while PSM2 or MSH6 mutations often contribute to PMS2 or MSH6 loss only. Hall et al[25] also reported that gene mutation accompanied by MLH1 and MSH2 loss result in PMS2 and MSH6 degradation, but because of the binding properties of MMR heterodimeric complexes, PMS2 and MSH6 mutations do not result in MLH1 and MSH2 deterioration. The use of both MSH2 and MLH1 monoclonal antibodies in IHC can be used to evaluate the MSI status of tumors compared to expensive and time-consuming molecular tests, such as PCR and DNA sequencing. Also, IHC is 92.3% sensitive and 100% specific for screening DNA MMR defects by detecting MLH1 and MSH2. Therefore, IHC can be an alternative technique for identifying defective genes when PCR is not available.[26] We found no association between the MMR expression with gender, tumor diameter, diagnosis, tumor grade, and site of tumor. Most patients in the young age group (<40 years old) had loss of MLH1 and MSH2 expression. Younger CRC patients with MMR deficiency or loss of protein expression have an increased risk of developing hereditary colorectal cancer syndromes.[27] In addition, rectum was identified as the most common site of tumor in this study of Nepalese CRC patients. Previous study by Patra et al[28] also reported that rectum as the most common site of tumor location in Indian colorectal cancer patients.

This is probably the first such study conducted in Nepal. Brahmins and Chhetris have the largest population in Nepal compared to other ethnic groups such as Magars, Mandals, Damais, and Chaudharis. However, due to the small number of CRC patients, the correlation between the MLH1 and MSH2 expression with ethnic groups could not be further demonstrated. In an underdeveloped country like Nepal, where molecular techniques are

not easily available and are unaffordable, IHC can be an alternative tool for the early diagnosis of various types of malignancies, including CRC. IHC analysis of MLH1 and MSH2 expression is rapid, cost-effective, accurate and widely used to analyze the MMR gene status in colorectal adenocarcinomas because preliminary testing is economical compared to MSI testing by molecular methods.[29,30] Genetic analysis of the MSI status is not only tedious and expensive but also requires specialized equipment that can only be operated by highly trained scientific professionals in sophisticated laboratories. Therefore, for mutations derived from truncation or degradation of antigens, IHC is an extremely reliable diagnostic tool for the screening method prior to further molecular techniques for mutation identification. However, IHC method cannot characterize cells expressing wild-type (WT) polypeptides and cells that express protein variants carrying inactive missense mutations that do not destabilize the proteins.[31]

This study had a few limitations. The IHC results could show some false-normal staining patterns.[26,32] Missense mutations in MLH1 account for more than one-third of all types of mutations, and this mutant protein is antigenically intact but catalytically inactive; therefore, IHC cannot distinguish between cells that express this mutant and the WT protein.[33,34] However, IHC of individual MMR proteins is being increasingly practiced in a clinical setting because the absence of immunoreactivity of a specific MMR protein can identify a possible mutated gene prior to mutational analysis.[24] The MMR protein expression profile of the Nepalese population shows that IHC for MMR genes can be introduced as a first-line screening method in most local laboratories for selected CRC patients. In addition, IHC should be followed by DNA testing for MSI to obtain a comprehensive picture of molecular abnormality. Screening of family members, especially young CRC patients, can further establish the genetic inheritance pattern of MMR protein mutations, improving the ability to distinguish possible carriers. Larger sample size should be used to further elucidate possible clinical characteristics associated with the loss of MMR protein expression with regard to Nepalese CRC patients.

In conclusion, IHC can be a potential screening method of determining the MMR expression profile of Nepalese CRC patients. IHC can be performed in local clinical laboratories to find MMR protein defects in selected CRC cases prior to expensive molecular tests. There is a prospect of introducing the IHC of MLH1 and MSH2 in every pathological laboratory for routine diagnosis of CRC.

Conflict of Interest

The authors affirm no conflict of interest in this study.

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