Research Article

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Use of immunohistochemical versus microsatellite analyses as markers for colorectal cancer

Kolorektal kanser için mikrosatellit analizine karşı immünohistokimyasal analizin marker olarak kullanılması

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Abstract

Objectives: Our aim was to determine how well immunohistochemical analysis identified colon cancer patients with microsatellite instability in Turkish patients.
Material and methods: Subjects were patients that underwent surgery for colorectal cancer in our institution between 2006 and 2011. Patients were grouped as: (1) suspected Lynch syndrome (n = 14), (2) familial colorectal cancer (n = 14), and (3) sporadic colorectal cancer groups (n = 14). Mismatch repair proteins were analyzed by a four antibody-panel immunohistochemistry. Microsatellite instability analysis was conducted on DNA samples using MSI-PCR followed by fragment analysis.
Results: The immunohistochemistry and PCR results had good concordance in 35/42 patients. Both microsatellite instability and at least one mismatch repair protein deficiency were detected in 11 patients, and both microsatellite stability and normal expression of mismatch repair proteins were detected in 24 patients. Test results were discordant in seven of the patients.
Conclusion: As it is not feasible to perform expensive molecular tests in healthcare units in many developing countries, the four antibody-panel immunohistochemistry is a reliable and affordable method for screening for colorectal cancer, including Lynch syndrome and sporadic cases when suspected.

Keywords: Colorectal cancer; Hereditary non-polyposis colorectal cancer; Lynch syndrome; Immunohistochemistry; Microsatellite instability.

Özet

Amaç: Bu çalışmada immünohistokimyasal analizin mikrosatellit instabilitesi olan kalıtımsal (Lynch sendromu) veya sporadik kolorektal kanser şüphesi bulunan Türk hastaları ne kadar iyi tanıyabildiğini değerlendirmek amaçlanmıştır.
Gereç ve Yöntem: Çalışmaya merkezimizde kolorektal kanser nedeniyle 2006 ve 2011 yılları arasında ameliyat edilen hastalar dâhil edilmiştir. Hastalar; (1) Lynch sendromu şüpheli grup (n:14), (2) Ailesel kolorektal kanser grubu (n:14), (3) Sporadik kolorektal kanser grubu (n:14) olarak gruplandırılmıştır. Mismatch repair proteinleri immünohistokimyasal olarak 4'lü panel ile değerlendirilmiştir. Fragman analizini takiben DNA örneklerinde PCR ile mikrosatellit instabilitesi analizi yapılmıştır.

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Bulgular: İmmünohistokimyasal analiz ve PCR sonuçları 42 vakanın 35'inde uyumlu çıkmıştır. Mikrosatellit instabilitesi ile birlikte en az bir mismatch repair protein kaybı 11 hastada saptanırken, mikrosatellit stabilitesi ile birlikte normal mismatch repair protein ekspresyonu 24 hastada saptanmıştır. 7 hastada uyumsuz sonuçlar bulunmuştur.
Sonuç: Gelişmekte olan ülkelerdeki sağlık kuruluşlarında pahalı moleküler yöntemlerin uygulanmasının elverişli olmaması durumunda 4'lü panel immünohistokimyasal analiz kalıtımsal (Lynch sendromu) veya sporadik kolorektal kanser şüphesi olan hastalarda tarama metodu olarak güvenilir ve uygulanabilir bir metottur.

Anahtar kelimeler: Kolorektal kanser; Herediter nonpolipozis kolorektal kanser; Lynch sendromu; Immünohistokimya; Mikrosatellit instabilitesi.

Introduction

Colorectal cancer (CRC) is one of the most frequent causes of cancer-related mortality [1] and is the third most common malignancy in Turkey [2]. Hereditary non-polyposis colorectal cancer or Lynch syndrome (LS), the most common hereditary form of CRC, is an autosomal-dominant syndrome predisposing the development of 3%–4% of all CRCs [3].

Patients with LS are prone to malignancies due to germ line heterozygous mutations in one or more of the mismatch repair (MMR) genes [4]. Following the loss of the other allele in somatic cells, the defective MMR gene loses its function and the neoplastic process starts. At least four MMR genes are found to be mutated in LS: mut L homolog 1 (MLH1), mut S homolog 2 and 6 (MSH2 and MSH6) and post meiotic segregation increased 2 (PMS2) [5].

Frequently, DNA replication errors causing microsatellite instability (MSI) occur in LS patients' tumor cells. Microsatellites are short (1–6 bases) and repeating DNA sequences that are distributed throughout the human genome. During DNA replication in a LS tumor cell, the defective MMR protein results in altered allele lengths of microsatellite regions due to deletions or insertions, which lead to MSI [3]. Thus MSI is a surrogate marker for MMR defects. MSI-polymerase chain reaction (PCR) analysis and MMR protein detection by immunochemistry (IHC) are the proposed screening methods for MMR defects and LS [6].

The diagnosis of LS depends on family history, pathologic findings, IHC, MSI analysis and detection of germline MMR gene mutations. Family history is still an important parameter for the diagnosis, and the Amsterdam I, Amsterdam II and the revised Bethesda Criteria rely heavily on family history to identify individuals at high risk for LS [7]. Moreover, early detection of LS leads to 65%–70% decrease in morbidity and mortality. Additionally, various prophylactic surgeries can be recommended in the case of mutation carriers [8]. However, MSI and MMR gene mutation analyses are costly and time-consuming, making these tests a financial burden for developing countries. Therefore, there is a need for a cheap, quick, and reliable screening algorithm for LS.

MSI is present in approximately 10%–13% of sporadic CRCs [8] and similarly MMR genes are involved in the 10%–15% of the sporadic CRCs [9].

In the present study, we performed a four antibody-panel IHC in parallel with MSI-PCR analysis. Our hypothesis was IHC can identify Lynch syndrome suspected patients' MSI status well enough for using as a first line screening method for Lynch syndrome especially in developing countries. Our aim was to determine how well IHC analysis identified colon cancer patients with microsatellite instability (MSI) and thus suspected LS in a population of Turkish patients.

Materials and methods

This study has been conducted according to the principles established in Helsinki. This study is approved by Ethics Committee of Ankara University (approval no: 29-603).

Patients and clinicopathological analysis

Patients diagnosed between 2006 and 2011 and who received surgery for CRC in the two hospitals of Ankara University School of Medicine were evaluated in Department of Medical Genetics using the Amsterdam II criteria: (1) at least three or more relatives with LS-related cancer (excluding familial adenomatous polyposis syndrome), one of whom is a first-degree relative of the other two, (2) two successive affected generations, and (3) one or more of the LS-related cancers diagnosed before 50 years of age [10].

Fourteen patients that matched all three criteria were designated as the suspected LS group. Fourteen patients that had family history of CRC were randomly chosen and designated as the Familial CRC group. Fourteen patients that had no family history of CRC were randomly chosen and designated as the sporadic CRC group [11].

Paraffin blocks of resection specimens were retrieved from the archive of the Department of Pathology and used for both IHC and MSI analysis. Localization of tumors was defined as right colon (caecum, ascending colon, hepatic

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flexure and transverse colon), left colon (splenic flexure, descending colon and sigmoid colon) and rectum (15 cm proximal to anal verge) [12]. Staging of tumors was done according to the American Joint Committee on Cancer tumor-node-metastasis staging system [13].

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue samples of all 42 patients were re-evaluated. Histopathological classification was done according to the WHO 2010 classification [14]. The proportion of necrosis in the tumoral tissue was evaluated to determine the effect of preoperative chemo-radiotherapy. For every patient, one FFPE sample of tumor tissue and one FFPE sample of normal colon mucosa were selected, except one outside case that contained only tumor tissue. To evaluate the loss of MMR proteins, monoclonal MLH1 (Cellmarque clone G168-728, dilution 1/50), MSH2 (Cellmarque clone G219-129, dilution 1/50), MSH6 (Cellmarque clone 44, dilution 1/50) and PMS2 (Cellmarque clone MRQ-28, dilution 1/25) antibodies were used. Four micrometer sections were prepared from FFPE tumor and normal colon mucosal samples for IHC staining. Following incubation with each of the primary antibodies, visualization was achieved with an automated immune-stainer, Ventana XT, using the Ultraview Universal DAB Detection Kit and DAB chromogen (Ventana Medical Systems, Tucson, AZ, USA). Nuclear staining of the normal colon mucosal epithelium or the tumor cells and lymphocytes in the lamina propria were accepted as positive controls. The loss of nuclear staining in tumor cells were scored by estimating the ratio of the unstained cells among the stained neoplastic cells and grouped as follows: 0%–9% loss, 10%–25% loss, 26%–50% loss, 51%–75% loss and 76%–100% loss of nuclear staining in neoplastic cells. Examination and scoring of the IHC stained slides were done by two authors (IK, SY) who were blinded to the groups. As we found that loss of more than 75% of nuclear staining in tumor tissue was significantly correlated with the results of the MSI analysis, we accepted loss of more than 75% of the nuclear staining for MMR protein expression in tumor tissue as the cut-off for MMR protein deficiency [15].

DNA isolation and microsatellite instability analysis

Eight sections (5 mm thick) of FFPE tumor tissue blocks were used for DNA isolation. Areas of tumor cells were selected by microscopic dissection, and DNA isolation was performed using the Qiagen, QIAamp DNA FFPE tissue kit. At the same time, DNA extraction was also performed on the normal colon mucosal samples from each patient. DNA samples were amplified by using primers in the Promega MSI analysis kit (version 1.2). The loci evaluated in this study are shown in Table 1. The detection of a difference in one locus was considered as low MSI, while a difference in two or more of the five loci was considered as high MSI [16]. Electropherograms of tumor and normal tissues were compared using the Gene Mapper 4.0 (Applied Biosystems) program [17].

Table 1: The microsatellite loci evaluated in this study.

Locus	Gene	Genbank number	Allele size
NR-21	SLC7A8	XM_033393	94-101
BAT-26	hMSH2	U41210U04045	103-115
BAT-25	c-kit	L04143X06182	114-124
NR-24	Zinc Finger2	X60152	130-133
MONO-27		AC007684	142-154

Statistical analysis

The SPSS 11.5 package program was used for data analysis. Mean value±standard deviation (SD) for metric variables and, frequency (percentage) for categorical variables were employed as descriptive statistics. In order to compare the independent groups, χ^2-test and one-way analysis of variance was used. A $p<0.05$ was considered as statistically significant.

Results

Clinicopathological parameters

The clinicopathological parameters of groups are given in Table 2. The three groups of patients were not significantly different from each other for demographic and pathological parameters except for their age ($p<0.001$).

Immunohistochemistry

Pathologists had consensus in all samples. As shown in Table 3, two patients (14.3%) in the sporadic CRC group, five patients (35.7%) in the familial group and 10 patients (71.4%) in the suspected LS group had at least one MMR protein deficiency. A statistically significant difference was detected between the three groups ($p=0.008$). In paired comparisons, a statistically significant difference was

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Table 2: Clinicopathological parameters of groups.

	Sporadic CRC	Familial CRC	Suspected LS
Number of patients	14	14	14
Age (mean ± SD)[a]	62.71 ± 8.862	45.93 ± 10.745	44.5 ± 15.058
Gender (%/n)			
Male	78.6% (n:11)	64.3% n (n:9)	71.4% (n:10)
Female	21.4% (n:3)	35.7% (n:5)	28.6% (n:4)
Localization (%/n)			
Right colon	42.85% (n:6)	42.85% (n:6)	42.85% (n:6)
Left colon	14.28% (n:2)	21.42% (n:3)	14.28% (n:2)
Rectum	42.85% (n:6)	35.71% (n:5)	42.85% (n:6)
Tumor grade (%/n)			
I	0% (n:0)	21.42% (n:3)	7.14% (n:1)
II	78.57% (n:11)	35.71% (n:5)	57.14% (n:8)
III	21.42% (n:3)	42.85% (n:6)	35.71% (n:5)
Stage (%/n)			
0	0% (n:0)	7.14% (n:1)	7.14% (n:1)
I	14.28% (n:2)	21.42% (n:3)	28.57% (n:4)
II	21.42% (n:3)	50% (n:7)	21.42% (n:3)
III	28.57% (n:4)	14.28% (n:2)	28.57% (n:4)
IV	35.71% (n:5)	7.14% (n:1)	14.28% (n:2)

[a]$p < 0.001$. CRC, Colorectal cancer; LS, Lynch syndrome; SD, standard deviation; n, number.

detected between the suspected LS group and the sporadic CRC group ($p = 0.002$). Representative sections of MMR staining loss are shown in Figure 1. Of the 10 patients with MMR loss in the suspected LS group, six patients showed loss of expression for both MLH1 and PMS2, two patients showed loss of expression for both MSH2 and MSH6, one patient lacked only MSH6, and one patient lacked only PMS2. Of the five patients with MMR loss in the familial CRC group, three patients showed loss of staining for MLH1 and PMS2 and two showed only loss of staining for MSH6. Of the two patients with MMR loss in the sporadic CRC group, one patient showed loss of expression for MSH2 and MLH1 and another showed loss of expression only for MSH6.

MSI-PCR

According to MSI analysis, as shown in Table 3, one patient (7.1%) in the sporadic CRC group, four patients (28.6%) in the familial CRC group and nine patients (64.3%) in the suspected LS group had either high or low MSI. A statistically significant difference was detected between the patient groups as established by the Amsterdam criteria ($p = 0.007$). Nine of 14 patients in suspected LS group showed high MSI by PCR. In the familial CRC group, 2/14 patients had low MSI and two patients had high MSI. One patient in the sporadic CRC group showed high MSI.

Comparison of IHC and PCR Results

As shown in Table 4, the IHC and PCR results had good concordance in 35/42 patients. Both MSI and at least one MMR protein deficiency were detected in 11 patients, and both microsatellite stability (MSS) and normal expression of MMR proteins were detected in 24 patients. Table 5 shows results of the patients (n:7) which had discordant test results. One patient in the suspected LS group had MSI, but there was no abnormality seen using IHC, and four patients had MSS, but showed loss of expression of one or more MMR proteins, two patients had low-MSI with loss of expression of one or more MMR proteins.

We compared the sensitivity and specificity of two-panel (staining for MLH1 and MSH2) versus four-panel (staining for MLH1, PMS2, MSH2 and MSH6) antibody tests in detecting either high MSI or any MSI (high MSI or low MSI). As shown in Table 6, Two-panel tests had a sensitivity of 75% and specificity of 90% for detecting high MSI, and a sensitivity of 83% and specificity of 87% for detecting any MSI. Four-panel tests had a sensitivity of 65% and specificity of 96% for detecting high MSI, and a sensitivity of 77% and specificity of 96% for detecting any MSI.

Discussion

Contemporary molecular medicine enables the optimization of treatment strategies according to the molecular profiles of CRC subtypes. However, identification of CRC

Table 3: Mismatch repair protein loss as determined by immunohistochemistry and microsatellite instability as determined by polymerase chain reaction in patient groups.

Groups	MMR loss ratio (%)	MSI ratio (%)	High MSI n	Low MSI n	MSS n
Sporadic CRC	2/14 (14.3%)	1/14 (7.1%)	1	0	13
Familial CRC	5/14 (35.7%)	2/14 (14.3%)	2	2	10
Suspected LS	10/14 (71.4%)	9/14 (64.3%)	9	0	5

MMR, Mismatch repair; MSI, microsatellite instability; MSS, microsatellite stabile; CRC, colorectal cancer; LS, Lynch syndrome; n, number.

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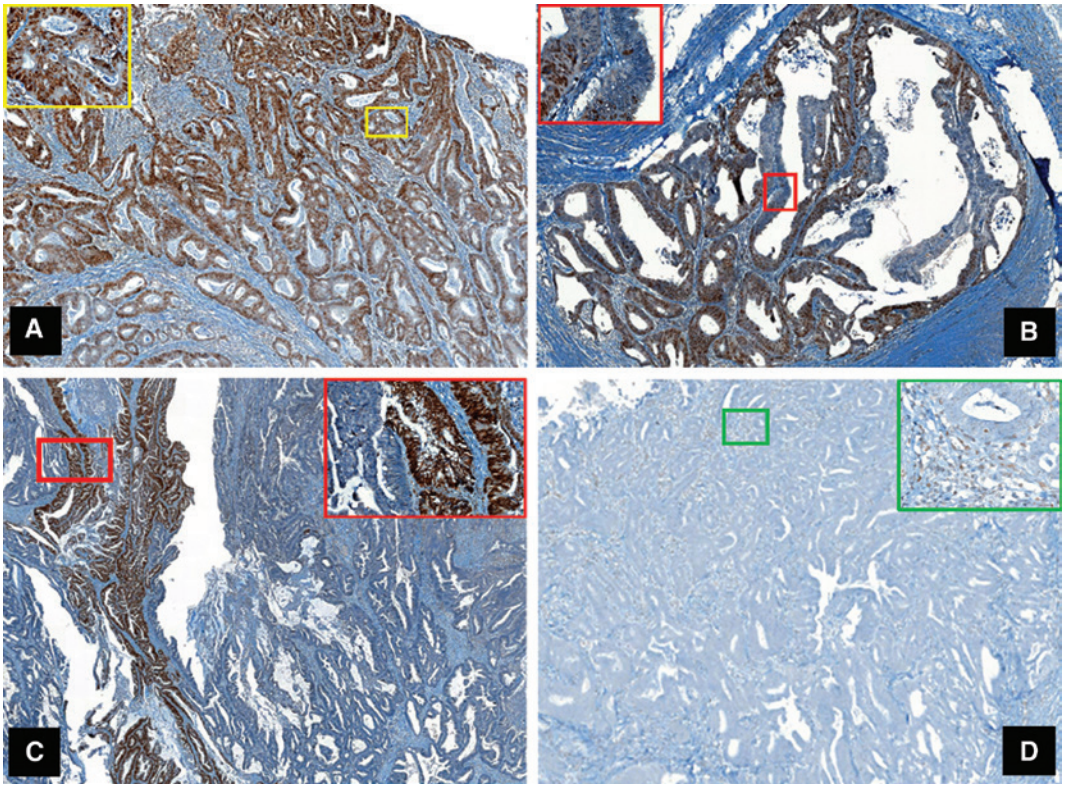


Figure 1: Representative sections of mismatch repair (MMR) staining loss.
(A) Normal staining of a MMR protein in tumor cell nuclei. (Familial colorectal cancer group case – MSH6). (B) Loss of less than 75% of nuclear staining for the MMR proteins immunohistochemistry (IHC) evaluation. (Sporadic colorectal cancer group case – MSH6). (C) Loss of more than 75% nuclear staining for any MMR protein was categorized as "MMR protein loss" in the IHC evaluation. (Familial colorectal cancer group case – MSH6). (D) Complete loss of a MMR protein in tumor cells. (Suspected Lynch Syndrome group case – MLH1).

Table 4: Correlation of polymerase chain reactionand immunohistochemistry results in all patients.

	MMR loss n	MMR normal n	Total n (%)
High-MSI n	11	1	12 (28.6%)
MSS + low-MSI n	6	24	30 (71.4%)
Total n (%)	17 (40.5%)	25 (59.5%)	42 (100%)

MMR, Mismatch repair; MSI, microsatellite instability; MSS, microsatellite stabile; n, number.

subtypes at the molecular level requires advanced infrastructure, which is not possible to implement in every healthcare unit. The most accurate diagnosis of LS is via MMR gene mutation analysis, however, MSI is often used as a surrogate marker for MMR gene mutation and LS. However, both of these methods are expensive and may not be affordable in many healthcare settings. In this study, our aim was to determine how well immunohistochemical analysis, which is a relatively simple and inexpensive

Table 5: Cases with discordance between the four-panel immunohistocehmistry and polymerase chain reaction results.

Group	Patient no.	Age	MSS/MSI by PCR	Protein expression by IHC			
				MLH1	PMS2	MSH2	MSH6
Sporadic CRC	5	59	MSS	–	+	–	+
	21	57	Low-MSI	–	–	+	+
Familial CRC	23	44	Low-MSI	+	+	+	–
	28	32	MSS	+	+	+	–
	35	30	MSS	+	+	–	–
Suspected LS	39	61	MSS	+	+	+	–
	40	41	High-MSI	+	+	+	+

IHC, Immunohistochemistry; MSS, microsatellite stabile; MSI, microsatellite instability; CRC, colorectal cancer; LS, Lynch syndrome; MLH1, mut L homolog; MSH2, mut S homolog 2; MSH6, mut S homolog 6; PMS2, post meiotic segregation increased 2.

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Table 6: Sensitivity and specificity of two versus four antibody immunohistochemistry panels in detecting microsatellite instability.

2-Panel detection of high MSI		
Any loss in 2-panel IHC	High MSI	Sensitivity
12/42	9/12	75%
Normal 2-panel IHC	Low MSI or MSS	Specificity
30/42	27/30	90%
2-Panel detection of high or low MSI		
Any loss in 2-panel IHC	High or Low MSI	Sensitivity
12/42	10/12	83%
Normal 2-panel IHC	MSS	Specificity
30/42	26/30	87%
4-Panel detection of high MSI		
Any loss in 4-panel IHC	High MSI	Sensitivity
17/42	11/17	65%
Normal 4-panel IHC	Low MSI or MSS	Specificity
25/42	24	96%
4-Panel detection of high or low MSI		
Any loss in 4-panel IHC	High or Low MSI	Sensitivity
17/42	13/17	77%
Normal 4-panel IHC	MSS	Specificity
25/42	24/25	96%

2-Panel IHC, MLH1 and MSH2; 4-panel IHC, MLH1, PMS2, MSH2 and MSH6; MSI, microsatellite instability; MSS, microsatellite stabile.

testing method, identified CRC patients with MSI and thus suspected LS in Turkish patients. We found that the four-panel IHC identified 35/42 (83.3%) patients correctly using MSI-PCR as our comparator. Of the seven patients with IHC results discordant with the MSI-PCR results, six were false positives and one was a false negative.

Lindor et al. [18] used IHC for MLH1 and MSH2 in 1144 colorectal cancer patients and reported that the predictive value of normal IHC for MSS or low MSI phenotype was 96.7%, whereas the predictive value of abnormal IHC for high MSI was 100%. When mixed groups of CRC patients were considered, IHC (for MLH1 and MSH2) had 77%–100% sensitivity for the detection of high MSI and 99% specificity [15]. Generally, both low MSI and MSS are considered as a single subset, but this issue has been recently debated [19]. We found low MSI in two patients with loss of at least one of the MMR proteins as detected by IHC, suggesting that further evaluation might be needed for patients with low MSI.

Cawkwell et al. [20] showed that the expression of MLH1 or MSH2 was completely absent in the high MSI phenotype tumors in their study, and they suggested that investigation of the minor MMR enzymes (PMS1, PMS2, hMSH6/GTBP, hMSH3) would not be required. However in our study, two patients with high MSI showed defects in either MSH6 or PMS2 and one patient with low MSI showed a defect in only MSH6. Our results are in agreement with the detailed study by Lindor et al., which concluded that if IHC is performed only for MLH1 and MSH2, then some cases with MSI can be missed. In addition, Shia reported that using a four MMR antibody-panel (MLH1, MSH2, PMS2, MLH6) for IHC, instead of a two antibody-panel (MLH1 and MSH2), significantly increases the sensitivity of IHC in predicting germline mutation to a level which is virtually equivalent to MSI testing [6].

In a study by South et al., all cases of CRC were prospectively stained for these 4 MMR proteins and 57 out of 270 (21.1%) patients lacked expression in one or more of these proteins. South et al. [5] suggested that routine evaluation of CRC cases for MMR proteins is feasible regardless of age at onset or family history. In a study conducted by Karahan et al. [21], loss of expression of one or more of these four MMR proteins was found in 24 out of 186 (12.9%) CRC cases. In our study, this ratio was 40.5% (17/42 patients) when all of the patients were considered. In a study by Erdamar et al. [9], routine examination of sporadic CRC patients detected loss of MLH1 or MSH2 protein expression in 32 out of 74 patients (43.2%). In our study, we found a defect in MLH1 or MSH2 protein expression in one out of 14 sporadic CRC patients (7.1%). The difference in our ratios from literature can be explained by the patient selection criteria used in these studies as well as some technical aspects of the tissue fixation and processing.

In patients who had a pre-diagnosis of LS using the Amsterdam II criteria, MSI was detected by PCR in 10 out of 28 (35.7%) patients, and MLH1 or MSH2 protein expression defects were detected in all 10 patients [22]. In our study, we showed MSI in nine out of 14 (64.3%) patients with suspected LS, and 7/9 (77.7%) of these patients had MLH1 or MSH2 expression defects. One patient with high MSI had an expression defect only for PMS2. These differences in results can be explained by insufficient number of patients and recording errors in pedigrees in our country.

Several factors may account for the discordance found between the results of the IHC panel and the MSI-PCR analysis in our study. In general, the success of the four-panel IHC as a diagnostic tool may vary due to the fixation and processing procedures of the tissues [18]. The discordance in cases with MSS, but some apparent loss of MMR protein expression, might be explained by variations in staining quality and challenges of the IHC procedures [23] or difficulties in detecting unusually low rate of MSI due to technical problems [24]. Another cause of discordance may be whether or not the patient received neoadjuvant therapy, which can induce loss of MSH6 expression [25]. This may have been the case for patient number 39 in our suspected LS group, who received preoperative radiation and showed

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MSS but with loss of MSH6 expression. Patient number 40 in the suspected LS group showed high MSI, but no loss of MMR protein expression was initially seen by IHC. However, when the slides for this patient were reassessed, there was 50%–75% loss in MLH1 staining in the tumor tissue. This emphasizes the need for good standardization of the IHC evaluation criteria for MMR loss. Finally, MSI evaluation is used as a surrogate marker for more expensive MMR gene sequencing and mutation analysis. Discrepancies between MSI and MMR gene mutation could explain some of the discrepancies between MSI and MMR protein expression. If discordant test results in IHC and MSI testing occurs, patients should undergo further evaluation [26].

Our study had some shortcomings. First, this study was retrospective in nature and paraffin-embedded tissue samples were used for laboratory tests, reflecting real-life clinical practice. Second, we were not able to make a definitive diagnosis by germline MMR gene mutation analyses. Despite this, our study is one of the rare studies on this subject that has been conducted in our country.

In conclusion, it is widely accepted that all CRC patients should be tested for MSI/MMR gene defects using IHC, PCR or both at the same time regardless of any parameter [27]. Recently, Kidambi et al. [28] conducted a study addressing this issue, and they found similar outcomes between universal and selective screening for LS. In our opinion, routine expensive MSI-PCR analysis can be unfeasible for many health care units in our country or in developing countries. We suggest that if LS is suspected, then patients should be screened by a four antibody-panel IHC, and loss of nuclear staining in more than 75% of neoplastic cells can be accepted as a suggestive for LS. Molecular tests should be performed only if necessary, for example in cases where adjuvant therapy may influence the results.

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