Minimal microsatellite shift in microsatellite instability high endometrial cancer: a significant pitfall in diagnostic interpretation

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Abstract

Mismatch-repair deficiency testing plays a critical role in the identification of proband in Lynch Syndrome families and triaging patients with high stage or recurrent solid malignancies for check point inhibitor (Pembrolizumab) immunotherapy. We compared microsatellite shift patterns of microsatellite instability PCR analysis at 5 NCI recommended loci between microsatellite instability high endometrial carcinoma ($n = 50$) and microsatellite instability high colorectal cancer ($n = 19$). The endometrial cancer cohort included 45 endometrioid, 1 serous, and 4 clear cell carcinomas. Overall, 52% (26/50) of microsatellite instability high endometrial cancers showed minimal microsatellite shift (defined as a one to three nucleotide repeat shift at an involved locus) observed at least at one locus. Among microsatellite instability high endometrial cancers with minimal microsatellite shift, the frequencies at each involved locus were *D2S123* (21/21, 100%), *D17S250* (10/11, 89%), *D5S346* (11/12, 92%), *BAT25* (9/12, 80%), and *BAT26* (8/21, 45%). Noticeably, 11 of the 26 cases (42%) showed only minimal shift. Among microsatellite instability high endometrial cancers with minimal microsatellite shift, 65% (17/26) had combined *MLH1* and *PMS2* loss, 8% (2/26) had combined *MSH2* and *MSH6* loss, 13% (3/26) had *MSH6* loss and 15% (4/26) had loss of *PMS2* by immunohistochemistry. In contrast, only 16% (3/19) had minimal microsatellite shift seen in colorectal cancer cohort with corresponding loss of *MLH1/PMS2, MSH2/MSH6,* or *MSH6*. Overall, 15% (7/50) of microsatellite instability high endometrial carcinomas showed isolated loss of *MSH6* in contrast to 7% (1/15) seen in microsatellite instability high colorectal carcinomas. In conclusion, microsatellite instability high endometrial carcinomas have a significantly higher frequency of minimal microsatellite shift that coincides with a high percentage of combined loss of *MLH1/PMS2*. Microsatellite instability high endometrial cancers also have more frequent loss of *MSH-6*. Diagnostically, recognition of minimal microsatellite shift is crucial for accurate interpretation of microsatellite instability PCR data of endometrial carcinoma.

Introduction

Lynch syndrome is an autosomal dominant genetic disorder that predisposes the patient to various types of cancer, especially colorectal and endometrial carcinomas [1–3]. The increased risk of developing these cancers is due to impaired DNA mismatch repair leading to subsequent genetic instability. Microsatellite instability testing and analysis of mismatch-repair protein expression of colorectal

and increasingly endometrial cancers play a critical role in the identification of proband of a Lynch syndrome family [4]. Multiplex polymerase chain reaction-based method is a well-established molecular method to interrogate the microsatellite status, often at the five NCI-recommended loci, with a reported test sensitivity of 93% in identifying tumors with mismatch repair deficiency [5]. Immunohistochemistry testing evaluates the expression of four mismatch repair proteins (*MLH1, PMS2, MSH2,* and *MSH6*), with a reported test sensitivity of over 92% [6]. Immunohistochemistry has an advantage over microsatellite instability PCR testing in its ability to identify specific mismatch repair gene deficiency, and therefore to guide subsequent comprehensive gene sequencing to identify the causative mutation [7]. Moreover, mismatch repair deficiency testing has recently become a critical companion diagnostic procedure in patients with high stage or recurrent

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solid malignancies for immune check point inhibitor (e.g., Pembrolizumab) therapy [8, 9].

In contrast to the well-established colorectal cancer screening algorithm, strategies for cost-effective Lynch syndrome screening of endometrial cancer patients are currently evolving [10] and microsatellite instability high endometrial cancers have been found to prosses distinct pathogenetic differences from colorectal cancers. For example, *BRAF* mutation is exceedingly rare in endometrial cancers with *MLH1* promoter hypermethylation [11] and germline mutations of *MSH6* are more often seen in endometrial cancers than colorectal tumors [12]. The aim of this study was to compare the microsatellite shift patterns by microsatellite instability PCR in correlation with mismatch repair protein expression by immunohistochemistry between microsatellite instability high endometrial carcinoma and microsatellite instability high colorectal cancer.

Materials and methods

Specimens

The study cohorts included 50 microsatellite instability high endometrial cancers that were identified from 263 endometrial cancers (Table 1) that consecutively underwent combined mismatch repair protein immunohistochemistry and microsatellite instability PCR testing at authors' institution. For comparative analysis, 19 microsatellite instability high colorectal cancers were randomly selected from our departmental archives. Hematoxylin and eosin (H&E) stained sections and mismatch repair protein immunohistochemical slides were systemically reviewed for each case. Clinicopathological parameters and microsatellite instability

Table 1 Clinicopathological characteristics of MSI-H endometrial cancer cohort

Age (median age)	45 to 80 (62) years	
	Category	No.
Histological type	Endometrioid	45
	Serous	1
	Clear cell	4
Histological grade[a]	FIGO 1	6
	FIGO 2	29
	FIGO 3	10
Pathological stage[b]	FIGO Stage I	35
	FIGO Stage II	5
	FIGO Stage III	6
	FIGO Stage IV	2

[a]Endometrioid carcinomas only

[b]No staging surgery performed in 3 cases of endometrioid carcinoma

PCR data were revisited and compared between the two cohorts. The study was approved by the institutional review board.

MSI testing by PCR and capillary electrophoresis

DNA was extracted from tumor tissue and normal tissue of each case according to the pressure treatment and DNA extraction protocol described previously [13]. Microsatellite status was interrogated by microsatellite instability PCR testing at 5 NCI-recommended loci (*D2S123, D5S346, D17S250, BAT25,* and *BAT26*). The PCR primers are summarized in Table 1. PCR was performed as follow: denaturation was at 94 °C for 12 min, followed by 31 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min. The last cycle was followed by an extra extension step at 72 °C for 1 min, then the product was held at 4 °C. PCR products were analyzed by capillary electrophoresis using an ABI 3500 Genetic Analyzer (Applied Biosystems, USA). Status of microsatellite loci was compared between the tumor and its paired non-neoplastic tissue. Microsatellite instability high was defined by the presence of microsatellite instability at two or more loci. microsatellite instability low was defined by the presence of only one unstable locus. Microsatellite stable was defined by the absence of instability at all five loci. Minimal microsatellite shift was defined by shift of 1 to 3 microsatellite nucleotide repeats at an involved locus (Fig. 2a) and major shift was defined by more than 3 microsatellite repeat shifts (Fig. 2b).
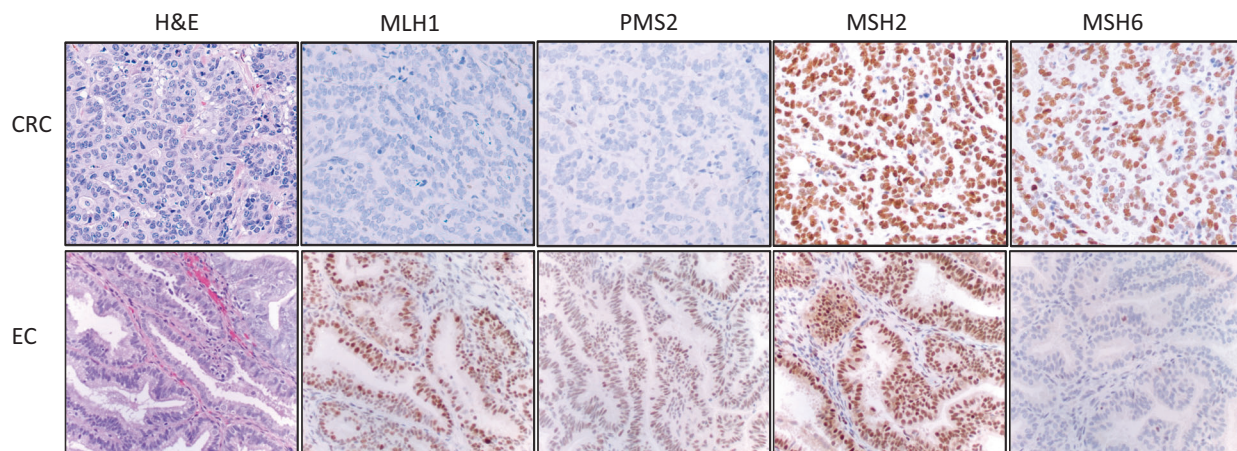
MMR gene expression by immunohistochemistry analysis

Mismatch repair protein immunohistochemistry was performed on 4-μm sections of formalin-fixed, paraffin-embedded tumor tissue block that were selected after reviewing HE stained sections to ensure tissue adequacy in each case. After incubation at 60 °C for 2 h, the tissue sections were deparaffinized in xylene twice, followed by 5 min each in 99.5% and 95% ethanol and 5 min in distilled water. Tissue sections were then stained for four mismatch repair protein antibodies including *MLH1, PMS2, MSH2,* and *MSH6* (Ventana, prediluted) with an incubation time of 60 min. DAKO EnVision + kit (DAKO, Carpinteria, CA) was used for visualization. Positive and negative controls (normal colonic mucosa) were included. Complete lack of nuclear staining of invasive carcinoma cells in the presence of positive staining of inflammatory or stromal cells on the same tissue section was scored as mismatch repair deficient (Fig. 1).

Fig. 1 Mismatch Repair Protein Immunohistochemistry. Top Panel: Combined loss of *MLH1* and *PMS2* with retained expression of *MSH2* and *MSH6* in colorectal adenocarcinoma. Lower Panel: Loss of *MSH6* and retained expression of *MLH1, PMS2,* and *MSH2* in a FIGO grade 1 endometrial endometrioid adenocarcinoma

Results

Clinicopathological characteristics of study cohorts

The microsatellite instability high endometrial cancer study cohort included 45 endometrioid, 1 serous and 4 clear cell carcinomas (Table 1). The age of the patients at diagnosis ranged from 45 to 80 years with a median age of 62 years. Histologic grading of the 45 endometrioid carcinomas included 6 FIGO grade 1, 29 FIGO grade 2 and 10 FIGO grade 3 tumors. Forty-seven patients underwent staging surgery with final pathological stage consisting of 35 stage I (75%), 5 stage II (11%), 6 stage III (13%), and 2 stage IV (4%) tumors.

The microsatellite instability high colorectal cancer cohort included 19 cases (15 males and 4 females). The age of the patients at diagnosis ranged from 43 to 87 years with a median age of 70 years. All patients had pathologic stage III disease at the time of surgery.

High frequency of minimal microsatellite shift in microsatellite instability high endometrial cancer

Among microsatellite instability high endometrial cancer cases, microsatellite instability was present at 2 or 3 loci in 66% (33 of 50 cases) and 4 or 5 loci in 34% (17 of 50 cases) of cases (Table 2). Microsatellite instability high colorectal cancers showed comparable microsatellite instability with 79% of cases (15 of 19) involving 2 or 3 microsatellite loci and 21% of cases (4 of 19) involving 4 or 5 microsatellite loci.

Although both minimal and major microsatellite shifts (Fig. 2a) were observed in the two study cohorts, microsatellite instability high endometrial carcinoma showed a higher frequency of minimal shift involving at least one of the five loci (52%, 26 of 50 cases), in contrast to only 16% of microsatellite instability high colorectal cancers (3 of 19 cases) demonstrating minimal microsatellite shift (Table 2).

Among the 26 microsatellite instability high endometrial cancers with minimal microsatellite shift, the minimal shift involved one locus in 6 cases, 2 loci in 10 cases, 3 loci in 8 cases, 4 loci in 2 cases, and 5 loci in none (Table 3). Eleven of these cases (42%, 11/26) demonstrated only minimal shift at all unstable loci. There were significant variations among the five microsatellite loci involved by minimal shift (Table 3). The frequency of minimal shift at a microsatellite locus that became unstable were 100% at *D2S123* (21/21 cases, Fig. 3), 91% at *D17S250* (10/11 cases), 92% at *D5S346* (11/12 cases), 75% at *BAT25* (9/12 cases), and 38% at *BAT26* (8/21 cases).

Combined loss of *MLH1* and *PMS2* coincides with minimal microsatellite shift in MSI-H endometrial cancer

Most endometrial carcinoma cases (48 of 50) and colorectal cancer cases (15 of 19) had mismatch repair protein immunohistochemistry performed on the same tumor block analyzed by microsatellite instability PCR. As expected (Table 2), the most common abnormal immunohistochemistry result was the combined loss of *MLH1* and *PMS2* proteins in both endometrial cancer (62.5%) and colorectal cancer (53%) (31). Five endometrial cancers (10%) and 4 colorectal cancers (27%) had combined loss of *MSH2* and *MSH6*. Six endometrial cancers (13%) and 2 colorectal cancers (13%) lost *PMS2* expression alone and 7 endometrial cancers (15%) and 1 colorectal cancer (7%) had *MSH6* loss alone (Table 2, Fig. 1). Among microsatellite instability high endometrial cancers with minimal microsatellite shift, 65% (17/26) showed combined *MLH1* and

Table 2 Summary of microsatellite instability PCR testing and mismatch repair protein immunohistochemistry

			Endometrial cancers (N = 50)	Colorectal cancers (N = 19)
MSI	No. Loci involved	2 to 3 Unstable Loci	33 (66%)	15 (79%)
		4 to 5 Unstable Loci	17 (34%)	4 (21%)
	Shift pattern	Minimal Shift	26 (52%)	3 (16%)
		Major Shift	24 (48%)	16 (84%)
MMR	*MLH1/PMS2* loss	Overall	30 (63%)	8 (53%)
		With minimal shift	17 (65%)[a]	1 (33%)[a]
	MSH2/MSH6 loss	Overall	5 (10%)	4 (27%)
		With minimal shift	2 (8%)[a]	1 (33%)[a]
	PMS2 loss	Overall	6 (13%)	2 (13%)
		With minimal shift	4 (15%)[a]	0[a]
	MSH6 loss	Overall	7 (15%)	1 (7%)
		With minimal shift	3 (12%)[a]	1 (33%)[a]
	Not available		2	4

[a]Percentages are numbers calculated based the total number of cases with minimal microsatellite shift, i.e., 26 endometrial carcinomas and 3 colorectal carcinomas

PMS2 loss, 8% (2/26) had combined *MSH2* and *MSH6* loss, 15% (4/26) and 13% (3/26) showed individual loss of *MSH6* or *PMS2*, respectively (Table 2). Moreover, it is noted that 43% (3/7 cases) of the *MSH6* deficient endometrial carcinomas showed minimal microsatellite shift (Table 2). In contrast, among the 19 microsatellite instability high colorectal cancer cohort, 3 cases had minimal microsatellite shift, corresponding to 1 case of combined loss of *MLH1/PMS2*, 1 case of combined loss of *MSH2/MSH6* and 1 *MSH6* loss alone.

Discussion

The goal of Lynch syndrome screening is to identify involved families, and therefore to provide surveillance for susceptible family members and to prevent additional Lynch syndrome-associated malignancies in the index patient [14]. Beyond Lynch syndrome screening, microsatellite instability testing has recently acquired unprecedented prognostic and predictive value in guiding tumor immunotherapy of patients with advanced microsatellite instability high solid tumors [15–18]. The two most common Lynch syndrome-associated cancers are colon and endometrial cancers with a lifetime risk between 52–82% and 25–60%, respectively [2, 19]. Clinicopathologically, Lynch syndrome-associated endometrial cancer is seen in often young patients with a mean age of 48–55 years [20]. The morphology of Lynch syndrome-associated endometrial cancer is characterized by peritumoral lymphocytes, tumor infiltrating lymphocytes, tumor location in the lower uterine segment, and high histologic grade [21–24]. These gross and histological features may be helpful in guiding patient selection for mismatch repair deficiency testing. Microsatellite instability high endometrial cancers are predominantly of endometrioid histologic subtype of both inherited (Lynch syndrome) and sporadic mismatch repair deficient tumors [24]. Clear cell and serous carcinoma are rarely observed in Lynch syndrome patients [25, 26]. Consistently, 90% (45 of 50 cases) in our study cohort were endometrioid adenocarcinomas. Clear cell carcinoma was seen in 8% (4 of 50 cases) and serous carcinoma was seen in one case. Recently, universal screening for Lynch syndrome of all newly diagnosed endometrial cancer patients has been advocated in several clinical recommendations. Our institution has adopted a screening algorithm to reflex all newly diagnosed endometrial cancers for simultaneous microsatellite instability PCR and mismatch repair protein immunohistochemical testing.

In this study, microsatellite instability high endometrial cancers demonstrate a high frequency of minimal microsatellite shift. In a previous comparative study of microsatellite instability profiles of endometrial and colorectal tumors, it was observed, that the size of deletions/insertions differs between colorectal and endometrial carcinomas, in that the latter had shorter alteration or minimal nucleotide shift [27–29]. However, such difference in microsatellite instability nucleotide shift in endometrial carcinoma has not been a common knowledge among practicing molecular pathologists as mismatch repair deficiency testing of endometrial cancer has only become a standard clinical practice very recently. While there are high concordance rates between immunohistochemistry and PCR for colorectal cancer, microsatellite instability PCR testing of
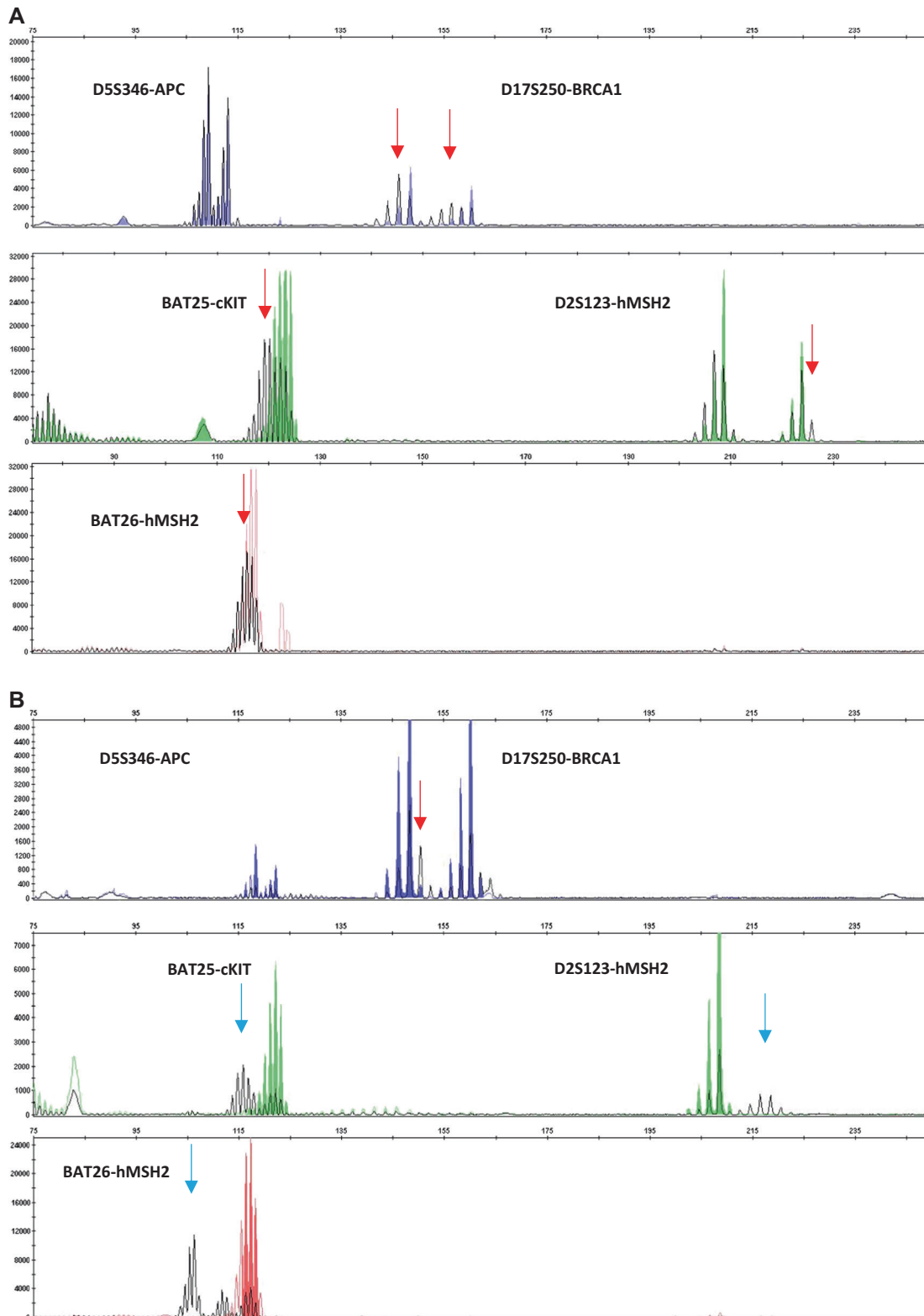
Fig. 2 Microsatellite instability testing by analysis of PCR products amplified at 5 NCI recommended loci. a: Minimal microsatellite shift at four of 5 microsatellite loci (red arrows) in an endometrial endometrioid adenocarcinoma. b: Major microsatellite shifts (blue arrows) and minimal microsatellite shifts (red arrow) in a colorectal carcinoma)

Table 3 Minimal microsatellite shift patterns at 5 NCI recommended loci in microsatellite instability high endometrial carcinomas (N = 26)

#	MSI Loci/No. repeat shift					Summary of shift pattern		
	BAT26	D2S123	D17S250	D5S346	BAT25	# Loci minimal shift	#Loci major shift	% Loci minimal shift
1	5	0	0	1	0	1	1	50%
2	9	3	2	2	2	4	1	80%
3	8	1	0	1	0	2	1	67%
4	2	2	2	0	0	3	0	100%
5	9	5	0	0	1	1	2	33%
6	9	3	1	3	4	3	2	60%
7	6	3	3	2	3	4	1	80%
8	2	0	0	2	0	2	0	100%
9	6	2	0	2	3	3	1	80%
10	0	2	3	0	0	2	0	100%
11	3	0	0	0	2	2	0	100%
12	0	1	0	5	0	1	1	50%
13	0	2	3	0	0	2	0	100%
14	6	2	2	0	2	3	1	75%
15	12	2	0	0	0	1	1	50%
16	0	2	2	0	0	2	2	100%
17	5	2	3	0	2	3	1	75%
18	2	2	2	0	0	3	0	100%
19	0	2	5	0	0	1	1	50%
20	5	2	0	2	5	2	2	50%
21	2	2	0	1	0	3	0	100%
22	2	1	0	1	0	3	0	100%
23	2	0	0	0	2	2	0	100%
24	4	0	0	0	2	1	1	50%
25	8	2	0	2	8	2	2	50%
26	2	2	0	0	0	2	0	100%

endometrial cancer for Lynch syndrome screening has been reported to have a high frequency of false negativity [6, 30], with a particularly low detection sensitivity of *MSH6* deficient endometrial cancer [5]. It is possible, that the high frequency of minimal nucleotide shift in endometrial cancers may have contributed to the reported high false negative rate as a result of misinterpretation of minimal shift as stable microsatellite. Corroborating our study, a recent comparative investigation of microsatellite instability PCR profiles between endometrial and colorectal cancer aimed to identify causes of the reported higher false negative rate with microsatellite instability PCR testing [31]. It was found, that 53% of microsatellite instability high endometrial cancers had a mean absolute shift of 1 to 2 nucleotide repeats (minimal shift) in contrast to only 20% of microsatellite instability high colorectal cancers showing minimal shift, similar to our findings (minimal repeat shift in 52% of microsatellite instability high endometrial cancers vs. 16% in colorectal cancer cases). Taken together, overlooking minimal microsatellite shifting may potentially lead to an incorrect assignment of microsatellite instability high endometrial cancer as microsatellite stable or microsatellite instability low in 42% (21/50) of our study cases (Table 3). It has been suggested that microsatellite instability PCR testing of tissue samples with less than 30% of tumor content may not be able to detect the minimal nucleotide shift, further increasing the likelihood of false negativity [31]. Moreover, the standard NCI five microsatellite instability loci may not provide the most sensitive amplicons for microsatellite instability PCR testing. It has been recently advocated that testing additional mononucleotide repeat loci may improve the detection sensitivity by PCR [32, 33].

Overall, in our study, 42% (11/26) of cases of endometrial cancer demonstrated only minimal shift and no major shift was observed. It is worth noting, that among microsatellite instability high endometrial cancers, certain loci are more prone to be involved by minimal microsatellite shift (Table 3), particularly *D2S123* (21/21, 100%), *D17S250* (10/11, 91%), and *D5S346* (11/12, 92%). The

Fig. 3 Microsatellite shift patterns at *D2S123* Locus in microsatellite instability high endometrial cancer (orange, *N* = 50) and colorectal cancer (blue, *N* = 19)
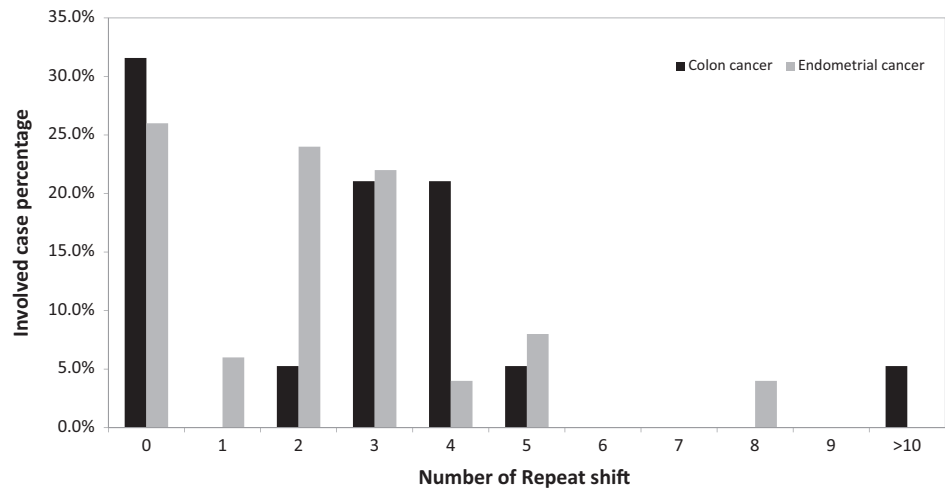
reason for the high frequency of minimal shift in microsatellite instability high endometrial carcinomas is unclear. It has been hypothesized, that the difference in microsatellite instability profiles between microsatellite instability high endometrial and colorectal cancer may be due to more rapid turnover of DNA replication in colorectal cancer, compared to endometrial cancer [31]. Taken together, our data further emphasize, that the high frequency of minimal microsatellite shift is a potential cause of false-negative microsatellite instability PCR result when testing microsatellite instability high endometrial cancers. Awareness of this potential pitfall is essential for accurate interpretation. Careful assessment of small shift peaks and the overall peak patterns, particularly at *D2S123, D17S250* and *D5S346* loci, are important for accurate interpretation of microsatellite instability PCR data in endometrial carcinoma.

Unlike colorectal cancer, female *MSH6* mutation carriers have the highest risk for developing endometrial cancer in older patients (61%), compared to those with *MLH1* (25%) and *MSH2* mutations (49%) [34]. While hypermethylation of *MLH1* promoter is common in sporadic endometrial cancer [35] similar to microsatellite instability high colorectal cancers, *BRAF* V600E mutation is very uncommon in sporadic endometrial carcinomas with *MLH1* promoter hypermethylation [11]. Corroborating the literature, our study has confirmed that microsatellite instability high endometrial carcinomas have more frequent minimal microsatellite shift, further emphasizing the genetic differences between microsatellite instability high endometrial carcinoma and microsatellite instability high colorectal carcinomas. It should be noted that such frequent minimal microsatellite shift observed in endometrial cancer has not been reported in other types of microsatellite instability high malignancies. Nevertheless, molecular pathologists should pay close attention to the subtle microsatellite shifting and interpret them as unstable microsatellite loci when testing all tumor types.

The United States Multi-Society Task Force has published a consensus statement regarding screening, genetic evaluation and management for Lynch syndrome-associated colorectal cancers [36]. However, strategies for cost-effective screening and subsequent genetic evaluation of endometrial cancer patients are still evolving [10] with varying approaches of integrating mismatch repair protein immunohistochemistry and microsatellite instability PCR testing into the screening algorithms. Mismatch repair protein immunohistochemistry is preferred at some institutions as a primary screening method, supplemented by microsatellite instability PCR testing [37]. The advantages of mismatch repair protein immunohistochemistry include gene specific recognition when abnormal loss of expression of mismatch repair genes is observed, and the interpretation is familiar to pathologists. However, it has been increasingly recognized that interpretation of mismatch repair protein immunohistochemistry of endometrial carcinoma can be problematic. Firstly, similar to colorectal cancers, weak or equivocal staining patterns of mismatch repair protein immunohistochemistry due to technical or tissue fixation issues can lead to significant interobserver variability [38]. Second, not infrequently the foci of mismatch repair deficient invasive carcinoma are closely admixed with atypical endometrial hyperplasia, that may retain normal expression of mismatch repair genes, leading to false negative interpretation. Thirdly, in rare cases, missense mutations of one of the mismatch repair genes may result in a nonfunctional protein but normal immunostaining pattern, leading to false negativity [39]. Lastly, while non-neoplastic cells express nuclear staining of mismatch repair proteins, abnormal cytoplasmic staining of tumor cells may be misinterpreted as normal expression [40].

Microsatellite instability PCR is a sensitive method for the detection of mismatch repair deficient tumors. The limitations of molecular microsatellite instability testing include the requirement of paired normal tissue for testing

and inability to specify which mismatch repair gene is involved when a tumor has mismatch repair deficiency. Recent studies indicate a high concordance rate between mismatch repair protein immunohistochemistry and microsatellite instability PCR in the workup of endometrial cancer [41]. It is important to recognize that both mismatch repair protein immunohistochemistry and microsatellite instability PCR share similar testing sensitivity of 91 to 93% and both miss 7–9% of mismatch repair deficient/ Lynch syndrome-related cancers [10, 37]. Our study has also confirmed a higher frequency of *MSH6* loss in microsatellite instability high endometrial carcinomas. Loss of *MSH6* frequently leads to minimal microsatellite instability in our study (43%, 3/7 cases), that may be erroneously misinterpreted as either microsatellite instability low or microsatellite stable tumor. While minimal microsatellite shift may be overlooked and misinterpreted as a false negative PCR result, the limitations of immunohistochemistry interpretation of *MSH6* must also be taken into consideration. *MSH6* expression level may diminish after chemotherapy or radiation treatment in microsatellite stable tumors, leading to false positive results [39, 42]. Therefore, complimentary co-testing by microsatellite instability PCR and mismatch repair protein immunohistochemistry likely offers the most sensitive and specific approach to capture mismatch repair deficient endometrial carcinomas.

In conclusion, there are fundamental pathogenetic differences between microsatellite instability high endometrial carcinomas and microsatellite instability high colorectal cancers. Compared to colorectal cancers, microsatellite instability high endometrial carcinomas incur a higher frequency of minimal microsatellite shift that coincides with a higher percentage of combined loss of *MLH1/PMS*2. Microsatellite instability high endometrial cancers also have more frequent loss of *MSH6*. Diagnostically, recognition of minimal microsatellite shift is crucial for accurate interpretation of microsatellite instability PCR data for Lynch syndrome screening and guiding check point inhibitor immunotherapy for patients with high stage or recurrent endometrial cancers.

Compliance with ethical standards

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