

ORIGINAL PAPER

THE CONCORDANCE OF DNA MISMATCH REPAIR PROTEIN BETWEEN ENDOSCOPIC BIOPSIES AND SURGICAL SPECIMENS AND INTER-OBSERVERS VARIATIONS IN COLORECTAL CANCER PATIENTS: REFLECTIONS FROM ENDOSCOPE DOCTORS

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Previous studies showed that the yield of immunohistochemistry (IHC) staining on endoscopic biopsies may be as good as on surgically removed tissues. However, we noted that some patients showed inconsistent DNA mismatch repair protein (MMRP) stains between biopsies and surgical specimens. In this study, we aimed to investigate factors which are related to the consistence of MMRP evaluation between two pathologists or between different tissues. Two pathologists were asked to diagnose 4 MMRP, both on endoscopic biopsies and surgical materials, in 51 colorectal cancer (CRC) patients, using a single blind method. The consistence of two specimens and inter-observers' variances across different pathologists were compared respectively and the factors related to this variability were analyzed. Among the 816 paired MMRP, 804 (98.5%) pairs showed concordant IHC stains between biopsies and surgical materials, the agreement was almost perfect for MSH6 and PMS2 ($\kappa = 0.85, 0.85$ separately); 804 (98.5%) pairs showed concordant IHC stains between two pathologists, the inter-observer agreement was almost perfect for MSH6 and PMS2 ($\kappa = 0.85, 0.88$ separately). Clinical and pathological characteristics analysis showed that biopsy number and TNM stage were related to the variations. Inter-observers variations should be taken into account during MMRP testing in colorectal cancers. Generous endoscopic biopsies could improve the accuracy of endoscopic biopsy for MMRP detection which can be used histological tool in the evaluation of CRC and a promising new prognostic factor for these patients.

Key words: colorectal cancer, endoscopic biopsy, surgical specimens, DNA mismatch repair protein.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths around the world [1]. Every year, 1,000,000 new CRC cases and 700,000 mortalities caused by CRC are registered [2]. Cumulative

genetics and epigenetic alterations, such as DNA mismatch repair (MMR) and cell cycle regulators' genes participate in the development of CRC [3]. MMR deficient (dMMR), mainly due to the negative expression of mismatch repair proteins (MLH1, MSH2, PMS1 and/or PMS2) [4], associates with

distinct clinicopathological features of CRC, including poorly differentiated tumor cells, presence of mucin, lymphocytic infiltrate and predominantly proximal colon cancer. Patients with dMMR may reflect better overall survival rate and better response to 5-FU based therapies [5].

In recent guidelines, it was suggested that all CRC patients should be tested 4 MMRP through immunohistochemistry (IHC) staining [6, 7]. Previous studies have shown that the yield of IHC staining on endoscopic biopsies may be as good as on surgically resected tissues [8, 9, 10, 11]. Biopsy samples may provide superior staining with a faster and more thorough fixation, given the sensitivity of immunostaining associates with the degree of tissue fixation. However, we noted that IHC staining for MMRP on endoscopic biopsies was not entirely consistent with the same staining on resection specimens in some patients [12]. As the first step for further genetic evaluation, proper interpretation of IHC staining will have a profound impact on selected treatment strategy [13]. So it is very important to ensure the accuracy of MMRP detection on endoscopic biopsies, especially for terminal stage CRC that can't be resected on. In this study, we ask two pathologists to diagnose 4 MMRP both on endoscopic biopsies and surgical materials in CRC patients, record the consistence between two specimens and inter-observers' variations respectively and analyze the factors related to the variations.

Material and methods

Study design

Between March 2013 to September 2017, CRC patients both taken endoscopy biopsy and surgical resection were consecutively recruited. Patients were included if the following criteria were fulfilled: 1) primary colorectal cancer diagnosed by pathology; 2) both endoscopic biopsy and radical resection of colorectal cancer were performed, and MMRP was detected in both the biopsy and surgical specimens; 3) no chemotherapy, radiotherapy or other antitumor regimens before the operation. Patients' demographic features, clinical details and histological parameters of the tumors were recorded. The MMRP (MLH1, MSH2, MSH6 and PMS2) of endoscopic/surgical specimens was assigned to two senior pathologists by random distribution with single blind method, that means the pathologists didn't know the pairing of the specimens in advance. The inter-observers' variation analysis was carried out by the researcher.

Immunohistochemistry staining for MMRP

MMRP, included MLH1, MSH2, MSH6 and PMS2 performed on both preoperative endoscopic

biopsies and subsequent surgical resection specimens were analyzed. Thin (5 μ m) sections of representative blocks were taken from the areas with colon tumor cells > 50%. Following antigen retrieval in repair solution EnVisiona (Dako, Denmark), the mouse monoclonal antibodies against MLH1, MSH2, MSH6 and PMS2 (Dako Company, Denmark) were added, incubated at room temperature for 20 min, phosphate buffered saline (PBS) rinsed 3 times for 5 min. Incubations were performed with a secondary antibody, followed by staining (Diaminobenzidine as a chromogen) and nuclear counterstain (by hematoxylin). Phosphate buffered saline (PBS) was used as negative control and normal colorectal mucosa epithelium as positive control. Interpretation of the stains was performed according to the methods described by Shia *et al.* [13] and Barrow *et al.* [14]: "positive expression" showed the overall nuclear staining in tumor cells and internal non-neoplastic cells; "negative expression" was defined as the epithelial cells within the tumor tissue lacked nuclear staining while the surrounding stromal cells were positive for MMR staining; staining in less than 10% of the tumor with only a weak or moderate intensity was described as "focal positive expression". pMMR was used to describe tumor tissue with all MMRP present, while dMMR represents those showing deficiency in at least one of the 4 MMRP [12].

Statistical analysis

The agreement between endoscopic biopsies and surgical resections or inter-observers' variation were evaluated by using ICC (0-0.2: slight reliability, 0.2-0.4: fair reliability, 0.4-0.6: moderate reliability, 0.6-0.8: substantial reliability, > 0.8: almost perfect reliability) [15]. Factors associated with the consistence between specimens in detecting MMRP were analyzed by constructing a 2-2 matrix (consistent group vs inconsistent group). Two-sided p-values of < 0.05 were considered as significant. Statistical analyses were performed with IBM SPSS Statistics 20.0 (SPSS Inc, Chicago, Ill).

Results

Patient demographics

Fifty-one patients with CRC were included as the training cohort. Mean patient age was 66.4 years, with an age range of 43-94 years. Twenty-four (47.1%) patients were female, and 27 (52.9%) were male. Tumor locations were as follows: proximal colon – 19 (37.3%), distal colon – 17 (33.3%), rectum – 15 (29.4%) (Table I).

Table I. Patients' demographic features, clinical details and histological parameters of the tumors

PARAMETER	STRATA	TRAINING COHORT (N = 51)
Mean age (years) \pm SD	23.08	66.4 (10.5)
(Range)	46.15	43-94
Gender, n (%)	Female	24 (47.1)
	Male	27 (52.9)
Tumor site, n (%)	Proximal	19 (37.3)
	Distal	17 (33.3)
	Rectum	15 (29.4)
Tumor size, n (%)	\leq 2 cm	5 (9.8)
	2-5 cm	17 (33.3)
	> 5 cm	29 (56.9)
TNM, n (%)	I-II	19 (37.3)
	III-IV	32 (62.7)
LVI, n (%)	Absent	27 (52.9)
	Present	24 (47.1)
EMVI, n (%)	Absent	34 (66.7)
	Present	17 (33.3)
Margin, n (%)	Expansile	44 (86.3)
	Infiltrative	7 (13.7)

dMMR – DNA mismatch repair deficient; *pMMR* – DNA mismatch repair proficient; TNM – tumor-node-metastasis; LVI – lymphovascular invasion; EMVI – extramural venous invasion

The consistence between two specimens for each MMRP

To observe the consistency between endoscopic biopsies and surgical specimens, it was found that among the 816 paired MMRP, 804 (98.5%) pairs showed concordant endoscopic and operative material stains, either positive (788, 96.6%), focal positive (6, 0.7%) or negative (10, 1.2%). The agreement was almost perfect for MSH6 and PMS2 ($\kappa = 0.85, 0.85$ separately), substantial for MLH1 and MSH2 ($\kappa = 0.75, 0.66$ separately; Table II).

Inter-observers' variability for each MMRP

Among the 816 (102 \times 4) paired MMRP, 804 (98.5%) pairs showed concordant IHC stains between two pathologists, either positive (790, 98.3%), focal positive (4, 0.5%) or negative (10, 1.2%). The inter-observers' agreement was almost perfect for MSH6 and PMS2 ($\kappa = 0.85, 0.88$ separately), substantial for MLH1 and MSH2 ($\kappa = 0.74, 0.71$ separately; Table III). Specimens with intra-observer variability were collected from 5 patients. The patients' demographics, MMRP status in endoscopic biopsies and resected specimens, MMRP status diagnosed by two pathologists were shown in Table IV.

Table II. Agreement between biopsy and surgical material for all MMRP

MMRP	ENDOSCOPY BIOPSY	SURGICAL MATERIAL			TOTAL	κ
		NEGATIVE	FOCAL	POSITIVE		
MLH1	Negative	2	0	2	4	0.75 (substantial)
	Focal	0	2	0	2	
	Positive	0	0	198	198	
	Total	2	2	200	204	
MSH2	Negative	2	0	0	2	0.66 (substantial)
	Focal	1	2	0	2	
	Positive	1	0	198	200	
	Total	4	2	200	204	
MSH6	Negative	2	2	0	4	0.85 (almost perfect)
	Focal	0	0	0	0	
	Positive	0	0	200	200	
	Total	2	2	200	204	
PMS2	Negative	4	2	0	6	0.85 (almost perfect)
	Focal	0	2	0	2	
	Positive	1	0	195	196	
	Total	5	4	195	204	

Table III. Inter-observer variability for all MMRP

MMRP	PATHOLOGIST A	PATHOLOGIST B			TOTAL	κ
		NEGATIVE	FOCAL	POSITIVE		
MLH1	Negative	2	0	0	2	0.74 (substantial)
	Focal	0	2	0	2	
	Positive	2	0	198	200	
	Total	4	2	198	204	
MSH2	Negative	2	0	0	2	0.71 (substantial)
	Focal	1	0	2	2	
	Positive	0	1	198	200	
	Total	4	0	200	204	
MSH6	Negative	2	2	0	4	0.85 (almost perfect)
	Focal	0	0	0	0	
	Positive	0	0	200	200	
	Total	2	2	200	204	
PMS2	Negative	4	2	0	6	0.88 (almost perfect)
	Focal	0	2	0	2	
	Positive	0	1	195	196	
	Total	4	5	195	204	

Table IV. The 5 patients' demographics, MMRP status in endoscopic biopsies and resected specimens, MMRP status diagnosed by two pathologists

NO.	AGE	SEX	TUMOUR SITE	TUMOUR SIZE	TNM STAGE	MMRP NEGATIVE (bx) or focal	MMRP NEGATIVE (rs) or focal	PATHOLOGIST A	PATHOLOGIST B
1	82	M	HF	2	I	MLH1, PMS2	MSH2	MSH2	MLH1, PMS2
2	72	F	Caecum	6	II	MLH1, MSH2*	MSH2	MSH2*	MLH1, MSH2
3	76	M	Rectum	6	II	PMS2	PMS2*	PMS2	PMS2*
4	59	F	Rectum	4	II	MSH6	MSH6*	MSH6	MSH6*
5	61	M	Descend	10	IV	MSH6, PMS2	MSH6*, PMS2*	MSH6, PMS2	MSH6*, PMS2*

*bx – biopsy; MMRP – mismatch repair protein; Mut – mutated; rs – resection specimen; HF – hepatic flexure; * focal positive*

Factors associated with inter-observers' variability

According to the consistency of MMRP between endoscopic biopsy and operative specimens or between two pathologists, 51 patients were divided into two groups: consistent group (46 patients) which was defined as a consistent MMRP result between two specimens, while inconsistent group (5 patients) was described as inconsistent MMRP results. Clinical and pathological characteristics analysis showed that more patients in the consistent group had over 4 biopsies taken than those in the inconsistent group (76.1%

vs. 20.0%, $p = 0.036$). The accuracy of endoscopic biopsy specimens increased among patients with TNM stage III-IV CRC than that with stage I-II (78.3% vs. 20.0%, $p = 0.025$; Table V).

Discussion

Colorectal cancer (CRC) is a heterogeneous group of diseases both from the morphological and molecular point of view [16]. As far as we know, CRC is a heterogeneous clinical entity characterized by multiple molecular subtypes. Two main subtypes are 'classic' chromosomal instability pathway and microsatellite

Table V. Comparison of histopathological features according to mismatch repair protein status consistence in colorectal cancer

PARAMETER	STRATA	N	CONSISTENT GROUP (N = 46)	INCONSISTENT GROUP (N = 5)	P-VALUE
Mean age (years) \pm SD		51	66.3 (10.6)	67.0 (10.5)	0.9#
(Range)		51	43-94	52-82	
Gender, n (%)	Female	24	22 (47.8)	2 (40.0)	1.0
	Male	27	24 (52.1)	3 (60.0)	
Site, n (%)	Proximal	19	17 (37.0)	2 (40.0)	0.851*
	Distal	17	16 (34.8)	1 (20.0)	
	Rectum	15	13 (28.3)	2 (40.0)	
Size, n (%)	\leq 5 cm	5	4 (8.7)	1 (20.0)	0.617*
	2-5 cm	17	16 (34.8)	1 (20.0)	
	> 5 cm	29	26 (56.5)	3 (60.0)	
Biopsy number, n (%)	\leq 4	15	11 (23.9)	4 (80.0)	0.036
	> 4	36	35 (76.1)	1 (20.0)	
TNM, n (%)	I-II	14	10 (21.7)	4 (80.0)	0.025
	III-IV	37	36 (78.3)	1 (20.0)	
LVI, n (%)	Absent	27	24 (52.2)	3 (60.0)	1.0
	Present	24	22 (47.8)	2 (40.0)	
EMV, n (%)	Absent	34	32 (69.6)	2 (40.0)	0.405
	Present	17	14 (30.4)	3 (60.0)	
Margin, n (%)	Expansile	44	41 (89.1)	3 (60.0)	0.133*
	Infiltrative	7	5 (10.9)	2 (40.0)	

#Independent samples Student's t-test; *Fisher's exact test; TNM – tumor-node-metastasis; LVI – lymphovascular invasion; EMVI – extramural venous invasion

instability pathway (MSI). Microsatellite instability is reflective of a deficient mismatch repair system (dMMR), which may be due to either sporadic or germline mutations in the relevant mismatch repair (MMR) gene [17]. In China, IHC staining detection of MMRP was used in most laboratories to represent MSI owing to its simplicity and veracity [18].

According to MMR status, CRC was divided into two distinct types: MMR proficient (pMMR) defined as positive expression of all MMRP (MLH1, MSH2, MSH6 and PMS2) while MMR deficient (dMMR) characterized by one or more negative expression of MMRP (mostly MLH1 or MSH2, rarely MSH6 or PMS2) [14, 15]. dMMR is observed in 10-20% CRC patients [19] with several distinct clinicopathological features, such as predominantly proximal colon cancer, poorly differentiated, mucin tumor cells and lymphocytic infiltrate. Due to its influence in CRC chemotherapy, dMMR was served as a predictive factor to 5-FU based treatment in CRC patients [20, 21].

MMR status is frequently determined by IHC for MMRP on surgical specimens [10]. A number of

studies have shown that the yield of IHC staining performed on endoscopic biopsies may be as good as operation materials [8]. Vilkin *et al.* found that from a procedural perspective given the sensitivity of immunostaining to the degree of tissue fixation, endoscopic biopsy material may produce superior staining based on faster and more thorough fixation [8, 9], so they suggested that for rectal cancer, endoscopic material rather than operative material should serve as the primary material for IHC test [9].

However, these were retrospective researches collected from histopathology electronic database and didn't consider inter-observers' variances across different pathologists. The purpose of this study was to evaluate the consistency of endoscopic and surgical specimens for MMRP detection between different pathologists, and analyze factors associated with the variations.

In this study, we asked two pathologists to diagnose 4 MMRP both on endoscopic biopsies and surgical materials in 51 CRC patients respectively and found that among the paired MMRP, 804 (98.5%) pairs showed concordant IHC stains between two

pathologists, or between endoscopic and operative material stains, the agreement was almost perfect for MSH6 and PMS2.

Five patients showed inconsistent MMRP, either between different specimens or between two pathologists. In order to identify the clinical, pathologic, and molecular features related to inter-observers' variations in MMRP detection in CRC patients, patients were divided into two groups according to the consistency of MMRP between two specimens: consistent group and inconsistent group. We believe that the issues exist in endoscopic specimens are biopsy samples represent only a small proportion of a tumor and may erroneously classify the MMR status by virtue of inadequate sampling. Clinical and pathological characteristics analysis verified our hypothesis: the consistence of endoscopy biopsy was higher when the number of biopsy debris was more than 4 pieces. In previous studies, generous tumor sampling at the time of biopsy was suggested to reduce the risk of sampling limitations [11, 22, 23]. In this study, more than 4 biopsy pieces were suggested to ensure the accuracy of MMRP detection for the first time. Besides, CRC patients with TNM III-IV stage showed high consistency of MMRP detection due to fewer adenomas in advanced CRC[24], which confirmed the previous conclusion that endoscopic specimens are feasible for the diagnosis of MMRP in advanced CRC patients.

In this research, single-blind method was used to analyze inter-observers' consistence between two pathologists for MMRP detection on endoscopy and surgical specimens for the first time. However, several limitations need to be considered in interpreting our findings. First, measurement errors were inevitable because of the small sample size. Second, although it was a single blind study, clinicopathological data was collected from electronic medical records, selection biases need to be considered. By increasing the number of patients, selection bias and measurement errors were expected to limited.

Conclusions

In summary, this study showed that inter-observers' variations should be taken account when test mismatch repair proteins on colorectal cancers. Generous endoscopic biopsies could improve the accuracy of endoscopic biopsy for MMRP detection.

The authors declare no conflict of interest.

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