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ORIGINAL RESEARCH

Detection of DNA Mismatch Repair Protein Abnormalities in Sudanese Colorectal Cancer Patients Using Immunohistochemical Methods

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Abstract

Purpose DNA mismatch repair (MMR) protein abnormalities among 42 Sudanese colorectal cancer (CRC) patients were assessed.

Methods Sections were stained by immunohistochemical method to assess the abnormalities of MLH1, MSH2, MSH6, and PMS2.

Results Of the 42 included cases, 34 (80.95%) were MMR protein positive for all MMR proteins under assessment, 3 (7.14%) MSH2 inadequate, and 1 (2.38%) MSH6 inadequate. Abnormal MMR protein expression was found in 4 (9.5%) cases. Of these, 2 (50%) were MSH2 and MSH6 negative and 2 (50%) were MLH1 and PMS2 negative. Regarding microsatellite instability (MSI) results, the three cases that were MSH2 inadequate and positive for the rest by immunohistochemistry (IHC) showed stable results with both BAT 25 and 26. The case that was MSH6 inadequate showed stable results with both BAT 25 and 26. The 2 cases with MSH2- and MSH6-negative results were unstable with both BAT 25 and 26. Of the two cases that were MLH1 and PMS2 negative, one case showed non-evaluable results with both BAT 25 and 26 while the other case was unstable with BAT 26 and not evaluable with BAT 25.

Conclusions The percentage of MMR protein-negative cases in Sudanese CRC patients appears to be relatively low compared to what is generally reported in certain studies in different countries. Furthermore, MLH1 and PMS2 and MSH2 and MSH6 abnormal expression detected by IHC seems to be the most common form of MMR protein abnormalities in Sudanese CRC patients. Concerning the results of IHC, MLH1 and MSH2 seem to be the most inactivated MMR genes in Sudanese CRC patients.

Keywords Colorectal cancer · Immunohistochemistry · Mismatch repair proteins · Sudan

Introduction

Worldwide, CRC was the third most common cancer in 2008 with 1.233 million newly diagnosed cases (9.7% of the total) and about 608.000 deaths, which makes it the fourth cause of cancer deaths [1]. In Sudan, CRC represents 6.4% of the total number of malignant tumors and the second most common malignant gastrointestinal tumor (126 out of 374 malignant gastrointestinal tumors, 33.69%) after esophageal cancer (37.7%) during the period from 2000 to 2004 according to a published data from one pathology center in Khartoum [2].

According to the data reported from different articles, microsatellite instability (MSI) (notably the high levels) is present in almost 15% of CRC [3–5]. This is due to germline mutation in one of the MMR genes (mainly MLH1, MSH2, MSH6, or PMS2) or MLH1 epigenetic silencing [4]. Of these 15% CRCs with MSI, 12% are due to acquired hypermethylation of the MLH1 gene promoter while 3% are related to HNPCC [6].

Furthermore, MSI is present in over 90% of hereditary nonpolyposis colorectal cancer (HNPCC) patients in which MSI caused by defects in DNA MMR [7].

CRCs with MSI or mismatch repair deficient (dMMR) thought to have no or poorer benefit from adjuvant 5-fluorouracil (5-FU)-based chemotherapy compared to CRCs

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with microsatellite stable (MSS) [8–10]. However, a study found that any advantage of 5-FU among dMMR stage III tumors is suggested to be limited to tumors with suspected germline mutations compared to sporadic CRCs [11].

The availability of antibodies to MMR proteins has offered an alternative method to molecular techniques for recognizing dMMR CRCs [12]. IHC can be a specific, sensitive, fast, and cost-effective method for identifying MMR defects [13].

In IHC, MLH1, MSH2, MSH6, and PMS2 protein antibodies give an idea about the MMR system functionality [4].

Loss of expression of any of these proteins indicates the presence of dMMR and detects the gene that is most probably to harbor a germline mutation or inactivated due to another cause [4].

Positive staining by IHC for MMR protein is defined as the presence of nuclear staining in any malignant cells, while the adjacent normal epithelium of the colon and lymphocytes serve as internal controls [14].

The sections considered negative for MMR protein when all malignant cells show complete loss of staining for the examined MMR protein while there is a nuclear staining in the adjacent normal cells [14].

In the current study, we aimed to assess the frequency of DNA MMR protein abnormalities among Sudanese CRC patients mainly by detection of four MMR protein expression (*MLH1*, *MSH2*, MSH6, and *PMS2*) by immunohistochemical method and to determine the type of abnormal MMR protein expression among Sudanese CRC patients.

Materials and Methods

Study Samples

In this study, CRC cases were retrieved from the records of two Histopathology laboratories in Khartoum, Sudan. Paraffin sections were cut from paraffin wax embedded tissue blocks. The total number of included cases was 42.

Laboratory Procedures

Cutting

Special slides for IHC (SuperFrost® plus, DIAPATH) as well as ordinary frosted end slides were used. Using a rotary microtome, 3- and 10-μm sections were cut.

Immunohistochemical Analysis

The sections were examined for MMR protein expression of MLH1, MSH2, MSH6, and PMS2 using anti-MLH1, MSH2, MSH6 (mouse monoclonal antibodies-CELL MARQUE, USA), and anti-PMS2 (rabbit monoclonal antibody-CELL MARQUE, USA). IHC was performed using a fully automated slide preparation system (BenchMark XT, Ventana, USA), and the staining of two sections was repeated again using another fully automated slide preparation system (BenchMark ULTRA, Ventana, USA).

Positive staining is defined as the presence of nuclear staining in any percentage of malignant cells, while nuclear staining in adjacent normal cells serve as internal positive control. Negative staining is defined as the complete absence of nuclear staining in malignant cells while normal cells show positive nuclear staining. MMR protein-positive cases also indicated that the cases are proficient MMR (pMMR), and MMR protein-negative cases indicated that the cases are dMMR [4].

Immunohistochemical staining in some sections was inadequate to provide a good evaluation. In such instances, immunohistochemical analysis was repeated in another section, when available, from the same case. The repetition was performed using a prolonged time of antigen retrieval, and two inadequately stained sections were repeated for MSH2 and MSH6 using the two fully automated slide preparation systems. Sections were treated in an oven, at 60–65 °C—up to 30 min approximately, before repeating the immunohistochemical analysis with the antigen retrieval modification several. In BenchMark XT, the time of treatment in cell conditioning 1 (CC1) for

Table 1 MMR protein expression by IHC and MSI status

Expression of MMR protein	*n*	%	MSI analysis	
			BAT 25	BAT 26
MLH1 and PMS2 negative (MMR protein—cases)	2	4.76	1—not evaluable	Unstable
			2—not evaluable	Not evaluable
MSH2 and MSH6 negative (MMR protein—cases)	2	4.76	Unstable	Unstable
All positive (MMR protein + cases)	34	80.95	–	–
MSH2 inadequate/MSH6, MLH1, PMS2 positive	3	7.14	Stable	Stable
MSH6 inadequate/MSH2, MLH1, PMS2 positive	1	2.38	Stable	Stable
Total	42	100*	–	

*The total percentage was rounded to the nearest tenth

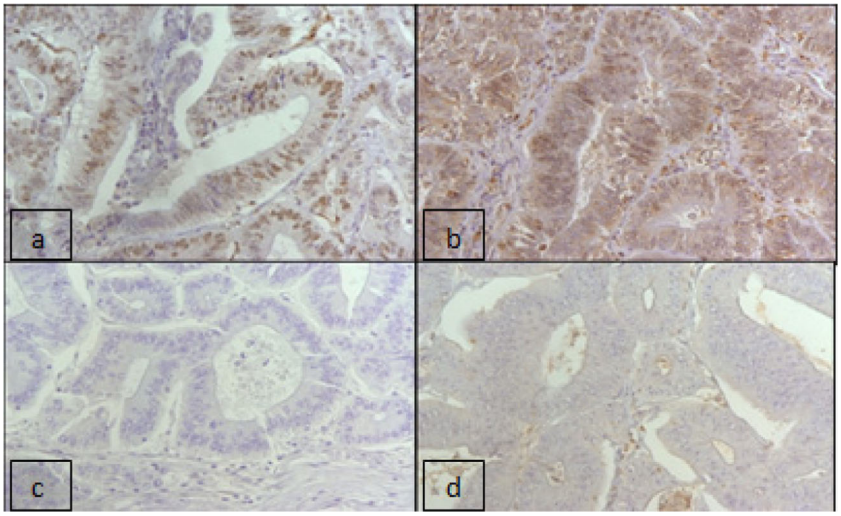

Fig. 1 Immunohistochemical staining results of MSH2, MSH6, MLH1, and PMS2 (shown at × 200). **a** MSH2 positive. **b** MSH6 positive. **c** MLH1 negative. **d** PMS2 negative

antigen retrieval was increased to 90 min for MSH2, MLH1, and MSH6. For PMS2, the time in cell conditioning 2 (CC2) for antigen retrieval was increased to 84 min. In BenchMark ULTRA, treatment in CC1 was done for 92 min for both MSH2 and MSH6. After repetition, a number of sections were evaluable. However, the immunohistochemical staining remained inadequate to allow a good evaluation in other sections. Therefore, MSI analysis was performed for these cases,

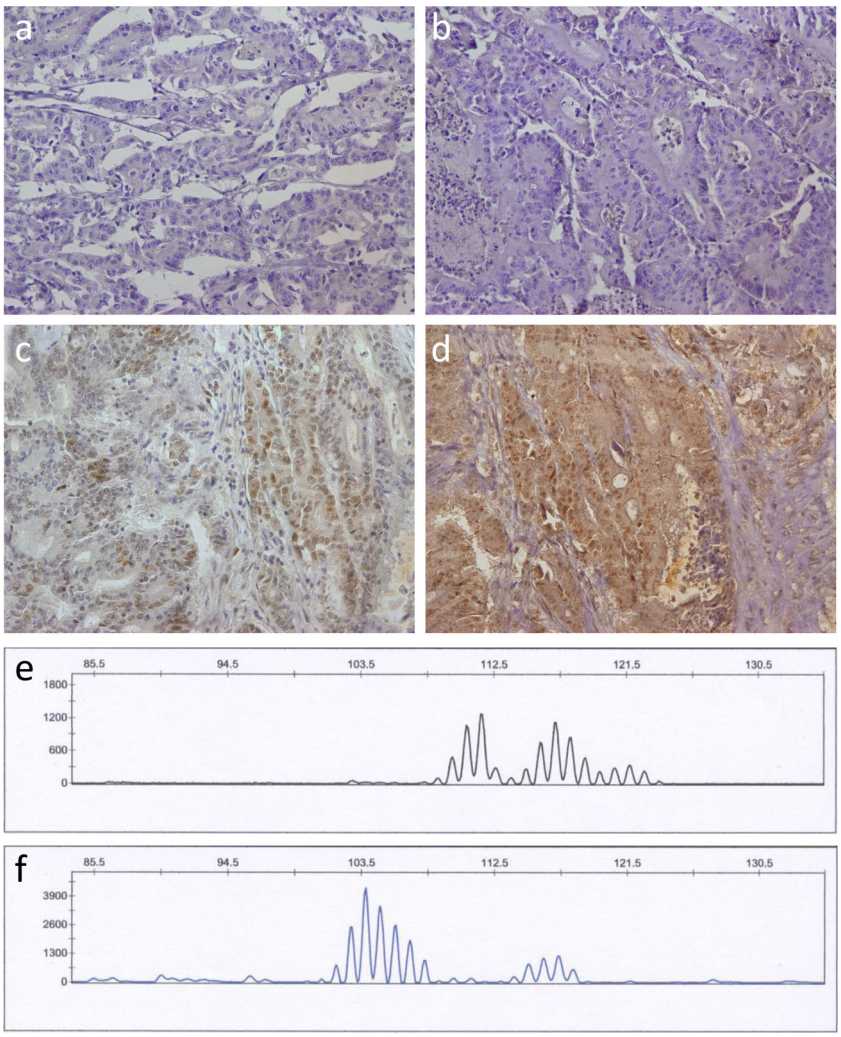

Fig. 2 Immunohistochemical staining results of MSH2, MSH6, MLH1, and PMS2 (shown at × 200) and MSI analysis of **a** MSH2 negative, **b** MSH6 negative, **c** MLH1 positive, and **d** PMS2 positive. Electropherograms for BAT25 and BAT26 from CRC tissue. **e** BAT 25 unstable. **f** BAT 26 unstable

Table 2 Description of study population by MMR status

MMR status	*n*	%
Mismatch repair-proficient (pMMR)/insufficient evidences of dMMR	38	90.4
dMMR	4	9.5
Total	42	100*

*The total percentage was rounded to the nearest tenth

which remained inadequate, as well as for those cases that showed negative staining results for any MMR protein.

Extraction of DNA

The areas with tumor cells were labeled. Then, DNA was extracted from formalin-fixed, paraffin-embedded tissues from unstained sections, hematoxylin & eosin-stained sections, or immunohistochemical-stained sections of different thicknesses and number, varying from 3 to 10 μm of 1–3 sections. The extraction was done using the kit "QIAamp® DNA FFPE Tissue" (Qiagen®) according to the manufacturer instructions with some minor modifications.

MSI Analysis

BAT26 and BAT25 microsatellite markers were used mainly. Polymerase chain reaction (PCR) was done using the extracted DNA in a final volume of 6 μl, 1X Buffer, dNTPs (250 μM each), primers (0.1 μM each), MgCl2 (3.75 mM), and 1 unit of Taq polymerase (Roche). For PCR reaction, the first step was denaturation at 95 °C for 5 min before the addition of the polymerase, then 35 cycles (30 s at 95 °C, 30 s at the annealing temperature and 30 s at 72 °C), and 10-min final extension at 72 °C.

The PCR products were marked with fluorochrome. In which, the primer of BAT 25 was labeled with fluorochrome NED, while the primer of BAT 26 was labeled with FAM. Each sample was added to formamide and standard (ROX 500) and then transferred to capillary automated sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

The analysis was conducted using the program GenMapper V4.0 for data processing. Electropherograms can recognize MSI CRC by the presence of new shorter peaks due to the shortening of the adenine repeats in cancer cells [4]. In some instances, the electropherograms were not definite to provide a clear MSI results for one or more of the used MSI markers. Such results were considered non-evaluable.

Table 3 Immunohistochemical patterns of dMMR CRCs

Immunohistochemical patterns of dMMR cases	*n*	%
MLH1/PMS2 negative	2	50
MSH2/MSH6 negative	2	50
Total	4	100

Results

In this study, we aimed to assess the MMR protein abnormalities in Sudanese CRC patients. MMR proteins were assessed by IHC in 42 Sudanese CRC patients.

Twenty-five (59.5%) of the study population were males, and 17 (40.4%) were females. Male to female ratio was 1.47:1. The ages of the study population were between 20 and 85 years (the age of 4 patients was not provided), and the mean age was 56.1 year. The majority of the study population were among age groups 50–59 and 60–69 years representing 7 (18.4%) for each, followed by 70–79 and 80–89 constituting 6 (15.7%) for each, followed by age groups 20–29, 30–39, and 40–49 representing 4 (10.5%) for each. Furthermore, the ages of 12 (31.5%) of the CRC patients were less than 50 years.

Regarding the grade of differentiation (assessed in sections stained by either hematoxylin & eosin or IHC and obtained from one block of each case), 11 (26.1%) of the cases were poorly differentiated and classified as high grade (HG), comparing to 31 (73.8%) well/moderately differentiated classified as low grade (LG).

Table 1 and Figs. 1 and 2 (for selected cases) show the MMR protein expression by IHC and MSI status. Of the 42 included cases, 34 (80.95%) were MMR protein positive for MLH1, PMS2, MSH2, and MSH6, 4 (9.5%) MMR protein negative (2 (4.76%) MLH1 and PMS2 negative and 2 (4.76%) MSH2 and MSH6 negative), 3 (7.14%) MSH2 inadequate and positive for the rest, and 1 (2.38%) MSH6 inadequate and positive for the others.

Regarding MSI results, the three cases that were MSH2 inadequate and positive for the rest by IHC showed stable results with both BAT 25 and 26. The two cases that showed MSH2 and MSH6 negative results were unstable with both BAT 25 and 26. Of the two cases that were MLH1 and PMS2 negative, one of them showed non-evaluable results with BAT 25 and 26, while the other case was unstable with BAT 26 and not evaluable with BAT 25. The case that was MSH6 inadequate and positive for the others showed stable results with both BAT 25 and 26.

Table 4 Description of study population by MMR protein expression and gender

MMR protein status	Male		Female		Total
	n	%	*n*	%	
MMR protein −	4	16	0	0	4
MMR protein +/insufficient evidences of MMR protein −	21	84	17	100	38
Total	25	100	17	100	42

Table 5 Description of study population by MMR protein expression and grade of differentiation

Grade of differentiation	MMR protein −		MMR protein +/insufficient evidences of MMR protein −	
	n	%	*n*	%
Poorly differentiated (HG)	0	0	11	29*
Well/moderately differentiated (LG)	4	100	27	71
Total	4	100	38	100

*The value was rounded to the nearest tenth

Concerning the MMR status based on the immunohistochemical results, MMR protein-negative cases (which considered dMMR) were detected among 4 (9.5%) of the included cases as shown in Table 2.

Immunohistochemical patterns of these four dMMR cases were 2 (50%) MLH1/PMS2 negative and 2 (50%) MSH2/MSH6 negative, as shown in Table 3. All MMR protein-negative cases were among males. In which 4 (16%) of the males were MMR protein negative while no MMR protein-negative cases were detected among females as shown in Table 4.

All MMR protein-negative cases were LG, which refer to well/moderately differentiated CRC, as shown in Table 5.

Discussion

In this study, we aimed to assess the DNA MMR protein abnormalities among Sudanese CRC patients. The total number of included cases was 42.

Sections were cut and assessed by IHC for the expression of 4 MMR proteins (*MLH1*, *MSH2*, MSH6, and *PMS2*). Furthermore, MSI analysis using mainly BAT 25 and 26 was performed for cases that showed negative or inadequate staining results by IHC.

In the current study, 4 (9.5%) MMR protein-negative CRC cases were detected by IHC. Different studies showed higher percentages of MMR protein abnormalities by IHC compared to our study. Khoo et al. (2013) found that the percentage of abnormal IHC staining for MLH1, MSH2, and MSH6 expression was (14.4%) among 298 Malaysian CRC cases [15]. Furthermore, our finding in the current study is less than what was detected by Lanza et al. (2006) in Ferrara, Italy. In their study, 718 colorectal adenocarcinoma patients were studied. Of whom, 114 (15.9%) were found with abnormal expression of MMR protein. Of these 114 cases with abnormal expression, 18 were MSH2 negative and 96 were MLH1 negative [16].

Moreover, Jensen et al. (2008) in Denmark found that 39 (14.9%) out of 262 CRC cases were MMR deficient by IHC or MSI analysis [17]. However, other studies found less percentages compared to our finding. A study from Spain by Jover et al. (2004) reported that the percentage of MMR-defective CRC cases was 7.6%. In their study, 172 CRC cases were studied. MSI analysis was performed by BAT 26, while IHC was performed using antibodies against MLH1 and MSH2 proteins. MSI was detected in 13 (7.6%) cases and all exhibited loss of expression of one of the two examined proteins (11 MLH1 negative and 2 MSH2 negative) [18]. De Jesus-Monge et al. (2010) examined the MMR protein expression among Hispanics from Puerto Rico. In their study, MLH1

Table 6 A summary of some studies regarding MMR protein expression examined by IHC

Authors and year	Country	Number of included cases	MMR protein-negative CRCs		Examined MMR proteins
			n	%	
Molaei et al. (2010) [22]	Iran	343	48	14	*MLH1*, *MSH2*, *PMS2*, and *MSH6*
Erdamar et al. (2007) [23]	Turkey	74	34	45.9	*MLH1* and *MSH2*
Coggins et al. (2005) [21]	England	732	57	7.78	*MLH1* and *MSH2*
Wright and Stewart (2003) [24]	New Zealand	458	89	19.4	*MLH1* and *MSH2*
Jin et al. (2008) [25]	China	146	32	21.9	*MLH1*, *MSH2*, and *MSH6*
Ashktorab et al. (2008) [26]	Oman	49	8	16.3	*MLH1* and *MSH2*
Kheirelseid et al. (2013) [20]	Ireland	33	3	9.09	*MLH1*, *MSH2*, and *PMS2*
Lindor et al. (2002) [13]	USA	1144	326	28.4	*MLH1* and *MSH2*

and MSH2 protein were examined by IHC in 164 CRC cases. Of these cases, 7 (4.3%) were negative [19]. Table 6 shows a summary of some other studies from different countries including the percentage of MMR protein-negative cases assessed by IHC. Most of these studies reported higher percentages of MMR protein-negative CRC cases compared to our study. However, the result obtained by Kheirelseid et al. (2013) was almost near to our finding [20], while the finding of Coggins et al. (2005) was less than our finding [21].

Furthermore, another study from Ghana, which is one of the West African countries, showed a high percentage (41%) of microsatellite instability-high (MSI-H) CRC cases. Kria Ben Mahmoud et al. (2012) examined the MSI status among 150 CRCs from Tunisians patients. They found that 15% of these CRCs were MSI-H [27].

Regarding the grade of differentiation of MMR protein-negative CRC cases in the current study, all these cases were LG, which refers to well/moderately differentiated CRC. However, this finding requires additional evaluation using a larger sample size to find out the accurate association between MMR protein-negative cases and the grade of differentiation in Sudanese patients. Nevertheless, this observation opposes other published data. Lanza et al. (2006) found that MMR protein-negative CRCs are characterized by poor differentiation [16]. Furthermore, Khoo et al. (2013) reported that CRC cases with MMR defect were frequently found poorly differentiated compared to those cases with no defect in MMR [15].

In our study, the results of IHC in several sections were inadequate to allow a good assessment. In such instances, the immunohistochemical analysis was repeated using successive antigen retrieval by increasing the time of this step. After repetition, a number of sections were evaluable. However, the immunohistochemical staining remained inadequate to provide a good evaluation in other sections. Raskin et al. (2013) found some difficulties in their study regarding the analysis of MMR proteins by IHC, which resulted in the presence of a limited number of CRC cases with sufficient immunohistochemical staining. They attributed these difficulties in achieving a good immunohistochemical staining to the prolonged time of fixation, which makes the tissue excessively dehydrated [28].

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Approval was obtained from the Ministry of Health—Khartoum state—Directorate of Research.

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