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Molecular Detection of Mononucleotide Biomarkers of Microsatellite Instability in Sporadic Colorectal Carcinoma Patients with Clinicopathological Correlation

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Abstract

Objectives: To identify the frequency and types of microsatellite instability among a group of sporadic CRC patients and to correlate the findings with clinicopathological characteristics.

Methods: During an 8-month period, all patients with sporadic CRC who attended to two teaching hospitals in Baghdad, Iraq were recruited to this cross-sectional study regardless of age, sex, ethnicity, or tumor characteristics. Demographic, clinical, and histopathological features were recorded. DNA was extracted from FFPE-blocks of the resected tumors and normal tissues. PCR amplification of five microsatellite mononucleotide repeat loci (*BAT25, BAT26, NR-21,* NR-24, and *MONO-27*) and 2 pentanucleotide repeat control markers (Penta C and Penta D) was performed to determine the MSI status. Capillary electrophoresis and Genetic Analyzer 3500 (Applied Biosystem, Japan) were used to separate and examine the products. Data were analyzed by Genescan software (Promega, USA). Instability of two or more loci is considered MSI-H.

Result: In this study, ages of the 45 recruited patients ranged between 20–80 years, with a mean ± SD of 55 ± 12.3 years; of them, 31(68.9%) were ≥50 years; 25 (55.6%) were males. Rectal bleeding was the most frequent presenting feature [22 (48.9%)] patients; 23 (51.1%) of CRCs were located at recto-sigmoid region, 29 (64.4%) were T3 tumors, 34(75.5%) were non-mucinous adenocarcinoma, 39(86.7%) were moderately differentiated, 17 (37.8%) patients had stage III tumors; and 25 (55.5%) had lymphovascular invasion. MSI-H was seen in 5/45 (11.1%) patients; 3(60%) of them were ≥50 years, 4(80%) were males, 3(60%) were smokers, 2 (40%) presented with intestinal obstruction and altered bowel habits each; 4(80%) had T3 tumors, 3(60%) had mucinous adenocarcinomas [$P = 0.004$], 2(40%) had stage II tumor and stage III each.

Conclusion: The frequency of MSI-H among the recruited patients with CRC was 5/45 (11.1%) and it was significantly associated with mucinous adenocarcinoma subtype. *NR-24* and *NR-21* were the most prevalent instable markers.

Keywords: Colorectal neoplasms, adenocarcinoma, microsatellite instability, polymerase chain reaction, iraq

Introduction

Colorectal cancer (CRC) is the second most prevalent cancer in women following breast cancer, and the third most frequent cancer in men following lung and prostate cancers.[1–3] Around the world, both incidence and mortality rates of CRC had significantly risen.[4] Rectal and colon cancer deaths are predicted by 2035 to reach 60% and 71.5%, respectively.[5] CRC is a heterogeneous disease caused by the interaction of environmental and genetic factors that turn healthy colonic and rectal cells into invasive cancer.[6] Several factors may increase the risk of developing CRC including age, environmental factors such as unhealthy diet, alcohol, obesity, smoking, digestive disorders (Crohn's disease and ulcerative colitis), and genetic factors such as familiar adenomatous polyposis (FAP), and hereditary non-polyposis colorectal cancer (HNPCC).[7–9] Chromosome instability, microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) are the three main types of genomic instability seen in CRC.[10] MSI, seen in 15% of CRC cases, can develop due to genetic or epigenetic changes. Genetic modification includes germline mutations in the *MMR* genes (*MLH1, MSH 2, MSH6,* and *PMS2*), which typically manifests in inherited CRCs e.g. HNPCC – Lynch syndrome. The second process involves an epigenetic alteration that results in hypermethylation of the *MLH1* gene's promoter region, which causes an accumulation of DNA mutations and the production of the mutant MMR protein. Three primary forms of colorectal cancer can be distinguished by MSI testing: MSI-high (MSI-H), MSI-low (SI-L) and MSI-stable (MSS).[11] MSI-H has particular clinical and prognostic significance for CRC.[12] Compared to other types of CRCs, MSI-H tumors exhibit less metastasis and respond better to immunotherapy than chemotherapy, particularly 5FU.[13] The aims of this study were to identify the frequency and types and of MSI among a group of sporadic CRC patients and to correlate MSI status with demographic and clinicopathological characteristics.

Methods

This is a cross-sectional study that recruited 45 patients with sporadic colorectal adenocarcinoma from two major teaching hospitals in Baghdad, Iraq, namely: Baghdad Teaching Hospital and Gastroenterology & Hepatology Teaching Hospital, Medical City between September 2021 and April 2022. All patients, regardless of age, sex, race, residence, tumor characteristics, who underwent surgical resection of their tumors, were enrolled to this study. The diagnosis and histopathologic findings were reviewed and confirmed by a second histopathologist. For each patient, the basic demographic, clinical and histopathologic findings were recorded from hospital records. Two slices were cut from the formalin-fixed, paraffin-embedded (FFPE) tissue blocks: one from the tumor with the highest concentration of tumor cells and the other from nearby

non-tumorous tissue (safe margin). DNA was extracted using the QIAamp DNA FFPE Tissue Kit® (50) by (QIAGEN / German), according to manufacturer's instructions. Five single nucleotide repeat loci (*BAT25, BAT26, NR-21, NR-24, and MONO-27*) and two pentanucleotide repeat control markers (Penta C and Penta D) were tested. The mononucleotide markers were used for MSI determination while the pentanucleotide markers were used for specimen identity i.e. the normal and the tumor specimens originate from the same individual.

The MSI Analysis System, Version 1.2 kit (Promega, USA), which is a fluorescent PCR-based assay, was used for detection of microsatellite instability. PCR amplification was carried out in the following steps: initial denaturation at 95°C for 11 min; 10 cycles at 94°C for 30 sec., 58°C for 30 sec., and 70°C for one min., followed by 20 cycles at 90°C for 30 sec., 58 °C for 30 sec., and 70°C for one min., and finally, an extension step at 60°C for 30 minutes. The PCR products were separated by capillary electrophoresis (CE) using Genetic Analyzer 3500 (Applied Biosystem, Japan). The data were exported and analyzed with Genescan software (Promega, USA) to determine MSI status.

Operational Definitions

MSI-High (MSI-H) is used when ≥2 of the 5 markers exhibit instability, MSI-Low (MSI-L) is used when only one of the five markers exhibits instability, and MS-Stable (MSS) is used when none of the markers exhibit instability.[11]

For the purpose of this study, both MSI-L and MSS were considered in one category, referred to as MSS, in accordance to ESMO guidelines as no clinical differences were observed between MSS and MSI-L tumors.[14]

Statistical Analysis

The computer software IBM-SPSS ver. 26 was used for statistical analysis. Qualitative data were described by frequency and percentage. Fisher Exact Test was used to determine if there are non-random associations between two categorical variables of small numbers. A *P*-value <0.05 was considered statistically significant.

Results

Patients' Characteristics

Ages of the 45 recruited patients ranged between 20–80 years, with a mean ± SD of 55 ± 12.3 years. Patients were divided into two age groups: <50 years [14 (31.1%)], and ≥50 years old [31 (68.9%)]. CRC was more frequently observed in males 25 (55.6%) than in females 20 (44.4%), with an M:F ratio of 1.25:1. The most frequent presenting feature was rectal bleeding as seen in 22 (48.9%) patients. Twenty (44.4%) patients were smokers, while only 2 (4.4%) drink alcohol. Twenty-three (51.1%) of CRCs were located at recto-sigmoid region, 29 (64.4%) were T3 tumors, 34 (75.5%) were non-mucinous adenocarcinoma, 39 (86.7%) were moderately differentiated, 17 (37.8%) patients had stage III while 14 (31.1%) had stage IV tumors; and 25 (55.5%) had lymphovascular invasion; Table 1.

Table 1. **Clinicopathological characteristics and MSI status of the 45 recruited CRC patients**

Parameter		Total No.	MSS No. (%)	MSI-H No. (%)	*P*- value
Age	<50	14	12(30 %)	2(40.0%)	0.639
	> = 50	31	28(70 %)	3(60%)	
Sex	Male	25	21(52.5%)	4(80%)	0.362
	Female	20	19(47.5%)	1(20%)	
Smoking	Non-smoker	25	23(57.5%)	2(40%)	0.642
	Smoker	20	17(42.5%)	3(60%)	
Alcohol consumption	No	43	38(95%)	5(100%)	1.0
	Yes	2	2(5%)	0(0%)	
Presentation	Rectal bleeding	22	22(55%)	0(0%)	0.15
	intestinal obstruction	8	6(15%)	2(40%)	
	Abdominal pain	6	6(15%)	0(0%)	
	Altered bowel habits	5	3(7.5%)	2(40%)	
	Anemia	2	2(5%)	0(0%)	
	Constipation	2	1(2.5%)	1(20%)	
Tumor location	Right-sided	11	9(22.5%)	2(40%)	0.255
	Lt-sided colon	11	9(22.5%)	2(40%)	
	Recto-sigmoid	23	22(55%)	1(20%)	
Histological subtype of adenocarcinoma	Non-mucinous	34	33(82.5%)	1(20%)	0.004*
	Mucinous	10	7(17.5%)	3(60%)	
	Signet ring	1	0(0%)	1(20%)	
L.V. invasion	Present	25	23(57.5%)	2(40%)	0.642
	Absent	20	17(42.5%)	3(60%)	
Tumor grade	Well	2	1(2.5%)	1(20%)	0.125
	Moderately	39	36(90%)	3(60%)	
	Poorly	4	3(7.5%)	1(20%)	
TNM-T stage	T1	1	1(2.5%)	0(0%)	0.664
	T2	6	5(12.5%)	1(20%)	
	T3	29	25(62.5%)	4(80%)	
	T4	9	9(22.5%)	0(0%)	
TNM-N stage	N0	15	13(32.5%)	2(40%)	0.751
	N1	16	15(37.5%)	1(20%)	
	N2	13	11(27.5%)	2(40%)	
	N3	1	1(2.5%)	0(0%)	
TNM-M stage	M0	30	26(65%)	4(80%)	0.651
	M1	15	14(35%)	1(20%)	

L.V. = Lymphovascular; TNM=Tumor, Node, Metastasis Staging System
* = Statistically significant (Fischer exact test)

MSI Status Results

In this cross-sectional study, 5/45 (11.1%) patients had MSI-H. Three (60%) of them were ≥50 years, 4(80%) were males, 3(60%) were smokers, 2 (40%) presented with intestinal obstruction and 2(40%) with altered bowel habits. Regarding the tumor characteristics, 4/5 (80%) had T3 tumors, 3(60%) had mucinous adenocarcinomas [$P = 0.004$], and 3(60%) with moderately-differentiated adenocarcinoma. Two (40%) had stage II tumor and 2(40%) with stage III, 2(40%) with lymphovascular invasion; Table 2.

Regarding the gene frequency of MSI panel, *NR-24* showed the highest percentage as detected in 4 (80%) patients, followed by *NR-21* that was detected in 3/5 (60%)], while all 3 remaining markers were detected in 2 (40%) patients each. Two (40%) patients showed the combination of *BAT26, NR-21,* and *NR-24* as the instable markers; Table 3.

Discussion

Various chromosomal and epigenetic alterations are seen in colorectal cancers. Repeated sequences known as microsatellites are highly susceptible to misalignment during replication, which raises their mutation rate to 1.2×10^{-3} [15] and causes MSI. Recent studies indicate that individuals with CRC who display MSI have a favourable prognosis, but is usually absent in 80–85% of CRCs.[16] MSI-H CRC is associated with immune checkpoint upregulation such as programmed cell death receptor (PD-1) and programmed death ligand (PDL-1), the expression of which is usually used as an indication to use immunotherapeutic PD-1 blocking agents e.g. pembrolizumab (Keytruda) and dostarlimab (Jemperli), the former is the first-line therapeutic agent in advanced metastatic or inoperable CRCs.[17]

MSI testing has remained an excellent therapeutic and prognostic tool for CRCs.[18] Three methods can be used to detect the deficient mismatch repair (dMMR) or MSI: immunohistochemistry (IHC), polymerase chain reaction (PCR) followed by capillary electrophoresis (CE), or next generation sequencing (NGS). There is a sufficiently compelling evidence to warrant the use of PCR as the most accurate and efficient method of MSI detection and is considered the gold standard test.[19] MSI testing is performed by comparing the length of the amplified product of the target microsatellite in cancer tissues with those of matched normal tissues via capillary electrophoresis.[19]

Only a few regional studies have reported MSI status among CRC patients, like UAE,[20] Egypt,[21] North part of Iran,[22] Turkey,[23] and India.[24] Assessment of MSI in different solid tumors, including CRCs, is still under research and remains out of the daily routine practice in many countries.

In this study, the mean age of the 45 CRC patients was 55 ± 12.3 years. These findings were similar to many locoregional studies from Baghdad-Iraq,[4] Turkey.[25] and Saudi Arabia.[26] The study also showed male predominance [25 (55.6%)]. These results are comparable to results of other studies from Al-Najaf city in Iraq[27] and Saudi Arabia.[28] The majority of CRCs [23 (51.1%)] were located in the rectosigmoid region, which is similar to a regional study from Saudi Arabia.[26] On histological examination, 34 (75.5%) CRCs were non-mucinous adenocarcinoma, and 39 (86.7%) were moderately differentiated; this is similar to another regional study from Northern Saudi Arabia[29] and Jordan.[30]

In this study, 5/45 (11%) sporadic CRCs showed MSI-H, which is less than the percentage reported in the northern parts of Iran (22.9%)[22] and Egypt (57.9%)[21] but comparable to results from different countries e.g. North America, Europe, and East Asia.[31]

For the 5 MSI-H patients, age ≥50 years was more frequently seen than <50 years, similar to another study.[32] There is a male predominance in both MSI-H and MSS groups,

Table 2. **Clinicopathological features of the five MSI-H CRC patients**

Parameter		No. of MSI-H (%) ($n = 5$)
Age	<50	2(40.0%)
	> = 50	3(60%)
Sex	Male	4(80%)
	Female	1(20%)
Smoking	Non-smoker	2(40%)
	Smoker	3(60%)
Alcohol consumption	No	5(100%)
	Yes	0(0%)
Presentation	intestinal obstruction	2(40%)
	Altered bowel habits	2(40%)
	Constipation	1(20%)
Tumor location	Right-sided	2(40%)
	Lt-sided colon	2(40%)
	Recto-sigmoid	1(20%)
Histological subtype of adenocarcinoma	Non-mucinous	1(20%)
	Mucinous	3(60%)
	Signet ring	1(20%)
L.V. invasion	Present	2(40%)
	Absent	3(60%)
Tumor grade	Well	1(20%)
	Moderately	3(60%)
	Poorly	1(20%)
TNM-T stage	T1	0(0%)
	T2	1(20%)
	T3	4(80%)
TNM-N stage	N0	2(40%)
	N1	1(20%)
	N2	2(40%)
TNM-M stage	M0	4(80%)
	M1	1(20%)
Gene frequency of MSI	*NR-24*	4(80%)
	NR-21	3(60%)
	BAT-26	2(40%)
	BAT-25	2(40%)
	MONO-27	2(40%)

L.V. =Lymphovascular; TNM=Tumor, Node, Metastasis Staging System

Table 3. **Types, frequencies, and patterns of the detected mononucleotide markers among 5 MSI-H CRC patients**

Mononucleotide marker	P1	P2	P3	P4	P5	Gene frequency
BAT25	+				+	2/5(40%)
BAT26		+	+			2/5(40%)
NR-21	+	+	+			3/5(60%)
NR-24		+	+	+	+	4/5(80%)
MONO-27	+			+		2/5(40%)
Total	3(60%)	3(60%)	3(60%)	2(40%)	2(40%)	

contrary to what was reported in another study,[31] but is comparable to findings from Iran and India and other parts of the world.[22, 24] They found a higher incidence of CRC in males compared to females and stated that young women (18–44 years) with CRC have a better survival outcome compared to men of the same age or compared to older women.[33] In addition, MSI behaves in a sex-dependent manner, where the mutation rate is five times higher in males than in females.[34]

Regarding smoking, 60% of MSI patients and only 42.5% of MSS patients smoke, with no statistically significant difference. This is consistent with another study conducted from university of Utah, USA.[35]

Statistical analysis showed no significant difference between MSI-H and MSS groups of CRC patients in terms of presenting features, tumor location, tumor grade, tumor stage, and lymphovascular invasion. Tumor type, however, showed a statistical significance in favour of mucinous adenocarcinomas in MSI-H; this latter finding is consistent with other studies.[36–37]

The small sample size of MSI-H in this study averts making comparisons and conclusions to these findings, awaiting larger-scale future studies on this topic. Generally, these findings are similar to other studies;[38–39] the advanced presentation is common in our population due to lack of a national screening programs.

NR-24 marker was the most frequently instable marker among the MSI-H patients, being reported in 4(80%) patients, followed by *NR-21*. A previous Iraqi study using a different panel of MSI genes reported that the most frequent instable marker among the MSI-H patients was *BAT-25* (48.93%) followed by *BAT-26* (44.68%).[37]

The type of tested MSI genes and the method used has changed with time, starting from dinucleotide markers that showed inconsistencies in results, to mononucleotide markers requiring comparison to normal tissues, to the more recent use of MSI kits e.g. Idylla MSI kit that is quick, fully automated, does not require normal tissues, and is as sensitive and specific as the currently used kits e.g. Promega MSI Analysis System version 1.2.[40]

A novel panel of seven monomorphic biomarkers (*ACVR2A, BTBD7, DIDO1, MRE11, RYR3, SEC31A,* and *SULF2*) is currently recommended to be used in a diagnostic test to identify the MSI status in CRC.[40]

The recent inclusion of MSI assessment in the investigation of newly diagnosed CRC patients in the specialized centers, would allow a clearer vision to the true incidence of MSI-H and characteristics among different population groups in Iraq in the near future.

Conclusion

The clinical benefits of detecting MSI status accurately and affordably are clear. The frequency of MSI in sporadic CRC patients in this study was 5/45 (11.1%) and was significantly associated with mucinous type. *NR-24* and *NR-21* were the most prevalent instable markers. These findings help physicians and oncologists decide whether to give immunotherapy or chemotherapy, and to predict prognosis based on result of MSI testing.

Declarations

Ethics Approval and Consent to Participate

- This study was approved by the ethics research committee at the Dept. of Pathology and Forensic Medicine, College of Medicine, University of Baghdad (Issue No. 200 at November, 10th, 2021).

Availability of Data and Material

- The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

- The authors declare that they have no competing interests.

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