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The Correlation Between Microsatellite Instability and the Features of Sporadic Colorectal Cancer in Sample of Iraqi Patients

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ABSTRACT

Microsatillite instability (MSI) is a mutational signature found in colorectal carcinoma (CRC), it represents about 12% in non inherited CRC. We aimed in this study to correlate between MSI and sporadic CRC Iraqi patients. A total of 47 patients with colorectal carcinoma were enrolled in this study, among these patients 26 (55%) males and 21(45%) females, with a range age from 37 years to 72 years, mean age (54.5 year). Microsatellite marker amplifications were performed as singleplex PCR reactions. Results were divided into three groups, group 1 with MSI $_{high}$ were constituted a frequency of 17 (36.17 %), and group 2 with MSS (16) (34.04 %) and group 3 with MSI $_{low}$ the frequency(14) (29.78 %). MSI $_{high}$ was expressed in high percentage (38.09%) in women comparted with male(34.61%),while its relation to age, results indicated that age group (≤ 50 years) showed high percentage (37.93%) compated with Group 1(≥50 years) (33.33%) and the realtion of MSI with morphological feature of the specimens showed that poorly diffrrentiated CRC specimens showed the highly percentage (47.07%) when comparted with well and moderatly differntiated cases. The mucinous type of CRC reveled 100% pecentage for MSI comparted with nonmucinous (27.65%) with siganficant difference. High frequancy of MSI was shown in right site (52.94%) with significant difference compared with MSI L and MSS. In conclusion: This study showed that there were a close association between MSI with female patients, old patients, and right site, mucinous and poorly differentiated CRC.

Indexing terms/Keywords

CRC, MSI, PCR.

Academic Discipline And Sub-Disciplines

Immunogenetics.

Subject Classification

Tumor Immunology.

Type (Method/Approach)

PCR-Experimental.

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INTRODUCTION

Colorectal cancer (CRC), is one of the most frequent malignancies in the Western world. Worldwide, approximately 1, 2 million people developed colorectal cancer in 2008 and the disease related mortality was about 36% (1) (2) The disease affects slightly more men than women and sporadic colon cancer is considered to be a disease of the elderly with a median age at diagnosis of 70 years (3).

Microsatellites are repeated DNA sequences, usually 1 to 10 nucleotides long, present throughout the genome. Instability is mostly characterized by single base-pair insertions or deletions in these repeat loci, causing widespread genomic instability due to the failure of the cell's mismatch repair (MMR) mechanism (4) (5) .A recognizable clinicopathological profile of MSI tumors has been established from clinical studies. CRC displaying MSI tend to be right sided and diagnosed at lower pathological stages compared with MSS cancers. The age distribution of MSI cancers follows a u shaped distribution, and sporadic MSI cases are generally diagnosed in older patients (>70 years of age) whereas familial cases are younger (<50 years of age)(6). Since many colon cancers demonstrate frame shift mutations at a small percentage of microsatellite repeats, the designation of a colon tumor as showing microsatellite instability depends on the detection of at least two unstable loci out of five from a panel of loci that were selected by a National Cancer Institute consensus conference (4).

MSI is also found in ~80% of adenomas of variable size from HNPCC patients, compared to12-18% of the sporadic tumors (7). MSI tumors are more often located in the proximal colon, poorly differentiated, and of a mucinous, or signet ring, histological type (8).Another common finding in MSI CRC is the presence of tumor-infiltrating T cells (9). MSI tumors have often been associated with a better patient prognosis compared with MSS (1). Three classes of MSI have been defined: MSI-H (MSI-high), MSI-L (MSI-low) and MSS (microsatellite stable) that are distinguished by the degree of instability found in a panel of five reference markers. The panel recommended by the National Cancer Institute consists of two mononucleotide (BAT25 and BAT26) and three dinucleotide (D5S346, D2S123 and D17S250) repeating units. MSI-H tumors exhibit instability in two or more markers whilst MSI-L tumors have instability in only one marker. Tumors lacking apparent instability can, however, be either MSI-L or MSS and so may require the analysis of additional markers for confirmation (4). **This study aims to identify for the first time in Iraq, types of MSI in sample of CRC Iraqi patients.**

Aterial And Methods

Specimens, were collected from GIT center, Baghdad hospital and private hospitals. In the period from 1-4-2013 to 1-2-2014, which more than three biopsies were obtained from grossly tumor areas, and surgery specimens obtain after surgery tumor removal both specimens from surgery and biopsies were fixed with 10% buffered formalinized saline, for preparation the paraffin embedded tissue blocks to histological molecular diagnostic methods DNA extraction from FFEP.

Histological evaluation specimens, slides from fixed paraffin embedded tissue blocks were stained with haematoxylin – eosin stain and subsequently evaluated by an experienced pathologist.

DNA extraction from FFPF ,QIAamp DNA FFPE tissue kit, (50) reaction Mineute columns, kits. DNA evaluation by Nano drop For an A260/A280 value of 1.5, the percentage of protein in the DNA preparation, for good PCR-SSP results, DNA is required with an A260/A280 quotient of 1.6 or greater. The sections of tumor tissue should contain more than 50% of neoplastic cells (14) in order to avoid false negatives. Microsatellite marker amplifications are performed as singleplex PCR reactions using DNA from tumor tissue. In accordance to the recommendations by the NCI. The validated five microsatellite panel is tested first. This latter panel is currently adopted by the institutions of the IMPACTS group in a diagnostic setting (6)

PCR PreMix*AccuPower*®

Stability: the powerful technology for convenient and easy to perform DNA amplification. It contains DNA polymerase, dNTPs, a tracking dye and reaction buffer in a premixed format, freeze-dried into a pellet. 25/100 bp Mixed DNA Ladder is specially designed for determining the size of double strand DNA from 25 to 2,000 base pairs. The DNA Ladder consists of 17 double strand DNA fragments ranging in size from 25 to 200 bp in 25 bp increments, the PCR reaction was performed in a final volume of 50 ml, containing:

0.5 µl b-globin both forward primer, reverse primer 30 pmol/ml. 0.3 pmol/µl final, 1 µl of diluted sample DNA, 20 µl mister mix, H2O to volume, Overlay the reaction mixture with 20 ml of mineral oil.

Thermal cycling: 94°C 10" + 5 × (94°C 60", 52°C 60", 72°C 60") + 35 × (94°C 30" 52°C 30" 72°C 30") + 72°C 5'.

Gel visualization: mix 10 ml of PCR product with 2 µl of 6× loading buffer; load on a 2% agarose gel prepared with 1× TBE containing 0.5 mg/ml ethidium bromide. Run at 80 V constant until bromophenol blue reaches 1/2 of the gel. Inspect under a UV source. A single band should be visible in the sample.

Thermal cycling: 94°C 10' + 5 × (94°C 60", 55°C 60", 72°C 60") + 35 × (94°C 30" 55°C 30" 72°C 30") + 72°C 5'.in all genes with same add of PCR reaction of b-globin.

Internal control

Since DNA extracted from FFPE can be variably degraded and may contain PCR inhibitors, we suggest performing a preliminary quality control to test if sample. DNA is suitable for MSI and to determine the optimal quantity for amplification. For this purpose, a 167 bp fragment of the b-globin gene is amplified. Since b-globin gene is present in all the cells (it

never undergoes deletions) and is not polymorphic, it is a suitable target for the control PCR.(15) The size of the amplified fragment (167 bp) reflects the length of the largest PCR product that can be obtained.

Positive control for b-globin: DNA from normal human lymphocytes, 50 ng/ml.(16)

PCR primers

Primers: Lyophilized primers should be dissolved in a small volume of distilled water or to make a concentrated (e.g. 300 pmol/µl) stock solution. Prepare small aliquots of working solutions containing 30 pmol/ml to avoid repeated thawing and freezing.

Marker Name	Genomic Position	Sequences (5¢–3¢)	*T* °m	(bp)
b-globin	11p15.5	F: ACACAACTGTGTTCACTAGC R: GAAAATAGACCAATAGGCAG	53.4 53.9	167

Marker Name	Genomic Position	Sequences (5¢–3¢)	*T* °m	(bp)
D2S123	2p16-2p16	F: ACATTGCTGGAAGTTCTGGC R: CCTTTCTGACTTGGATACCA	62.6 57.5	121–141
D5S346	5q21-5q22	F: ACTCACTCTAGTGATAAATCGGG R: AGCAGATAAGACAGTATTACTAGTT	58.7 52.1	96–122
D17S250	17q11.2-17q12	F: GGAAGAATCAAATAGACAAT R: GCTGGCCATATATATATTTAAACC	50.6 57.2	151–169
BAT25	4q12-4q12	F: TCGCCTCCAAGAATGTAAGT R: TCTGCATTTTAACTATGGCTC	59.7 57.0	124
BAT26	2p16.3-2p16.3	F: TGACTACTTTTGACTTCAGCC R: AACCATTCAACATTTTTAACCC	57. 59.0	121

Statistical methods:

The significance of differences in proportions was analyzed by the chi-square test. Fisher's exact test was used when there was a cell with a number less than 5. Entry of data into the computer and the chi-square and Fisher exact tests were performed with SPSS version 15 and P values equal or less than 0.05 were considered statistically significant.

Results

Sex and age distribution

Total of 47 patients with colorectal carcinoma were collected among of these patients 26(55%) males and 21(45%) females figure (1), with a range age from 37 years to 72 years, mean age (54.5 year), with 1:1.2 ratio between female and male. The patients' age were classified into two group first 50≥ years (38.29%), second group 50≤ years 29 (61.70%) fig. (2).

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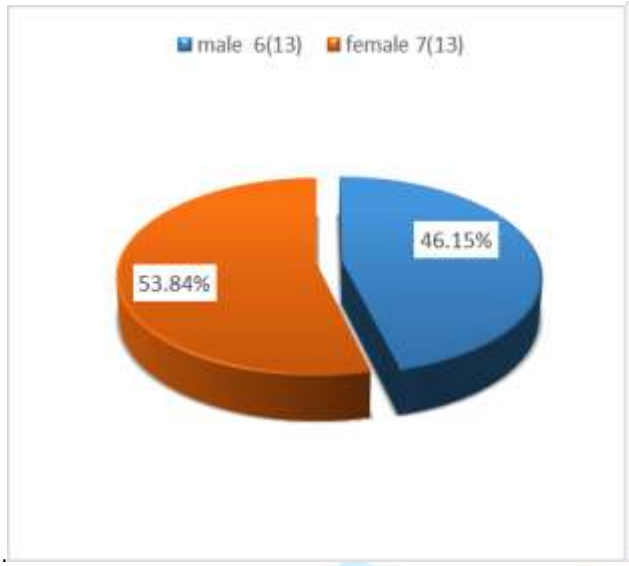

Figure 1. The sex distribution of CRC

Figure 2 the age group frequency in CRC

Morphological differentiation, as regards with grades of colorectal carcinoma, it was observed that (14.89%) cases of well differentiated, (53.19%), moderate differentiated and (53.19%), and (31.91%) poorly differentiated Figure 3.

Site of tumor: Patients with CRC were classified according to the site of tumor location as shown in figure (4), right site consistent (51.06 %) of all cases and patients, with left site (36.17 %) and (12.76 %) in the rectum from all cases.

Figure 3. The morphological distribution of CRC

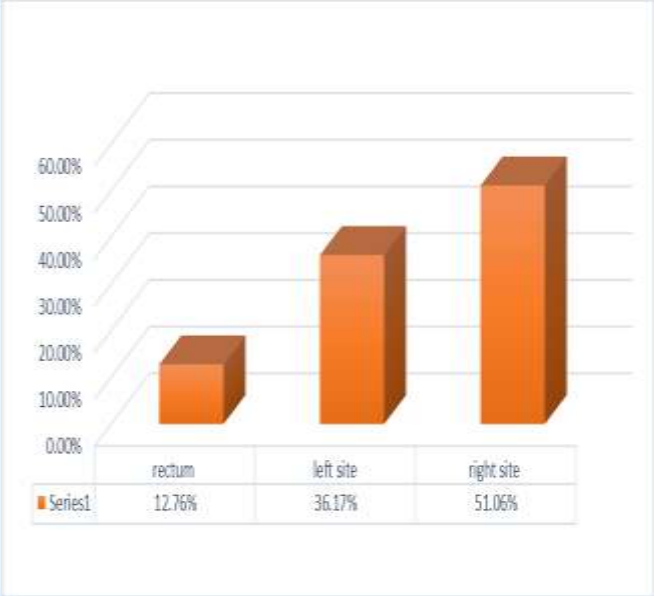

Figure4. Site of tumor distribution in CRC.

MSI Distributions

Microsatellite marker amplifications were performed as singleplex PCR reactions. The results were divided in to three groups, group 1 with MSI $_{high}$ were constituted a frequency of 17 (36.17 %), and group 2 with MSS (16) (34.04 %) and group 3 with MSI $_{low}$ the frequency(14) (29.78 %) figure.5.

As shown in the figure 6, the internal control B-globin was appeared in the region 167bp while D5S346 reviled at the region 122 bp figure 7, BAT26 in 121bp figure 8, BAT25 in 124 bp figure 9, D17S250 in 169bp figure 10 and D2S123 in 141bp figure 11.

MSI Related With Frequency of Microsatellite Panel Genes.

MSI $_{high}$ and MSI $_{low}$ showed close associated with frequency of the microsatellite genes panel in all cases, the highly percentage was show in BAT25 48.93%, while 44.68% in both BAT26, D5S346, 19.14% in D17S250 and 25.23% in D2S123 12(47) table 1.

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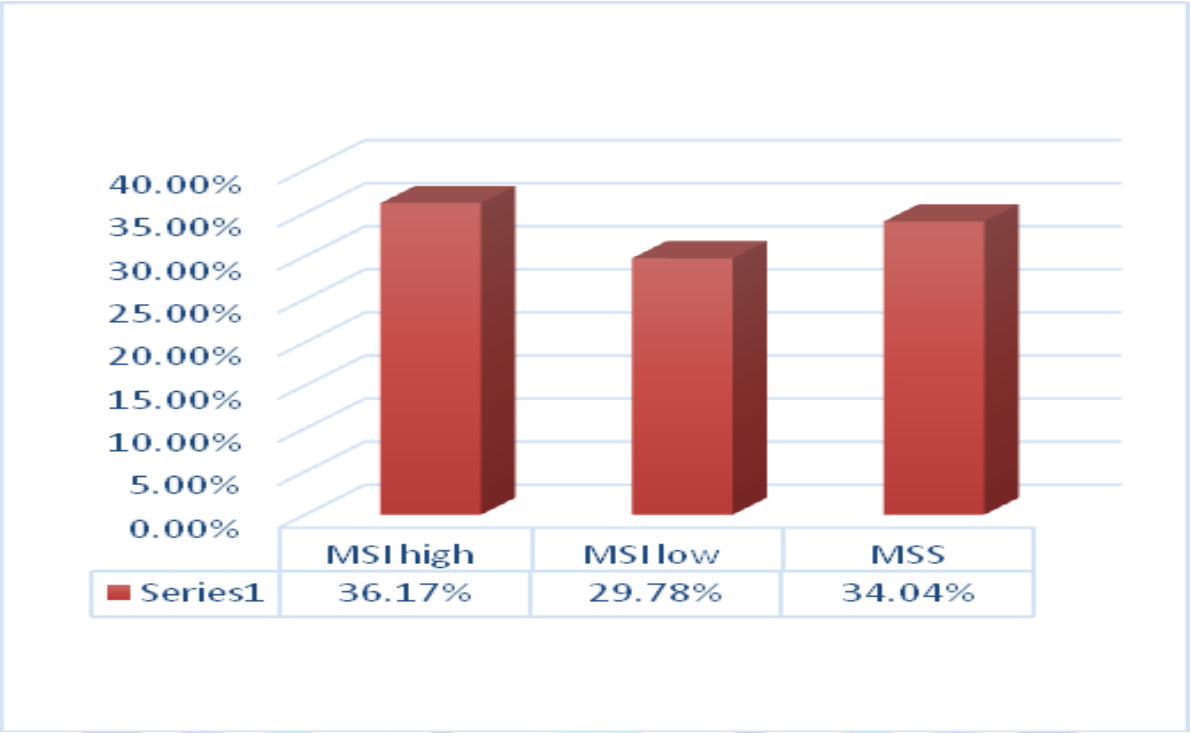

Figure 5. MSI ($_{high, low and stable}$) in groups of CRC patients.

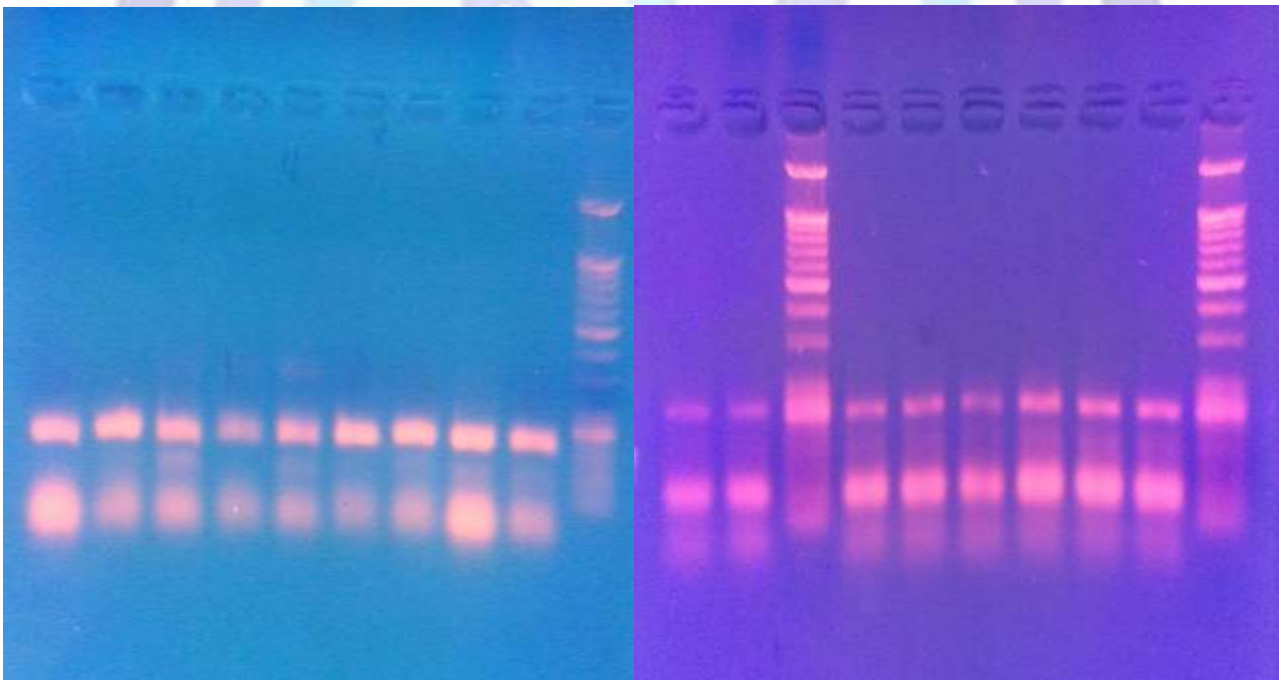

Fig. 7 Gel electrophoresis (2% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for D5S346 was appeared in 122 bp, 25/100bp DNA ladder was used.

Fig. 6 Gel electrophoresis (2% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for b-globin 167bp , 25/100bp. DNA ladder was used

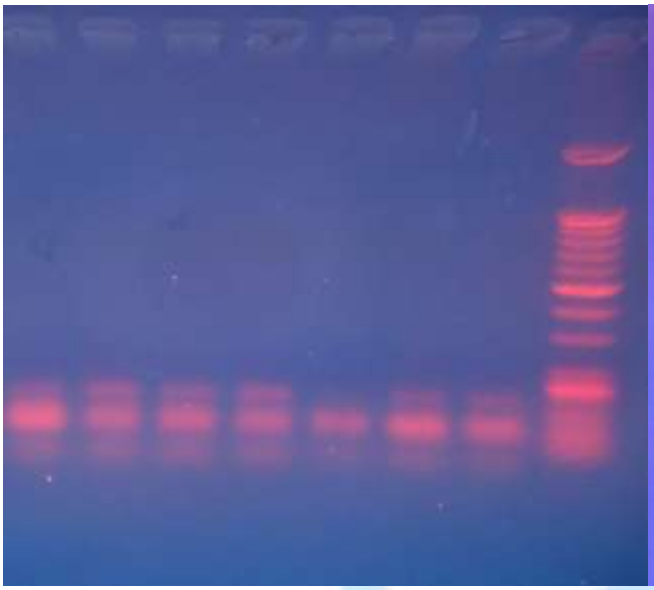

Fig. 9 Gel electrophoresis (2% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for BAT25 was shown in 124bp, 25/100bp DNA ladder was used

.

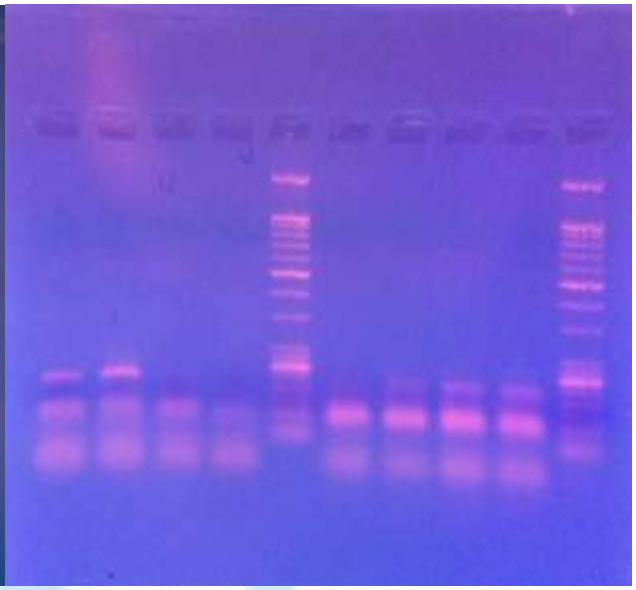

Fig. 8 Gel electrophoresis (2% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for BAT26 was shown in 121bp 25/100bp DNA ladder was used.

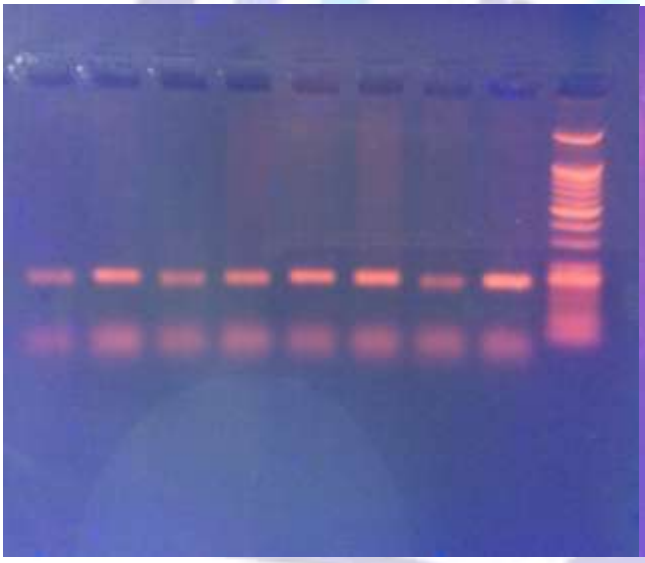

Fig. 11 Gel electrophoresis (2% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for D2S123 was shown in 141bp, 25/100bp DNA ladder was used.

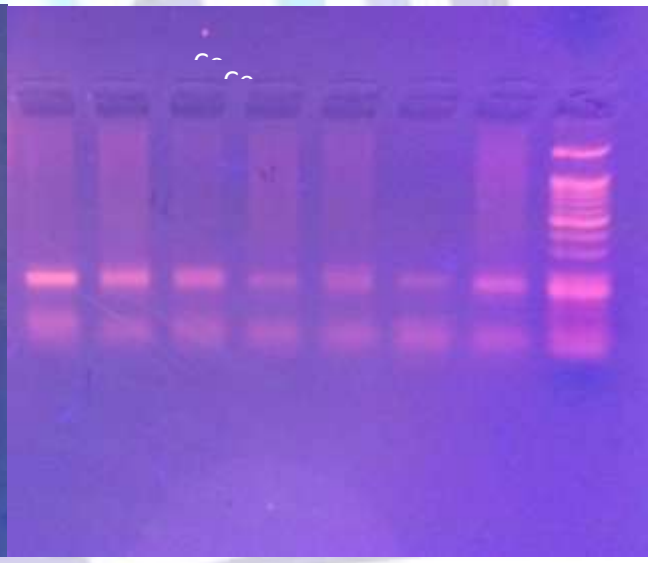

Fig. 10 Gel electrophoresis (2% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for D17S250 was shown in 169, 25/100bp DNA ladder was used

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Table 1 the Genes Fraquancy of Microsetillte Panle in Both MSI $_{High}$ and MSI $_{Low}$

NO	MSI status	Total NO/%	MSI $_{high}$ NO/%	MSI $_{low}$ NO/%	MSS NO/%	All cases	P value
1.	BAT26	21(44.68)	17(36.17)	4(8.51)	26(55.31)	47	P0.0004
2.	BAT25	23(48.93)	17(36.17)	6(12.76)	24(51.06)	47	0.005
3.	D5S346	21(44.68)	17(36.17)	4(8.51)	26(55.31)	47	P0.0004
4.	D17S250	9(19.14)	9(19.14)	0(0)	38(80.85)	47	P0.000
5.	D2S123	12(25.53)	12(25.53)	0(0)	35(74.46)	47	P0.0000

MSI related with sex, MSI $_{high}$ was signifcantly associated with female patients that constut 38.09%, while male patients account to 34.61% while, but male patients showed the high percentage 34.61% for expression of MSI $_{low.}$ Mss was shown to be expressed in high percentage in female patients 38.09% Table 4-2, but wih no signaficant defferent.

Table 2 The Sex Distribution in MSI $_{High,}$ MSI $_{Low}$ and MSS. .

Sex	MSI $_{high}$ NO/%.	MSI $_{low}$ NO/%.	MSS NO/%.	Total number	P value	X^2 Df=2
Male	9(34.61)	9(34.61)	8(30.76)	26	0.944	0.115
Female	8(38.09)	5(23.80)	8(38.09)	21	0.526	1.286
P-	0.732	0.131	1.000	47		Df=2

MSI Associated With Age

Patients were classified according to age in tow groups, Group 1which included patients with age less than 50 years was showed high percentage of expression for MSI $_{low}$ 38.33%, , followed by the MSI $_{high}$ (33.33%). The group 2 high associated with MSI $_{high}$ and MSS with 37.93%, table 4-3 with no significant different.

Table 3 MSI Associated with Age Group Patients of CRC

Age group	MSI $_{high}$ NO/%.	MSI $_{low}$ NO/%.	MSS NO/%.	Total	X2= 1.213
Group 1 ≥50	6(33.33)	7(38.88)	5(27.77)	18	
Group 2 ≤50	11(37.93)	7(24.13)	11(37.93)	29	
	17	14	16	47	P= 0.5452

MSI related with morphological feature , MSI$_{high}$ showed close associated with poorly defferentated cases 47.07%, followed by moderatly defferentated 35.26% and the showed well deffrrentated 17.64% table 4-4 but no signifacant deffernce between grade and MSI $_{high}$ and $_{low}$ and signaficant with MSS.

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Table 4 Morphological Distribuation in MSI $_{High,}$ MSI $_{Low}$ and MSS in CRC.

Grade	MSI $_{high}$ NO/%	MSI $_{low}$ NO/%	MSS NO//%	Total	P value	X^2 Df=2
well	3(17.64)	2(14.28)	2(12.5)	7	0.807	0.429
modreate	6(35.26)	7(50)	12(75)	25	0.156	3.720
poor	8(47.05)	5(35.17)	2(12.5)	15	0.067	5.400
P-	0.187	0.131	0.0001			
X^2 Df=2	3.353	4.071	18.750		0.214 Df=4	5.807

MSI related with muosinous and nonmuosinous CRC, MSI close association with mucinous cases of CRC showed high percentage of expression 100%, comparted with nonmucinous AC with 27.65% Figure 4-12, with highly significant drffrence.

Figure 4-12. MSI related with Muosinous and nonmuosinous CRC.

MSI related with site of tumor, MSI $_{high}$ was highly expressed in patients with right site colon location (52.94%), followed by left site (35.29%) where an rectum showed lower expression site frequency percentage (11.76%), as show in table 4-5, with significant deffrence in both MSI $_{high}$ and MSI $_{low}$.

Table 5 Realted of Site of Tumor With MSI $_{Low,}$ MSI $_{Low\ And}$ MSS

Site	MSI $_{high}$	MSI $_{low}$	MSS	Total	P value	X^2
Right	9(52.94)	7(50)	8(50)	24	0.829	0.375
Left	6(35.29)	6(42.85)	5(31.25)	17	0.916	0.176
Rectum	2(11.76)	1(7.15)	3(18.75)	6	0.472	1.500
Df=2	17	14	16	47		
P-value	0.038	0.036	0.168		Df=4	
X^2	6.529	6.643	3.563		0.893	1.111

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MSI $_{high}$ repeated gene, MSI$_{high}$ showed the expressed 4 genes in this panale high frequncy with 52.94% and follwing by 3 genes with 47.05%, and 5 genes reveiled 17.64% figure 4- 13 with signiffcant defference p = 0.012.

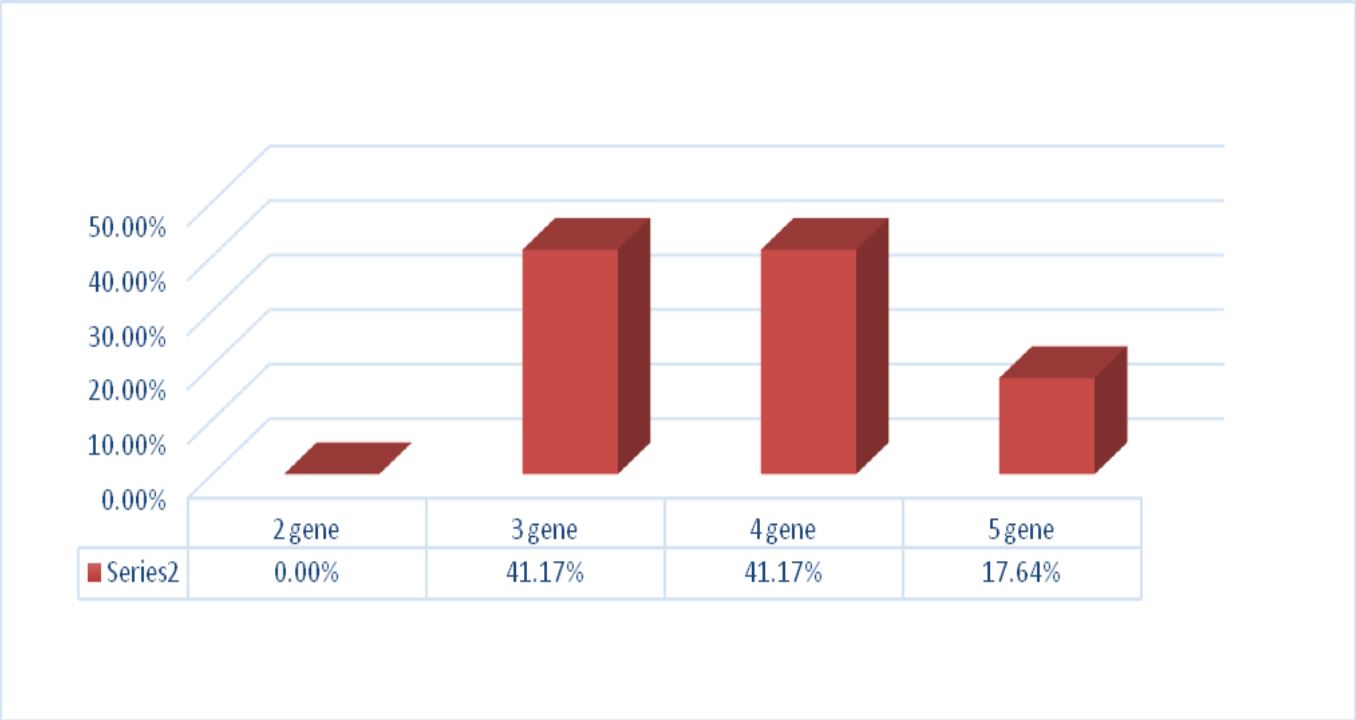

Fig. 13. MSI ^{High} Repeated Genes In Patient Of CRC.

Discussion

MSI the hallmark of hereditary nonpolyposis colorectal cancer (HNPCC), previously known as Lynch syndrome, occurs in approximately 15–25 % of sporadic tumors of the colorectum and other organs as well.(17)

CRC is a heterogeneous disease and MSI may serve as a predictive marker for the response of patients with CRC to specific therapies and as a guide to the selection of optimal therapy.(18)

In this study detection microsatellite colorectal cancer pathway, first MSI test 47 cases of tumor by used five microsatellite marker. The results divided in to three groups first MSI $_{high}$ with percentage (36.17 %), and second group MSI $_{low}$ was recorded in (29.78 %), while the third group MSS was shown in (34.04 %).

This result was agreement with some and different from other, in study of,(29) was demonstrated (43%) microsatellite instability-$_{high}$ (52.94%) were microsatellite stable (MSS) and 2(3.9%) were MSH $_{Low}$.

MSI markers on paraffin-embedded surgically resected tissues sporadic Iranian colorectal cancer patients MSI analysis revealed MSI-H (26.9%), MSI-L (16.4%) and MSS (56.7%) (20) while in study of (21) Microsatellite instability analysis showed (20.8%), MSS 58.4%, MSI-L 20.8%.

While in the study of (22) by used same Bethesda panel of sporadic CRCs in Czech patients the rate of MSI (20.4%), MSI L (3.9 %) and MSS (72.1%). in Italy patient with 10 markers used to detection MSI, the rate of MSI 25.0%, MSI L 29.5 % and MSS 45.5% In study of, (23) .

Some studies was shown less rate of MSI, in study of (24) the prevalence of MSI of sporadic CRC cases was 17%, and that of MSS was (83%), while in study of (25) was show MSI (14.73%), MSI L (8.21%) and MSS (77.05%) but in study of 756 patients from Omani patients were referred to (12.2%) the rate of MSI-H (26) In study (29) the rate of MSI-H (17.97%), MSI-L (12.58%) and MSS (68.53%).

Tumor classification is historically based on various clinical (eg, proximal versus distal), pathological (eg, mucinous versus nonmucinous; well-moderate versus poorly differentiated), and/or molecular features [eg, microsatellite instability status (MSI)-high versus microsatellite stable (MSS) (28) (29) (30)

This study close associated with female, old patients, right site, mucinous and poorly differentiation, because small number of patients some criteria appeared high percentage but not significant. According to the criteria, the results of current study was apperaed, MSI $_{high}$ in the femal high percentage 38.09% comparted with male 34.61%, while the relation with age group the Group 2 ≤ 50 was shown high percentage 37.93% compated with Group 1 ≥50 33.33% and the realted of MSI with morphological feature was show the highly percentage in poor deffrrentated 47.07% comparted with well and moderate. The mucinous MSI was reveled highly 4(4) with 100% pecentage comparted with nonmucinous MSI 13(43) with 27.65% with siganficant defference. The site of tuomr with MSI was appeared high frequancy in right site 52.94% with significant difference compared with MSI L and MSS.

This study agreement with (31) also observed this relationship when analyzed the location of tumors according to MSI status sporadic MSI are common in the proximal colon whereas MSI-L and MSS usually occur in the distal colon and the rectum, (20) (26) ((32)

The MSI status was associated with right-sided location,(24). (33) (34) (35)

Significant relationships were seen between microsatellite instability, young and old age.(36) In microsatellite stable tumors observed significant relationships, increased age, (37)

Sporadic CRC with MSI-High molecular features have a distinct phenotype, they are more common in older women.(35)

MSI-H CRCs also tend to occur more frequently in female CRC patients and with earlier onset of disease (<50 years) (35) while most patients in the MSI-H group were females (26) (20) CRC have a propensity to develop on in older women. (24) (34) Significant relationships were seen between microsatellite instability and female gender (36)

Significant relationships were seen between microsatellite instability poor histological differentiation and MSI CRCs tend to have prominent poor differentiation.(32) (36).(38). These features are found in both familial and sporadic MSI-H CRC cases occur predominantly in the poor differentiation related with MSI (11) (32) (37). (39) (40).

Mucinous CRC, a subtype of CRC, is characterized by the abundant production of extracellular mucins and accounts for 5– 15% of all CRCs (41).

CRC have a propensity to develop in a mucinous phenotype (11) (32) (33) (34) (37) (39) (40). while significantly higher percentages of mucinous CRC had MSI or CIMP (24).(26).

Pathological characteristics include increased lymphocytic infiltration (Crohn's disease-like reaction),).(11) (32) 33) (39) (40).

The high frequency of MSI-H suggests that should look at microsatellite instability prior to chemotherapy to determine the most appropriate chemotherapeutic strategy in our population.

With regard to MSI status and CRC patient survival, a systematic review of 32 studies that reported survival data on a total of 7642 colorectal cancer patients, including 1277 with MSI-H tumors, showed that MSI-H tumors were associated with better prognosis compared with MSS tumors (42) however, accumulating evidence has been suggested that MSI-high tumors are associated with good prognosis (43) (37)

CRC patients with MSI-H CRC status have better prognosis compared to non-MSI tumors, particularly among young patients (11) (33) (40) (44),(45) (46)

Molecular Correlates in CRC

The role of MSI-L as a subtype of molecular changes in CRC is controversial and tumors showing it resemble MSS tumors in clinical features (11) (40) (47)

Similar to the MSI-H tumors, MSI-L tumors also tend to occur more commonly in proximal colon, but their gender and family history associations are more similar to the MSS tumors (48)

Whether MSI-low (MSI-L) exists as a distinct phenotype from MSS has been controversial (47) (49)

Thus, a newer MSI marker panel has been designed to separate a substantial number of MSI-L tumors into MSS and MSI-H (49) (50)

MSI Related With Frequency of Microsatellite Panel Genes.

In this study the frequency of the microsatellite genes panel in all cases, the high percentage was recorded in BAT25 48.93%, while both BAT26, D5S346 was shown in 44.68%, while D17S250 was appeared in 19.14% and D2S123 in 25.23%.

Among MSI-H patients, instability in BAT-25 occurred in (83.3%), BAT 26 (72.2%), D2S123 in (61.1%) cases, D17S250 in (61.1%), and D5S346 in (44.4%).(20)

Samples positive for BAT25 instability (14%), BAT26, (21%) for BAT40, (18%) for D2S123, (16%) D17S250, and (14%) for D5S346.(21)

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