Colorectal carcinomas from Middle East

Molecular and tissue microarray analysis of genomic instability pathways

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ABSTRACT

الأهداف: على ضوء آخر التقارير المعنية بالتغيرات الجينية بين مختلف الأعراق و زيادة نسبة التزاوج بين الأقارب في السعودية. قمنا بتحليل عدم الاستقرار في مجموعة العوامل الوراثية عند أورام القولون و المستقيم. الهدف من الدراسة هي تقييم نسبة حدوث التغيرات (MSI)،أورام القولون الوراثي (HNPCC) التحولات لجين TP53 في أورام القولون و المستقيم في السعودية.

الطريقة: تم دراسة التغيرات للمتتابعات الصغيرة في أورام القولون و المستقيم في السعودية على ١٧٩ عينة عشوائية باستخدام PCR المجموعة المجهرية للأنسجة (IHC) للبروتينات MLH1 & MSH2كما تم دراسة التحولات للجين TP53باستخدام محلل التتابع للمواقع الجينية المفسرة ٥،٦،٧،٨.

النتائج: من ضمن التغيرات للمتتابعات الصغيرة (MSI) ل ١٥٠عينة من أورام القولون و المستقيم في السعودية ١٦٪ من الأورام المدروسة أظهرت عدم الاستقرار من النوع العالي MSH-H ١٩,٣٪ منهم أظهروا عدم استقرار من النوع المنخفض MSI-L ٦٤٪منهم أظهر استقرارا، القدرة على العيش بالنسبة للمجموعة MSI-H كانت أفضل من المجموعة MSI-L/MSS (P=0.0217) ٤٨٪ من MSI-H كانوا من النوع الوراثي ويمكن إيعاز ذلك لارتفاع نسبة التزاوج بين الأقارب في المجتمع السعودي، نسبة التحولات في جين TP53 كانت ٢٣,٩٪ بالنسبة للحالات التي تمت دراستها.

خاتمة: أكثر ما يميز أورام القولون المستقيم السعودية علو نسبة المتغيرات للمتتابعات الصغيرة الحاصلة لأسباب وراثية وقلة نسبة التحولات الجينية للجين TP53 مقارنة بالغرب وهذه المميزات تحتاج لمزيد من التقصي.

Objectives: To evaluate the overall incidence of microsatellite instability (MSI), hereditary non polyposis colorectal cancer, and tumor supressor gene (TP53) mutations in Saudi colorectal carcinomas.

Methods: We studied the MSI pathway in Saudi colorectal cancers (CRC) from 179 unselected patients using 2 methods: MSI by polymerase chain reaction, and immunohistochemistry detection of mutL homologs 1 and mutS homologs 2 proteins. The TP53 mutations were studied by sequencing exons 5, 6, 7, and 8.

Results: Of the 150 colorectal carcinomas analyzed for MSI, 16% of the tumors showed high level instability (MSI-H), 19.3% had low-level instability (MSI-L) and the remaining 64% tumors were stable. Survival of the MSI-H group was better as compared to the MSI-L or microsatellite stable group (p=0.0217). In the MSI-H group, 48% were familial MSI tumors, which could be attributable to the high incidence of consanguinity in the Saudi population. The TP53 mutations were found in 24% of the cases studied.

Conclusions: A high proportion of familial MSI cases and a lower incidence of TP53 mutations are some of the hallmarks of the Saudi colorectal carcinomas, which need to be explored further.

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The incidence of colorectal cancer (CRC) seems to be rising in the Kingdom of Saudi Arabia.[1] Consanguineous marriage is relatively common in the Kingdom and it might be expected that a number of cancers would be of hereditary type.[2] It has also been suggested that the CRC in the Kingdom presents at an earlier age is more aggressive and larger than similar cancer in the west. The study was carried out to see if there was any evidence to support the hypothesis that there were higher number of hereditary tumors in the Kingdom than in the west, to determine the incidence of hereditary non-polyposis colorectal cancer (HNPCC) which also might be expected to be higher, and to examine whether there are any genetic markers which differed from the west to explain the apparent aggression of tumors.[3] The CRC consists of 2 subtypes: the chromosomal instability (CIN) subtype associated with loss of function of the tumor suppressor gene TP53 resulting in accumulation of mutations,[4] and microsatellite instability (MSI) subtype, accounting for approximately 20% of the cases, characterized by inactivation of the mismatch repair (MMR) proteins.[5,6] The MSI, occurring in familial colorectal carcinomas has been termed HNPCC and accounts for 2-5% of all the CRC. Sporadic CRC arising from loss of DNA MMR protein accounts for 15% of all the cases. Identifying MSI pathway in CRC is of great importance as it helps in HNPCC proband identification. There are prognostic as well as evolving therapeutic differences between the 2 molecular subtypes: MSI-high (MSI-H) versus MSI-low/MSS (MSI-L/MSS).[7] Although mutations in MMR genes are believed to account for most HNPCC cases, methylation of the MMR gene sequences is the main underlying mechanism in most sporadic CRC with MSI-H phenotype.[8-10] The TP53 gene, located on the short arm of chromosome 17 (17p13.1), is involved in control of cell cycle, DNA repair, genomic plasticity, and programmed cell death.[11] The aim of this study was to evaluate the overall incidence of MSI-H, HNPCC, and TP53 mutations in Saudi colorectal carcinomas.

Methods. ***Patients and tumor samples.*** Archival paraffin blocks were collected from 179 patients diagnosed with colorectal carcinoma, and treated at King Faisal Specialist Hospital and Research Centre between January 1989 and December 2003. A detailed family history was obtained from the medical charts for all patients. Cancer pedigrees were traced backwards and laterally at least up to second-degree relatives. All the patients fulfilling the revised Bethesda criteria guidelines whose tumor showed MSI, and loss of expression of mutL homolog 1 (MLH1) or human mutS homolog 2 (MSH2) by immunohistochemistry (IHC) were classified into familial MSI or HNPCC cases.[12] The Institutional Review Board of the King Faisal Specialist Hospital and Research Centre approved the study.

Tissue microarray (TMA). The TMA construction was as described before.[13] Briefly, 3 cores of tissue cylinders with a diameter of 0.6 mm were punched from representative regions of each "donor" tumor block, and brought into recipient paraffin block using a modified semi-automatic robotic precision instrument (Beecher Instruments, Woodland, USA).

DNA extraction and purification. Genomic DNAs were extracted from paraffin embedded matched normal and neoplastic primary tissues using Gentra kit (Minneapolis, MN, USA) following the manufacturer's recommendation.

Microsatellite markers and analyses. Allelic imbalances were measured by performing MSI on all matched normal and tumor tissue by polymerase chain section (PCR) amplification. A reference panel of 5 pairs of microsatellite primers, comprising 2 mononucleotide microsatellite (BAT25, BAT26) and 3 dinucleotide microsatellites (D2S123, D5S346, and D17S250) were used to determine tumor MSI status.[6]

Mutational analysis of TP53 gene, PCR amplification. Exons 5-8 of the TP53 gene were amplified separately using the following primer sequences: exon-5-forward: 5 > G A C T T T C A A C T C T G T C T C 3 > , reverse: 5>CTGGGGACCC-CTGGGCAAC3>; exon-6-forward: 5>GAGACGACAGGGCTGGTT3>, reverse: 5>CCACTGACAACCACCCTT3>; exon-7-forward 5>CCAAGGCGCACTGGCCTC3>, reverse: 5>GCGGCAAGCAGAGGCTGG3>; and exon-8-forward: <CCTTACTG-CCTCTTGCTT3>, reverse 5>TGAATCTGAGGCATAACTGC3>. Sequencing was carried out on an automated DNA sequencer (Molecular Dynamics, Piscataway, NJ). Mutational analysis was carried out using DNA SEQMAN software (DNASTAR Inc., Madison, WI). Presence of mutation was considered when both strands showed base pair changes.

Immunohistochemistry. Colorectal carcinoma TMA section with positive control sections of normal colon and colorectal carcinoma were used for staining. The Dako mouse Envision System kit (DAKO, code No: K4001) was used as the secondary detection system with Diaminobenzidine as chromogen. Details of antibodies used, antigen retrieval steps, and staining are shown in **Table 1**. Staining was considered adequate where nuclear staining was seen in either stromal lymphocytes or in normal colonic epithelial cells in base of crypts. Loss of expression was recorded when none of the tumor nuclei stained for either MLH1 or MSH2, and these tumors were classified as MSI-IHC.

Statistical analysis was performed using SAS's (SAS Institute Inc.) JMP 5.1 software (Cary, NC, USA), and

all *p*-values reported are 2-tailed. Contingency analysis and Chi square tests were used to examine relationships between nominal variables. *P*-values less than 0.05 were regarded as statistically significant. Surviving curves were plotted according to the Kaplan-Meier method, and were analyzed by log-rank test.

Results. *MSI analysis by PCR amplification.* Of the 150 tumors, 25 (16.6%) showed allelic shifts in 2 or more loci, and were designated MSI-H. Of the 25 MSI-H cases, 12 were classified as HNPCC (fulfilling revised Bethesda criteria and were MSI-IHC), thus constituting 48% of the total MSI cases. Of these 12 HNPCC cases, MSH2 staining was negative in 8 cases, and MLH1 in 4 cases. Allelic shift in only one locus was demonstrated in 29 tumors (19.3%), and were designated as MSI-L. The remaining 96 cases (64%) designated as MSS showed no changes in any of the loci (**Figure 1**).

Table 1 - Antibodies used for immunohistochemistry study.

Antigen	Antibody clone	Dilution	Source	Antigen retrieval	Interpretation
MLH1	G-168-15	1:100	BD Pharmingen	Microwaved at 750 W for 5 minutes and 250 W for 15 minutes in ethylenediaminetetraacetic acid buffer solution, pH 9.0, followed by cooling for 15-20 minutes	Complete absence with an internal positive control
MSH2	FE-11	1:500	Zymed Lab		
p53	DO-7	1:50	Dako	Citrate buffer pH 6.0	Positive if >10% of tumor nuclei showed unequivocal staining

MLH1 - mutL homolog1, MSH2 - mutS homolog2, W - Watts

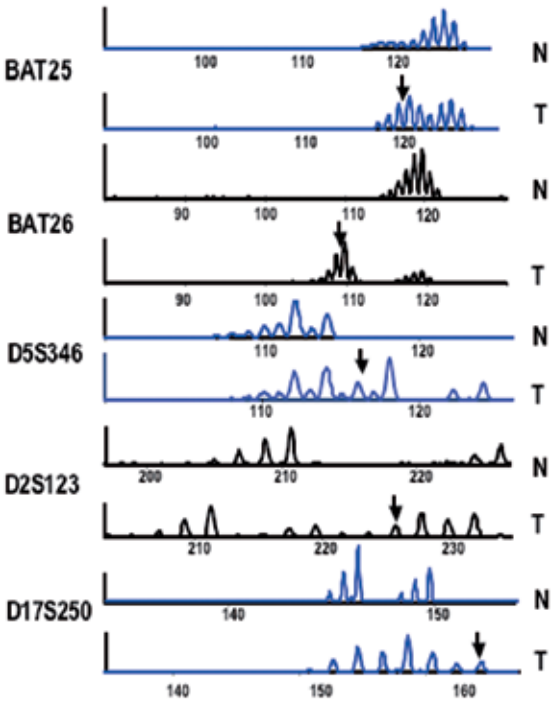

Figure 1 - Fragment pattern of a microsatellites instability (MSI) - high showing MSI at all 5 loci analyzed in the following order: BAT25, BAT26, D5S346, D2S123, and D17S250. (N - normal, T - tumor)

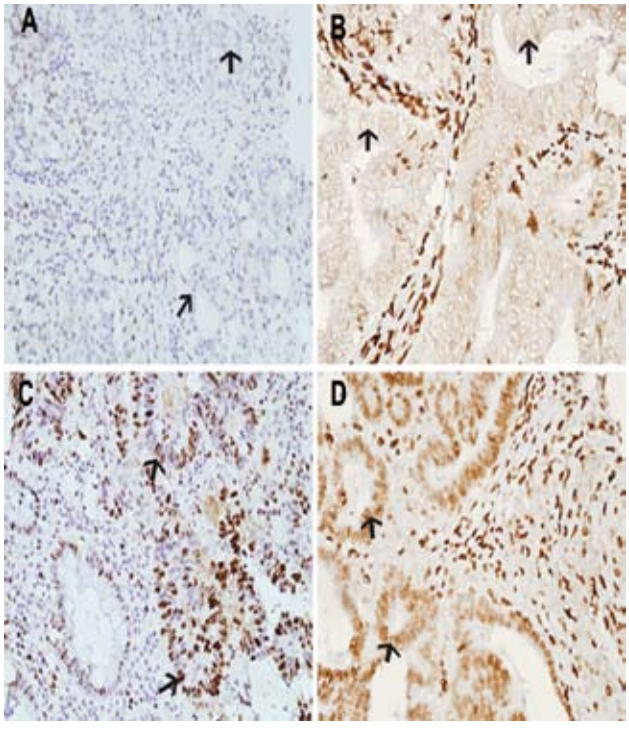

Figure 2 - Loss of expression of mismatch repair proteins **a)** mutS homolog 2 (hMSH2) and **b)** mutL homolog 1 (hMLH1), normal expression of **c)** hMSH2 and **d)** hMLH1. Internal positive control (lymphocytes and/or stromal fibroblast nuclei) are staining positive in all cases.

Immunohistochemistry. Among the 179 cases evaluated for IHC, 19 (11.2%) were classified as tumors showing loss of expression for MLH1 or MSH2, 150 (87.5%) as tumors showing expression for MLH1 and MSH2, and the remaining 10 cases were non contributory (**Figure 2**). Among the MSI group, a preponderance of MSH2 abnormalities was noted as 14 (73.6%) tumors showed absence of staining for MSH2. A further 5 tumors (26.3%) showed absence of staining for MLH1. Analyzable data for MSI molecular and MSI-IHC was available in 145 cases. Only 12 of the total 25 MSI-H cases showed loss of staining for either MLH1 (4 cases) or MSH2 (8 cases). Of the 19 discordant cases, 13 cases were MSI-H by PCR and showed expression for MLH1 or MSH2 by IHC (false negative cases). The remaining 6 discordant cases were MSS/MSI-L by PCR results however showed loss of expression for MLH1 or MSH2 by IHC (false positive

Table 2 - Correlation between MSI-H status and clinicopathological features in colorectal carcinoma.

Parameters			MSS/MSI-L		MSI-H		*P*-value
	n	(%)	n	(%)	n	(%)	
Age	150						
≤50 years	59	(39.3)	48	(81.4)	11	(18.6)	0.6026
>50 years	91	(60.7)	77	(84.6)	14	(15.4)	
Gender	150						
Male	77	(51.3)	65	(84.4)	12	(15.6)	0.7149
Female	73	(48.7)	60	(82.2)	13	(17.8)	
Tumors site	150						
Left colon	134	(89.3)	116	(86.6)	18	(13.4)	0.0062
Right colon	16	(10.7)	9	(56.3)	7	(43.7)	
Histological type	150						
Adenocarcinoma	144	(96.0)	120	(83.3)	24	(16.7)	1.000
Mucinous carcinoma	6	(4.0)	5	(83.3)	1	(16.7)	
Tumors size	125						
Less than 5 cm	68	(54.4)	61	(89.7)	7	(10.3)	0.0951
More than 5 cm	57	(45.6)	45	(78.9)	12	(21.1)	
Tumor stage (Duke's)	142						
A	26	(18.3)	22	(84.6)	4	(15.4)	0.0625
B	48	(33.8)	35	(72.9)	13	(27.1)	
C	68	(47.9)	61	(89.7)	7	(10.3)	
Differentiation	150						
Well	5	(3.3)	4	(80.0)	1	(20.0)	0.6064
Moderate	126	(84.0)	104	(82.5)	23	(17.5)	
Poor	19	(12.7)	17	(89.5)	2	(10.5)	
Lymph nodes	131						
Negative	70	(53.4)	54	(77.1)	16	(22.9)	0.0193
Positive	61	(46.6)	56	(91.8)	5	(8.2)	
p53 mutation	113						
Negative	86	(76.1)	71	(82.6)	15	(17.4)	0.0029
Positive	27	(23.9)	27	(100.0)	0	(0)	
MSI-IHC	145						
MSI	18	(12.4)	6	(33.3)	12	(66.7)	<0.0001
MSS	127	(87.6)	114	(89.8)	13	(10.2)	

MSS/MSI-L - microsatellite stable/microsatellite instability-low, MSI-H - microsatellite instability-high, MSI-IHC - miscrosatellite instability analysis by immunohistochemistry.

cases). The TP53 over expression by IHC was seen in 67.3% (95 of 141) of the colorectal carcinomas and correlated with TP53 mutation data (p=0.0421).

Clinicopathological parameters. The MSI-H tumors showed no correlation with age, gender, grade, or stage. However MSI-H tumors were more common in the right colon as compared to the left colon (p=0.0062) and a trend was seen with larger size (p=0.09). The MSI-H tumors were less likely to show lymph node metastasis (p=0.0193), and a trend was seen with larger size (p=0.09) as compared to MSS/MSI-L group (**Table 2**).

Frequency of TP53 mutations in CRC. The TP53 mutations were detected in 27 of the 113 carcinomas (23.8%). Total mutations of 52.5% of total mutations were detected in exon 8 (hot spot codon 273), while the remaining mutations were distributed between exons 5, 6, and 7. An inverse correlation was seen between MSI-H status and TP53 mutations, which was statistically significant (p=0.0029).

MSI-H versus MSS/MSI-L phenotype and survival. The median follow-up of the total study population was 31 months, and the maximum follow-up was 139 months. Twenty patients died (16 patients with MSI-S expression and 4 patients with MSI-L expression). The 25 patients with MSI-H expression had a significantly better overall survival (p=0.0321) than the 125 patients with MSS or MSI-L expression.

Discussion. This is a pilot study to characterize the genomic instability pathways in CRC, the fourth most common cancer in Saudi Arabia. Considering the impact of ethnic differences and a potential role of future targeted therapies, the findings in this paper will pave a way for future studies, which will elucidate the role of genomic instability in colon cancers in the Arab population. Findings of this study help in establishing screening strategies in CRC to identify the HNPCC cases, and also will have immediate therapeutic relevance. Overall, evidence of DNA MSI was found in 16.6% patients with colorectal carcinomas. This is within the range (12-24%) previously published for sporadic MSI-H colorectal carcinomas.[14] Studies have shown that MSI-H colorectal carcinomas are associated with a specific clinicopathological phenotype.[15,16] In our study we found that MSI-H tumors had a predilection to involve right colon (p=0.0062), had a lower risk of lymph node metastasis (p=0.0193), and presented with a less advanced Dukes stage (p=0.0625) as compared to MSS or MSI-L tumors. Examination of treatment outcomes for this relatively small and non-uniform cohort did show a survival advantage for MSI-H colorectal carcinomas as compared to MSI-L/MSS subset of colorectal carcinomas (p=0.0217). This is in concordance with earlier reports of MSI-H colorectal carcinomas, having a better survival as compared to MSI-L and MSS tumors.[17-20]

Evaluating cases for Amsterdam criteria posed unique difficulties.[21,22] The data of colon cancer collected addresses one important issue: due to lack of modern healthcare system up to the late 1970's it is impossible to depend on the information given by the patients, especially if they do not know the diagnosis from which their family member suffered or died. To overcome this problem, we defined those cases as HNPCC which fulfilled the revised Bethesda criteria and were MSI-H on MSI testing along with loss of expression of MLH1, or MSH2 protein by IHC. This definition was shown in a recent study to have a overall accuracy of 98.1%, with a sensitivity rate of 72.7% and a specificity of 98.1%.[23] Based on this study, we believe that 48% (12 cases) of our MSI-H groups belong to the HNPCC group. In these 12 MSI-H cases fulfilling the revised Bethesda guidelines, MLH1 was absent in 4 cases and MSH2 in 8 cases. Loss of MSH2 in the majority of the cases further lends credibility that all these cases were HNPCC. This high number of familial MSI cases could be attributed to founder mutation or the high incidence of consanguinity, which has been reported in the Saudi population to be 57.7% with regional variation from as low as 34% to an astounding high of 80.6%.[2] Soliman et al,[24,25] have earlier reported familial aggregation of colorectal carcinomas and have attributed it to consanguinity. Genetic testing was not performed due to limited resource of DNA extracted from archival paraffin embedded tissues, and few of the cases were referral cases with availability of only a single paraffin block. Similarly, fresh blood samples which can also be used for sequencing MMR genes, were not available. Lack of detailed family history to fulfill Amsterdam criteria and inability to do an exhaustive mutation analysis of MMR genes are thus some of the limitations of this study. There was no difference in the survival between the 2 subsets of MSI-L and MSS colorectal carcinomas. These data support recent studies suggesting that the molecular profiles of MSS and MSI-L tumors are indistinguishable.[26,27] In CRCs, TP53 gene mutations and allelic loss on 17p are genomic alterations that occur as late events in tumor progression. According to published reports in the literature, TP53 mutations have been described in about 40-50% of CRC.[11] The TP53 gene mutation incidence in our study is 24%, which is much lower than the range of 40-60% reported in the west. Similar prevalence figures of TP53 mutations (32.3%) have been reported in eastern Europe.[11] There was no difference in survival between the 2 subsets of TP53 gene mutations (p=0.2932). Conflicting evidence exits as to the prognostic significance of TP53 gene mutations expressed in CRCs.[28] The TP53 mutation

results showed an inverse relationship with MSI-H cases (p=0.0029) which coincides with published reports.[29-31] A key and novel finding of this study was MSI-H tumors with a very high probability of large proportion of HNPCC cases in Saudi colorectal carcinomas. The TP53 mutations were found at a lower frequency, and there was a significant inverse correlation between TP53 mutations and MSI.

In conclusion, a high proportion of familial MSI cases and a lower incidence of TP53 mutations are some of the hallmarks of the Saudi colorectal carcinomas, which need to be explored further.

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