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BRIEF ARTICLE

Somatic molecular changes and histo-pathological features of colorectal cancer in Tunisia

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Abstract

AIM: To determine correlations between family history, clinical features and mutational status of genes involved in the progression of colorectal cancer (CRC).

METHODS: Histo-pathological features and molecular changes [*KRAS*, *BRAF* and *CTNNB1* genes mutations, microsatellite instability (MSI) phenotype, expression of mismatch repair (MMR) and mucin (MUC) 5AC proteins, mutation and expression analysis of *TP53*, *MLH1* promoter hypermethylation analysis] were examined in a series of 51 unselected Tunisian CRC patients, 10 of them had a proven or probable hereditary disease, on the track of new tumoral markers for CRC susceptibility in Tunisian patients.

RESULTS: As expected, MSI and MMR expression loss were associated to the presence of familial CRC (75% *vs* 9%, $P < 0.001$). However, no significant associations have been detected between personal or familial cancer history and *KRAS* (codons 12 and 13) or *TP53* (exons 4-9) alterations. A significant inverse relationship has been observed between the presence of MSI and TP53 accumulation (10.0% *vs* 48.8%, $P = 0.0335$) in CRC tumors, suggesting different molecular pathways to CRC that in turn may reflect different environmental exposures. Interestingly, MUC5AC expression was significantly associated to the presence of MSI (46.7% *vs* 8.3%, $P = 0.0039$), MMR expression loss (46.7% *vs* 8.3%, $P = 0.0039$) and the presence of familial CRC (63% *vs* 23%, $P = 0.039$).

CONCLUSION: These findings suggest that MUC5AC expression analysis may be useful in the screening of Tunisian patients with high risk of CRC.

Key words: DNA mismatch repair; *KRAS*; *TP53*; Mucin 5AC

Core tip: This study reports, for the first time in Tunisia, the value of various histo-pathologic features and

somatic molecular changes [*BRAF*, *KRAS*, *CTNNB1*, *TP53*, mismatch repair (MMR) expression, microsatellite instability (MSI), MLH1 promoter methylation] in distinguishing patients with hereditary non polyposis colorectal cancer. Our results revealed that MUC5AC expression was significantly associated with the presence of MSI (46.7% *vs* 8.3%, P = 0.0039), MMR expression loss (46.7% *vs* 8.3%, P = 0.0039) and the presence of familial colorectal cancer (63% *vs* 23%, P = 0.039). These findings suggest that mucin 5AC expression analysis may be useful in the screening of Tunisian patients with high risk of colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is a complex biological process involving many genes. Intensive screening for genetic alteration in CRC led to the identification of at least two different molecular mechanisms implicated in CRC carcinogenesis: chromosomal (CIN) and microsatellite instabilities (MSI). The CIN pathway is found in about 80% of sporadic CRC and in familial adenomatous polyposis[1]. It involves chromosomal allelic losses[2,3]. The MSI pathway is found in most cases of hereditary non-polyposis colorectal cancer (HNPCC) and in 12% of sporadic CRC. It involves inactivation of DNA mismatch repair (*MMR*) genes. The presence of MMR deficiency leads to the accumulation of mutations in mononuclear tracts in the coding region of genes controlling cell cycle[4]. Although CRC shows genetic heterogeneity, the same four different signalling pathways could be implicated in tumor progression. The WNT/Wingless pathway could be activated through an *APC* mutation in CIN tumors or through a *CTNNB1* stabilizing mutation in MSI tumors[5]. *CTNNB1* and *APC* mutations were observed as early as the adenomatous stage of CRC neoplasia. The transforming growth factor beta (TGFβ) pathway is driven by *SMAD2* or *SMAD4* inactivating mutation in CIN tumors[6] or by a frame-shift mutation in the TGFβ type Ⅱ receptor in MSI tumors[7]. The RAS-MAP kinase pathway is activated by *KRAS* mutations in CIN[8] or by *BRAF* mutations in sporadic MSI tumors. Alteration of these genes correlated closely with the progression of the adenoma to cancer. The TP53 pathway is inactivated by *TP53* mutations in CIN tumors or by *BAX* inactivating mutation in MSI tumors. These alterations contribute to the adenoma-carcinoma transition. More recently, the existence of a third phenotype was suggested. The main alteration associated with this group of tumors is the hypermethylation of the promoter region of numerous genes, leading to their inactivation[9,10]. Activating somatic mutation of *BRAF* gene has been reported in 15% of sporadic tumors with MSI due to *MLH1* hypermethylation and never in tumors from HNPCC families with *MLH1* and *MSH2* germline mutations[11]. MMR germline mutations detections is an important supplement to HNPCC clinical diagnosis. It enables at-risk and mutation-positive relatives to be informed about their cancer risks and to benefit from intensive surveillance programs that have been proven to reduce the incidence of CRC[12]. However, germline tests are time-consuming and costly due to heterogeneity of mutations. In addition, MMR germline mutations are not always detected in Amsterdam positive families (sensibility, 50%-78%)[13]. The difference in somatic mutation status between sporadic CRC and HNPCC-related cancers may prove helpful in distinguishing HNPCC patients. In this study, we analysed for the first time in Tunisia the value of various histo-pathologic features and somatic mutations of 51 CRC cases in predicting CRC susceptibility.

MATERIALS AND METHODS

Patients and tissue specimens

Fifty-one formalin-fixed, paraffin embedded primary colorectal carcinomas and paired normal bowel of 51 different patients who had undergone colonic resection for the treatment of CRC were retrieved by retrospective review of the pathology archives. Ten of these patients were previously characterized for MMR germline mutations associated to Lynch syndrome[14]. Patients were evaluated according the revised Bethesda guidelines for the identification of HNPCC patients[15]. MSI testing, immunohistochemistry and somatic mutational analysis were performed in all patients regardless of age, personal or family history of cancer, and tumor characteristics.

DNA preparation

DNA was extracted from paraffin-embedded tissue samples of primary CRC and paired normal bowel using the DNeasy® tissue kit (Qiagen, Courtaboeuf, France).

MSI analysis

MSI was assessed using a set of five mononucleotide markers (BAT25, BAT26, NR21, NR22, NR24)[15,16].

Expression of MMR proteins

MMR was assessed by immunohistochemistry as previously described[16]. Immunohistochemistry for mucin (MUC) 5AC and TP53. Tumor sections were analysed using mouse monoclonal antibody against p53 (clone DO-7, Dakocytomation) and MUC5AC (clone CLH2, Novocastra). For TP53, a tumor was scored as TP53 overexpression-positive if nuclear staining was seen in more than 20% of the neoplastic cells in the absence of staining in the tumor adjacent cells. For MUC5AC, which is never expressed in normal colon mucosa[17], expression was interpreted as positive if more than 10% of tumor

Table 1 Clinical and histo-pathological characteristics of the 51 colorectal cancer patients *n* (%)

Characteristic	Patient
Age of onset of the first cancer (range) (yr)	51 (17-85)
⩽ 50	25 (49.0)
> 50	26 (51.0)
Sex	
Male	30 (58.8)
Female	21(41.2)
Site of the first CRC	
Right colon	14 (27.5)
Left colon	16 (31.4)
Rectum	21 (41.2)
TNM tumor stage	
Ⅰ	3 (5.9)
Ⅱ	24 (47.1)
Ⅲ	20 (39.2)
Ⅳ	3 (5.9)
Others	1 (2.0)
Degree of differentiation	
Well	33 (64.7)
Moderate	14 (27.5)
Poor	2 (3.9)
Mucinous CRC	2 (3.9)
Mucinous carcinoma type	
⩾ 50%	14 (27.5)
⩽ 50%	37 (72.5)
Signet ring cell carcinoma	2 (3.9)
Tumor infiltrating lymphocyte	
Crohn's–like reaction	2 (3.9)
Intra epithelial lymphocytes	1 (2.0)
Lymphoïde peritumoral reaction	10 (19.6)
Synchronous CRC	3 (5.9)
Metachronous CRC and HNPCC related cancer	3 (5.9)
Fulfillment of guidelines	
Amsterdam	3 (5.9)
Revised Bethesda	22 (43.1)
B1	22
B2	3
B3	11
B4	1
B5	1

CRC: Colorectal cancer; HNPCC: Hereditary nonpolyposis colorectal cancer; TNM: Tumor node metastasis.

cells displayed cytoplasmic staining in the absence of staining in the tumor adjacent cells.

TP53 mutations screening

Primers were designated for the coding regions and exon-intron boundaries of exons 5 to 8. Exons 4 and 9 were only analysed on those samples negative for mutations in exons 5-8. Primer sequences and polymerase chain reaction (PCR) conditions are available on request.

Mutation screening for KRAS, CTNNB1 and BRAF genes

KRAS (codon 12, 13), *BRAF* (exon 15) and *CTNNB1* (β-catenin) (exon 3) were screened in each CRC cancer using direct sequencing in forward and reverse orientations. Primer sequences and PCR conditions are available on request.

MLH1 promoter methylation assay

Genomic DNA obtained from paraffin-embedded tissue section was modified with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research) according to the specifications of the manufacturer. Primer sequences for methylation-specific PCR were modified from Grady *et al*[18].

Statistical analysis

Continuous variables are described as mean and range (min-max) and categorical variables as frequencies and percentages. The association between the different measured parameters was tested using non parametric tests. The difference between two independent groups was determinate by Mann-Whitney *U*-test and the significance of differences between more than two groups was calculated using Kruskal-Wallis test. Categorical data were compared by χ^2 appropriate or Fisher exact tests. A *P*-value < 0.05 was considered as statistically significant. Statistical analyses were performed with SPSS version 15.0 (SPSS, Chicago, IL, United States).

RESULTS

Clinical and pathological features

Demographic, clinical and tumor-related characteristics of the study group are summarised in Table 1. Twenty-five (49.0%) probands were under 50 years of age, including 12 (23.5%) under 40 years of age and 5 (9.8%) under 30 years of age; 26 (51%) aged more than 50 years, including 20 (39.2%) aged more than 60 years. Six patients (11.8%) had a personal history of synchronous/metachronous CRC tumors (4 cases) or previous primary CRC and HNPCC-related extracolonic tumors (2 cases). In 8 cases (16%), the proband was found to have at least one first-degree relative with CRC and/or HNPCC-related extracolonic cancers. In total, 25 (49%) of the 51 CRC patients belonged to families fulfilling the Amsterdam Criteria[19] for the clinical definition of HNPCC or fulfilled at least one criterion of the revised Bethesda criteria for the identification of HNPCC patients[15]. Criterion 1 was the most commonly satisfied Bethesda criterion (22/51, 43.1%). Clinical data analysis revealed that CRC was essentially right sided for patients having at least one first- or second-degree relative with CRC; whereas cancer was more frequently left sided or rectal for patients without a familial history of CRC ($P = 0.039$) (Table 2). However, no significant difference in tumor site was seen when Bethesda criteria where considered. The Bethesda-positives CRC tumors same to be associated to a more advanced stage of the disease ($P = 0.050$) (Table 2). However, no statistical difference has been seen when familial history was considered (Table 2).

Pattern and frequency of MSI

MSI-high (MSI-H) phenotype was detected in 10 (19.6%) of the 51 tested tumors. All the MSI tumors showed instability in all 5 analysed markers. Eight patients (8/25; 32%) were Bethesda-positives and only 2 (2/26; 8%) were Bethesda-negatives (Table 2). For the remaining 41 cases, the tumors were microsatellite stable (MSS) including 1 (1/3, 33%) Amsterdam I-positive patient and 16 (16/25,

Table 2 Statistical analysis of clinicopathological parameters of the 51 colorectal cancer studied tumors as a function of tumoral phenotype *n* (%)

	Mutation MMR -	Mutation MMR +	Family history of colorectal cancer		*P*	Ams - and Beth -	Ams - and Beth +	*P*	Ams (- or +) and Beth +	*P*
	(*n* = 6)	(*n* = 4)	Yes (*n* = 8)	No (*n* = 43)		(*n* = 26)	(*n* = 22)		(*n* = 25)	
Mutation geminale										
MMR+			4	0		0	2		4	
MMR-			3	3		0	5		6	
Site of tumor (CCR)					NS			NS		NS
Right colon	1	3	5 (63)	10 (23)		5 (19)	9 (41)		10 (40)	
Left colon	2	0	1 (13)	14 (33)		8 (31)	7 (32)		7 (28)	
Rectum	3	1	2 (25)	19 (44)		13 (50)	6 (27)		8 (32)	
Left colon + rectum	5	1	3 (38)	33 (77)	0.039	21 (81)	13 (59)	NS	15 (60)	NS
Right + left colon	3	3	6 (75)	24 (56)	NS	13 (50)	16 (73)	NS	17 (68)	NS
TNM Stage					NS			NS		NS
Ⅰ	0	1	1 (14)	2 (5)		2 (8)	0 (0)		1 (4)	
Ⅱ	1	0	2 (29)	23 (53)		16 (62)	9 (41)		9 (38)	
Ⅲ	4	3	4 (57)	15 (35)		7 (27)	11 (50)		12 (50)	
Ⅳ	0	0	0 (0)	3 (7)		1 (4)	2 (9)		2 (8)	
Ⅰ / Ⅱ	1	1	3 (43)	25 (58)	NS	18 (69)	9 (41)		10 (42)	
Ⅲ / Ⅳ	4	3	4 (57)	18 (42)		8 (31)	13 (59)	NS	14 (58)	0.050
Microsatellite instability					< 0.001			NS		0.038
MSI (MSI-L or MSI-H)	1	4	6 (75)	4 (9)		2 (8)	6 (27)		8 (32)	
MSS (MSI-L or MSS)	5	0	2 (25)	39 (91)		24 (92)	16 (73)		17 (68)	
Somatic mutations										
TP53	6	0	3 (38)	25 (64)	NS	14 (61)	13 (62)	NS	14 (58)	NS
KRAS	1	2	2 (25)	14 (33)	NS	9 (35)	5 (23)	NS	7 (28)	
BRAF	0	0	0 (0)	1 (2)		1 (4)	0 (5)		0 (0)	
CTNNB1	0	0	0 (0)	1 (2)		0 (0)	1 (5)		1 (4)	
Immunohistochemistry										
Loss of MMR	1	4	5 (50)	5 (50)	NS	2 (8)	6 (27)	NS	8 (32)	0.038
Overexpression of p53	5	0	2 (25)	19 (44)	NS	9 (35)	11 (50)	NS	12 (48)	NS
Overexpression of MUC5AC	2	3	5 (63)	10 (23)	0.039	6 (23)	8 (36)	NS	9 (36)	NS

Ams: Extented Amsterdam Ⅱ criteria; NS: Not statistically significant; Beth: Revised Bethesda Guidelines; MMR: Mismatch repair; CCR: Colorectal cancer; MSI: Microsatellite instability; MSS: Microsatellite stable; MUC: Mucin.

64%) Bethesda-positives patients. Six (27%) of the 22 Bethesda-positives Amsterdam-negatives patients showed MSI in tumor tissue. Hence, the sensitivity and the specificity of the Bethesda criteria in the prediction of MSI were 80% and 60%, respectively. MSI was also observed in 3 sporadic CRC cases (3/49, 6%). Other laboratories have demonstrated a frequency of MSI between 10% and 20% amongst sporadic CRC cases. Therefore, our results are comparable with results from other series[20,21]. CRC was diagnosed before 50 years of age in 80% (8/10) of the patients with MSI (Table 3). The mean age at tumor diagnosis in MSS patients was higher than in MSI patients [56.2 years (range 17-85 years) *vs* 42.4 years (range 18-72 years)] (Table 3). Two of the 10 MSI patients (20%) had synchronous/metachronous colorectal cancer and no one had additional extracolonic cancer. Mucinous colloid component was significantly more important in MSI-H tumors ($P = 0.0178$) (Table 3). No significant associations were observed between MSI phenotype and sex, tumor site and tumor node metastasis (TNM) stage (Table 3). However, MSI-H tumors have been reported to be more frequent in the proximal colon[22]. A *KRAS* somatic mutation was detected in 4 (4/10, 40%) MSI-H tumors (Table 4): 3 were located at codon 13 (*p*.Gly13Asp) and 1 was at codon 12 (*p*.Gly12Asp). No significant association has been detected between MSI and *KRAS* alterations (Table 4). However, an inverse correlation came to exist between MSI-H phenotype and TP53 overexpression ($P = 0.0335$) (Table 4). On the other hand, MUC5AC abnormal expression was significantly more frequent in MSI tumors compared to MSS tumors ($P = 0.0039$) (Table 4). This result was in accordance with data reported by Biemer-Hüttmann *et al*[23].

MMR protein expression

Forty-one of the 51 (80.4%) analysed CRCs exhibited normal MMR protein expression. Of the remaining 10 (19.6%) CRCs, 8 (80%) showed a combined MLH1 and PMS2 proteins expression loss suggesting an *MLH1* deleterious mutation, 1 (10%) showed a combined MSH2 and MSH6 proteins expression loss, hardly suggesting an *MSH2* deleterious mutation (or *MSH6*, eventually), while just 1 (10%) demonstrated loss of only MSH6 protein, suggesting an *MSH6* deleterious mutation. Two (2/10, 20%) of these patients were Amsterdam-positives whereas 8 (8/10, 80%) where Amsterdam-negatives (Table 2). Five patients with MMR proteins expression loss had a family history of cancer. Of the 8 cases with MLH1 expression loss, 2 (2/8, 25%) had an Amsterdam-positive family history. For MSH2 and/or MSH6 none of the 2 cases with expression loss had a cancer family history. The 10 tumors with MMR expression loss corresponded

Table 3 Comparison of the somatic phenotype and genotype as a function of the patient's clinical characteristics

	MSI (n = 10)	*KRAS* mutations (n = 16)	*TP53* mutations (n = 28)	TP53 overexpression (n = 21)	MUC5AC overexpression (n = 15)
Mean age at diagnosis (range), yr	45 (18-72)	51.5 (18-85)	48.5 (18-79)	49.5 (24-75)	50 (24-76)
Sex					
Males	80.00%	75.00%	57.10%	52.40%	66.70%
Females	20.00%	25.00%	42.90%	47.60%	33.30%
Tumor site[1]					
Proximal	50.00%	18.80%	21.40%	28.60%	33.30%
Distal	50.00%	81.30%	78.60%	71.40%	66.70%
TNM stage[2]					
I	10.00%	6.30%	0.00%	0.00%	0.00%
II	40.00%	43.80%	48.10%	38.10%	40.00%
III	50.00%	50.00%	44.40%	47.60%	53.30%
IV	0.00%	0.00%	7.40%	9.50%	6.70%

[1]Proximal, right colon; distal, left colon + rectum; [2]Tumor, node, metastasis (TNM) stage was unknown for one patient; MSI: Microsatellite instability; MUC: Mucin.

Table 4 Comparison of the microsatellite instability phenotype as a function of tumoral parameters

	MSI-H tumors (n = 10)	MSS tumors (n = 41)	P
MMR expression	100.00%	0.00%	< 0.0001[1]
KRAS mutations	40.00%	29.30%	NS
TP53 mutations	30.00%	67.60%	NS
TP53 surexpression	10.00%	48.80%	0.0335[1]
MUC5AC surexpression	70.00%	19.50%	0.0039[1]

[1]Fisher exact test. NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair; MSS: Microsatellite stable.

Table 5 Comparison of *TP53* somatic mutations as a function of tumoral parameters

	Presence of *TP53* mutations (n = 28)	Absence of *TP53* mutations (n = 19)	P
MSI	10.70%	36.80%	NS
MMR expression loss	10.70%	36.80%	NS
KRAS mutations	25.00%	47.40%	NS
TP53 surexpression	60.70%	21.10%	0.0090[1]
MUC5AC surexpression	21.40%	47.40%	NS

[1]Fisher exact test. NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair.

to the 10 MSI-H tumors. All the MSS tumors showed normal MMR proteins expression. Hence, immunohistochemical analysis had 100% sensitivity for the detection of tumors with high MSI. Of note, germline deleterious mutations in *MMR* genes had been reported in our previous studies[14,24] in 4 patients with MSI-H CRC tumors and MMR protein expression loss.

TP53 protein expression analysis

TP53 positive nuclear immunostaining was observed in the CRC tumors from 21 (21/51, 41%) patients. No significant association has been detected between TP53 overexpression and selection criteria (Amsterdam or Bethesda) or the presence of relatives with CRC (Table 2). Overall, no association has been detected between TP53 overexpression and the different clinical parameters, including age at tumor diagnosis, gender, TNM stage or tumor location (Table 3). All but one (20/21, 95.2%) of the CRC tumors with TP53 overexpression were MSS and showed normal MMR proteins expression. The remaining tumor was of MSI-H phenotype associated to a combined MLH1 and PMS2 proteins expression loss, suggesting an MMR deficiency. On other hand, we have noted a close correlation between *TP53* mutations and TP53 protein level (P = 0.0090) (Table 5), as previously reported[25,26]. The absence of mutation in the 4 tumors overexpressing TP53 may be due to a lack of sensibility of the utilized sequencing technique, which requires greater than 15%-20% of neoplastic cells burden in the analysed specimens. In addition, mutations may be located outside the screened exons (exons 4-9), which represent less than 5% of the *TP53* detected mutations[27].

Expression analysis of MUC5AC

Abnormal MUC5AC expression was identified in 15 CRC tumors (15/51, 29.41%), 6 of them showed mucinous colloid component ⩾ 50%. In 3 tumors, the stained area was limited to the focal glands. MUC5AC expression was significantly associated to the presence of personal and family history of CRC (P = 0.039) (Table 2). It is very interesting to note that abundant MUC5AC expression was seen in the tumor of 3 HNPCC subjects with deleterious germline *MMR* mutations[14]. However, we didn't detect any other significant association between MUC5AC expression and clinico-pathological characteristics (Table 6). Interestingly, MUC5AC expression was significantly associated to MSI phenotype and MMR proteins expression loss (P = 0.0039) (Table 6). In contrast, no significant association was detected between MUC5AC expression and *TP53* or *KRAS* genes mutations (Table 6).

TP53 mutations analysis

The *TP53* mutation analysis was possible in the CRC

Table 6 Comparison of mucin 5AC expression as a function of tumoral parameters

	MUC5AC expression (n = 15)	Absence of MUC5AC expression (n = 36)	P
MSI phenotype (n = 10)	46.70%	8.30%	0.0039[1]
MMR expression loss (n = 10)	46.70%	8.30%	0.0039[1]
TP53 mutations (n = 28)	40.00%	68.80%	NS
TP53 overexpression (n = 21)	33.30%	44.40%	NS
KRAS mutations (n = 16)	26.70%	33.30%	NS

[1]Fisher exact test. NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair.

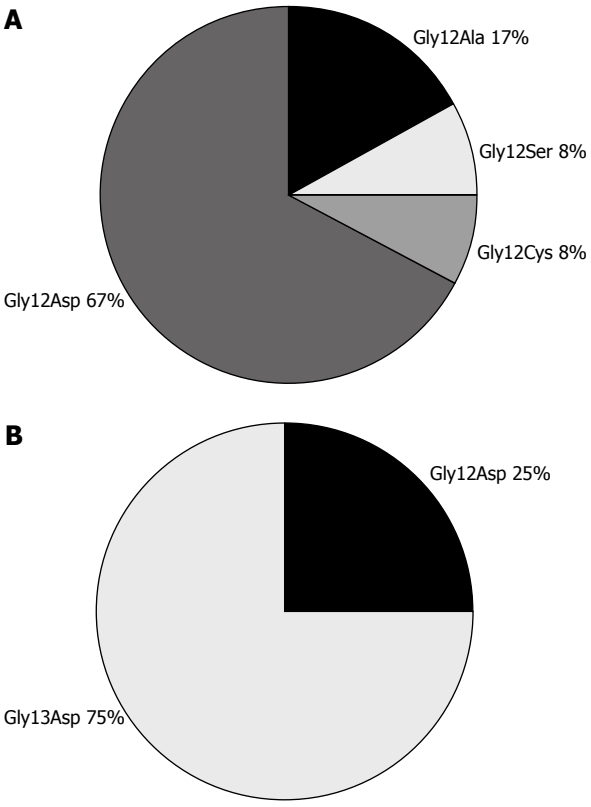

Figure 1 *KRAS* mutation spectrum as a function of tumoral microsatellite instability phenotype. A: Microsatellite stable (MSS); B: Microsatellite instability-high (MSI-H).

tumors of 47 patients. In total, a deleterious somatic mutation has been detected in 28 patients (28/47, 59.6%). Overall, there were no significant association of *TP53* mutations with Bethesda criteria, cancer family history (Table 2) or patient's clinical and histo-pathological data (Table 3). Particularly, we didn't detect any association between the presence of *TP53* mutations and tumor site (P = 0.0658) (Table 3). This was in contrast with data reported by other groups which showed that *TP53* mutations where more frequent in left-sided and rectal tumors[28-30]. A *KRAS* somatic mutation was identified in 7 (7/28, 25%) of the CRC tumors with *TP53* mutations (Table 5). All these mutations were G>A transitions

Table 7 Comparison *KRAS* somatic mutations as a function of tumoral parameters

	Presence of *KRAS* mutations (n = 16)	Absence of *KRAS* mutation (n = 35)	P
MSI	25.00%	17.10%	NS
MMR expression loss	25.00%	17.10%	NS
TP53 mutations	46.70%	65.60%	NS
TP53 overexpression	31.30%	45.70%	NS
MUC5AC overexpression	25.00%	31.40%	NS

NS: Not significant; MSI: Microsatellite instability; MUC: Mucin; MMR: Mismatch repair.

in codon 12 (5 mutations were p.Gly12Asp and 1 was p.Gly12Ser) and no mutation has been detected in codon 13.

KRAS somatic mutations

A *KRAS* mutations was identified in 16 (16/51, 31.5%) of all the CRC tumors. There was no significant association of *KRAS* mutations with Bethesda criteria, cancer family history (Table 2) and patient's clinical and histo-pathological data (Table 3). In addition, no significant association has been detected between *KRAS* mutations and the other tumoral parameters (Table 7). However, the mutation spectra same to be different between MSS and MSI tumors and more varied mutations have been detected in MSS tumors (Figure 1). Some amino acid changes were detected only in MSS tumors (Figure 1). Whereas, the *KRAS* mutation p.Gly13Asp have been detected only in MSI-H tumors in the absence of *TP53* mutations or TP53 overexpression (Figure 1).

BRAF mutations

The *BRAF* activating mutation c.1796A>T, p.Val600Glu was found in only 1 (1/51, 2%) stage Ⅱ non-mucinous and non-invasive tumor of the proximal colon of a 79 years old man with no cancer family history. This mutation was shown to be specific to sporadic CRC tumors due to *MLH1* promoter hypermethylation and absent in CRC tumors with MSS phenotype[31] and patients with *MLH1* or *MSH2* germline mutations[11]. Because we didn't examine the entire *BRAF* gene, we cannot rule out the presence of other mutations in the remaining CRC tumors. This tumor showed peritumoral lymphatic reaction, MSS phenotype, normal MMR proteins expression and abnormal MUC5AC expression. In addition, no somatic mutations in *KRAS* or *TP53* genes or overexpression of TP53 protein were detected in this tumor. According to some authors, this tumoral phenotype characterized CRC tumors arising from serrated polyps[32].

CTNNB1 mutations

Only 1 putative pathogenic somatic mutation was detected in an MSI-H CRC tumor (1/51, 2%). The change was a typical missense mutation causing alteration of serine at codon 45 (c.134C>T, p.Ser45Phe). This patient

was operated of TNM stage Ⅱ well differentiated and mucinous adenocarcinoma of the sigmoid at 36 years of age and didn't have any relatives with cancer. Tumor analysis detected a p.Gly13Asp *KRAS* mutation and a combined MLH1 and PMS2 proteins expression loss. These findings were in accordance with the specific affinity of *CTNNB1* mutation to MSI-H CRC tumors[5,33]. In addition, normal TP53 and MUC5AC proteins expression was detected in this tumor in the absence of *TP53* or *BRAF* somatic alterations. According to Young *et al*[34], this tumoral phenotype characterises CRC in Lynch syndrome, highlighting the presence of this syndrome in this patient.

MLH1 promoter methylation

Further analysis of *MLH1* promoter methylation status in the tumor of 4 patients showing MSI-H phenotype and MLH1 protein expression loss in the absence of *MLH1* deleterious somatic mutation by MLPA or sequencing, did not detect aberrant methylation discarding the hypothesis of sporadic cancers due to epigenetic inactivation of *MLH1* gene.

DISCUSSION

The detection of subjects at high risk of CRC remains problematic. It was essentially based on the family history of patients. Nevertheless, MSI testing and MMR protein expression analysis still the major screening tool for identifying HNPCC. In the present report, we have studied the phenotype and the genetic characteristics of the CRC tumors of 51 Tunisian non related patients selected according to the revised Bethesda criteria in order to compare the tumor phenotype due to MMR deficiency with somatic alterations in genes implicated in CRC tumorigenesis. Our aim was to define for each tumor the pathway of carcinogenesis and to identify new tumoral markers which may help in the diagnosis of CRC susceptibility and easy to use in medical practice. Clinical data analysis showed that CRC was essentially right sided in patients with first or second degree CRC relatives, whereas CRC was mostly distal (left colon and rectum) in patients without cancer family history. These findings are in accordance with data published[35,36]. As expected, genetic characteristics analysis of the 51 tumors showed that MSI phenotype and MMR expression loss were significantly associated to the presence of a CRC family history ($P < 0.001$). *TP53* mutations have been detected in 59.6% of the analysed patients. This finding was in agreement with previous studies in CRC, which reported *TP53* mutation frequencies between 50% and 70%[28-30]. Our study shows statistically inverse relationships between MSI and *TP53* alterations in CRC ($P = 0.0335$). This finding was in accordance with that reported by Samowitz *et al*[29]. This data highlights the hypothesis that MMR deficient CRC tumors evolve through a pathway that is independent of *TP53* gene. *KRAS* mutations were identified in 31.5% of all CRC tumors. This is consistent with previous reports that have identified *KRAS* mutations in 30%-45% of CRC tumors[8,29,37]. No significant association had been detected between MSI phenotype and *KRAS* alterations. However, the mutation spectrum was different between MSS and MSI-H tumors. In spite of our reduced number of tumors this finding was in accordance with that reported[8]. On the other hand, abnormal MUC5AC expression was found to be significantly associated to MSI phenotype ($P = 0.0039$) and CRC personal and family history ($P = 0.039$). In contrast, no significant association was detected between MUC5AC expression and *KRAS* or *TP53* genes mutations.

In conclusion, we suggest that MUC5AC expression analysis of CRC tumors may be useful in the screening of patients with high risk of CRC.

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COMMENTS

Background

This paper tend to make correlations between family history, clinical features and mutational status of genes involved in the progression of colorectal cancer (CRC) on the track of new tumoral markers for CRC susceptibility in Tunisian patients.

Research frontiers

Authors have screened 51 CRC tumors containing mixed hereditary non-polyposis colorectal cancer (HNPCC) and sporadic CRC Tunisian cases for somatic changes in *KRAS*, *BRAF* and *CTNNB1*, for microsatellite instability, for expression of mismatch repair (MMR) and mucin (MUC) 5AC, for mutation and expression of *TP53* and for *MLH1* promoter hypermethylation. They have also compared these molecular findings with clinical, pedigree and pathological data, regardless of age, personal or family history of cancer, and tumor characteristics.

Innovations and breakthroughs

In this study, authors report for the first time in Tunisia the value of various histo-pathologic features and somatic molecular changes in distinguishing patients with HNPCC from those with sporadic colorectal cancer in the aim to identify new tumoral markers of colorectal cancer susceptibility easy to use in the design of diagnostic, therapeutic and preventive strategies in Tunisia.

Applications

MUC5AC expression was significantly associated to the presence of the presence of familial CRC, microsatellite instability and MMR expression loss. This finding suggests that MUC5AC expression analysis may be useful in the screening of patients with high risk of CRC in Tunisia.

Peer review

Screening the subjects at risk of CRC is important. In this manuscript, the authors retrospectively reviewed the histo-pathological features, molecular changes and family history of 51 Tunisian CRC patients. Among many genetic and clinical variables, MUC5AC expression was concluded to be useful in the screening of patients at high risk of CRC susceptibility.

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