original article

Molecular characteristics of colorectal cancer in a Middle Eastern population in a single institution

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BACKGROUND: The few studies of the molecular biology of colorectal cancer (CRC) in Middle Eastern populations have included only small samples of patients.

OBJECTIVE: Evaluate the frequency and prognostic effect of RAS, BRAF, PIK3CA, PTEN, and EGFR somatic mutations as well as mismatch repair (MMR) deficiency in Lebanese Middle Eastern patients.

DESIGN: Retrospective single-center descriptive study.

SETTING: Lebanese Middle Eastern patients in a tertiary medical center.

METHODS: We included all patients diagnosed with CRC between January 2010 and December 2015, in whom RAS mutational status and the expression of MLH1 and MSH2 proteins were available.

MAIN OUTCOME MEASURES: Genetic mutations detected by direct sequencing while MMR protein expression was evaluated by immunohistochemistry.

SAMPLE SIZE: 645 patients.

RESULTS: RAS, BRAF, EGFR, PI3KCA, and PTEN mutation rates were 38.5%,12.9%, 0%, 11.1% and 0% respectively. The MMR deficiency rate was 20.6%. No factor was associated with RAS mutation whereas MMR-deficient tumors were less likely to be metastatic at diagnosis. Among patients with wild-type RAS females fared better than males (median overall survival [OS]=1734 vs 1079 days respectively, *P*=.015) even after adjustment for confounding factors by Cox regression analysis. This finding was not reproduced in the RAS-mutated group. The median OS of patients with MMR-deficient tumors was not reached, while the median OS was 2475 days in patients who had maintained expression of both MLH1 and MSH2.

CONCLUSION: The RAS mutation rate was similar to Western and East Asian countries, but not for the BRAF mutation and MMR deficiency. We also found a prognostic effect for sex in the RAS wild-type group, a finding worthy of further exploration.

LIMITATIONS: Retrospective, single center and small sample size. Expression of MSH6 and PMS2 not analyzed.

CONFLICT OF INTEREST: None.

Colorectal cancer (CRC) is a global burden; its incidence has increased significantly over the last decade.[1] Progress in the molecular biology of cancer has led to better understanding of the mutational landscape of CRC and the possibility of developing targeted therapies.[2,3] Adding anti-epidermal growth factor receptor (EGFR) antibodies to chemotherapy has shown a clear benefit in metastatic CRC.[4-7] However, genetic mutations conferring resistance to these drugs have been identified; the mutations mainly affect RAS (KRAS and NRAS) and BRAF genes.[5,6] Thus, genetic analysis of RAS and BRAF have become part of standard of care in metastatic CRC.[7]

Microsatellite instability (MSI) is a hypermutability status resulting from a deficient DNA mismatch repair (MMR).[8] MSI is mainly due to somatic hypermethylation of the promoter of the MLH1 gene, but can arise through inherited mutations in one of the four genes implicated in DNA repair (MLH1, MSH2, PMS2, and MSH6). It has become crucial to define the MSI status of a tumor, on the one hand to detect patients with Lynch syndrome and on the other hand to adapt the treatment.[6,9] Immunohistochemistry (IHC) is a valid method to detect MMR protein deficiency and serves as a surrogate for MSI with a high sensitivity and specificity.[6] The frequency of RAS and BRAF mutations as well as MMR deficiency have been reported in large epidemiological studies in western and Asian countries.[10-12] However, the few studies that have been reported in Middle Eastern populations included only small samples of patients.[13-20]

With the movement toward personalized cancer management, there has been significant interest in additional biomarkers such as PIK3CA, PTEN, and EGFR mutations. Some authors have suggested that these mutations could be associated with a lack of response to anti-EGFR antibodies.[21] These molecular alterations are potential targets for new drugs in the near future.[6] The aim of this descriptive study was to retrospectively report the frequency and the prognostic effect somatic mutations of RAS (KRAS, NRAS), BRAF, PIK3CA, PTEN and EGFR genes, as well as the frequency of MMR deficiency in Lebanese Middle Eastern patients in a real-life setting.

PATIENTS AND METHOD

This study retrospectively included all patients diagnosed with metastatic CRC in a single tertiary care Lebanese medical center, between January 2010 and December 2015, in whom RAS mutational status and the expression of MLH1 and MSH2 proteins were available. RAS mutations were investigated either at the time of diagnosis or during the course of the disease for the purpose of adjusting the treatment. The expression of MLH1 and MSH2 was analysed in patients who were suspected to have Lynch Syndrome, or in order to adapt the treatment. Finally, mutations of the BRAF, PIK3CA, EGFR, and PTEN genes were analysed in patients who had refractory metastatic CRC to look for a potentially targetable mutation. Ethical committee approval was unnecessary since there was no direct contact with patients.

Gene mutation analysis was performed on paraffin-embedded tissue. DNA was extracted from selected tumor tissue area (containing at least 50% neoplastic cells) after hematoxylin-eosin (H&E) staining. Deparaffinization was performed using xylene baths followed by 100% ethanol solution then by proteinase K digestion. DNA was isolated using the QIAamp DNA Extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions, followed by PCR using specific primers for KRAS and NRAS codons 12, 13, 59, 61, 117 and 146, BRAF exon 15, PIK3CA exon 9 and 20, EGFR exons 19 and 20, and PTEN exon 5 to 9.22 Mutations were detected by standard Sanger sequencing using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) and interpreted via the ABI 3130 Genetic Analyser. Each detected mutation was verified by two independent cycle sequencing PCR reactions and bidirectional DNA analysis.

The expression of MLH1 and MSH2 was evaluated by IHC on formalin-fixed paraffin-embedded tumor tissue and adjacent normal mucosa after deparaffinization of 5-µm thick sections. Mouse monoclonal antibodies against the MLH1 protein (clone G168–728; PharMingen, San Diego, CA) and MSH2 protein (clone FE11; Oncogene Research Products, Cambridge, MA) were used according to manufacturer's instructions, both diluted 1:100. Detection was made possible by the UltraVision streptavidin-biotin peroxidase detection kit (TP-060-HL; Lab Vision Corporation, Fremont, CA). The diaminobenzidine tetrachloride was used as chromogen during the peroxidase reaction. Slides were finally counterstained lightly with Mayer hematoxylin. Nuclear immunostaining of normal epithelial cells and lymphocytes served as internal positive controls. Samples were evaluated simultaneously by two pathologists according to international recommendations (diffuse 1+, focal or diffuse 2+, and 3+ defining positive staining).[23]

All information was collected from medical records including age at diagnosis, sex, primary tumor site (right ascending, transverse, left descending colon, sigmoid/high rectal area and middle/low rectum), macroscopic tumor size (defined as maximal diameter), grade, TNM stage according the AJCC staging system seventh edi-

tion,[24] number of positive lymph nodes, and number of resected lymph nodes.

Statistical analysis was performed using the IBM SPSS software version 22 (IBM, Armonk, NY). Continuous variables are represented by their means (or medians) and standard deviations. Discrete variables are represented by proportions.

RESULTS

The study included 644 patients with a male: female ratio of 1.35:1 and a mean (SD) age at diagnosis of 62 (14) years. All but 14 patients had surgical resection of the primary tumor. The sigmoid colon/high rectum and the right ascending colon were the most frequent primary tumor sites [n=192 (29.7%) and n=163 (25.3%) respectively], followed by the middle/lower part of the rectum [n=155 (24%)], the left descending colon [n=106 (16.4%)], and the transverse colon [n=30 (4.6%)]. At diagnosis, 58 (9%), 161 (25%), 206 (32%) and 220 (34%) patients had stage I, II, III, and IV cancers.

Table 1 shows the distribution of patients by biomarkers and **Table 2** shows the molecular biology of the tumor cells**.** The RAS mutation rate was equal to 38.5%, and the frequency of loss of expression of MSH2 or MLH1 proteins was equal to 20.6%. No factor was associated with RAS mutation (**Table 3**). MMR-deficient tumors (loss of expression of MLH1 or MSH2) were less likely to be metastatic at diagnosis and occurred more frequently in the right ascending colon (**Table 4**). We could not analyse the association between RAS mutations and MMR deficiency since the expression of MLH1 and MSH2 was maintained in all patients in whom RAS mutations and MMR status were analysed simultaneously. Due to the small number of patients harboring BRAF, PIK3CA, PTEN or EGFR mutations, we could not analyze the association between these biomarkers and tumor stage at diagnosis.

Survival

After a median follow up of 1770 days, median overall survival (OS) of the whole sample was 1556 days, 883 days in the RAS-mutated group versus 1149 days in the wild-type group (log Rank *P*=.90). Among the wild-type RAS group, females fared better than males (median OS: 1734 days vs 1079 days, respectively, log rank *P*=.015) even after adjustment for confounding factors (**Table 5**). This finding was not reproduced in the RAS-mutated group (median OS: 874 days vs 883 days in females and males respectively, log rank *P*=.42) (**Figure 1**). The median OS of patients with MMR-deficient tumors was not reached, while the median OS was 2475 days in patients who had maintained expression of both MLH1 and MSH2. The median OS of the 4 patients harboring a BRAF mutation was equal to 1100 vs 900 days in the wild type group (log rank *P*=.33). The median OS of the 3 patients harboring the PIK3CA mutation was equal to 800 vs 890 days in the wild type group (log rank *P*=.64). Since no patient had a PTEN or EGFR mutation, we could not compare the median OS between a wild-type and a mutated group for these two genes.

DISCUSSION

In this study, the RAS mutation rate was equal to 38.5%, which is in the range reported by large stud-

Table 1. Number of patients by biomarkers (n=644).

Patients by biomarkers	N (%)
RAS mutation analysis	222 (34.4)
RAS mutation analysis and MSH2 and MLH1 expression analysis	52 (8.1)
MSH2 and MLH1 expression analysis	370 (57.4)

Table 2. Molecular biology of tumor cells.

Gene	Type	Number of patients	Percentage
KRAS	Wild	172	62.8
	Mutated	102	37.2[a]
NRAS[b]	Wild	70	94.6
	Mutated	4	5.4
BRAF	Wild	27	87.1
	Mutated	4	12.9
PIK3CA	Wild	24	88.9
	Mutated	3	11.1
EGFR	Wild	22	100
	Mutated	0	0
PTEN	Wild	22	100
	Mutated	0	0
MLH1	Conserved	348	82.5
	Lost	74	17.5
MSH2	Conserved	409	96.9
	Lost	13	3.1

[a]Exon 2 codon 12 mutation in 70 cases (68.6%), exon 2 codon 13 mutation in 25 cases (24.5%), exon 4 codon 146 mutation in 4 cases (3.9%) and exon 3 codon 61 mutation in 3 cases (2.9%). [b]In patients with KRAS wild type mutation.

Table 3. Association between RAS status (KRAS and NRAS) and patient tumor characteristics.

		RAS status		*P*
		Wild, n (%)	Mutated, n (%)	
Male sex		98 (58.3)	64 (61.0)	.67[a]
Age at diagnosis: mean (standard deviation)		59 (17)	62 (13)	.08[b]
Size of the tumor at diagnosis (in cm)		4.5 (2.4)	5 (1.9)	.23[b]
Tumour localization	Right ascending colon	39 (24.4)	29 (28.2)	.67[a]
	Transverse colon	6 (3.8)	4 (3.9)	
	Left descending colon	29 (18.1)	13 (12.6)	
	Sigmoid colon/high rectum	59 (36.9)	35 (34)	
	Middle/lower rectum	27 (16.9)	22 (21.4)	
Tumor stage at diagnosis	T2	7 (6.2)	2 (3.1)	.61[a]
	T3	55 (48.7)	30 (46.9)	
	T4	51 (45.1)	32 (50)	
Node stage at diagnosis	N0	25 (21.9)	16 (27.1)	.56[a]
	N1	39 (34.2)	22 (37.3)	
	N2	50 (43.9)	21 (35.6)	
Number of positive lymph nodes		5 (7)	4 (5)	.28[b]
Lymph node ratio		0.3 (0.3)	0.2 (0.2)	.09[b]
Stage IV at diagnosis	No	82 (50)	42 (40.8)	.14[a]
	Yes	82 (50)	61 (59.2)	

[a]Chi-square test of independence. [b]Independent samples *t* test.

ies conducted in the USA,[10] Europe[11,25,26] and East Asia,[27,28] as well as in international surveys.[29] Besides, the frequency of the BRAF mutation and loss of MLH1 or MSH2 protein expression were also in the range reported in Western populations.[6,8,18,19] Conversely, BRAF mutations and MMR deficiency seem to be much less frequent in East Asia.[30-32] The fact that the frequency of mutated-RAS was similar between different populations whereas the rates of mutated-BRAF and MMR deficiency differed is intriguing. Colorectal carcinogenesis is influenced by both genetic and environmental factors.[25,33,34] It is induced by multiple pathways, among others the serrated and the epigenetic pathways. The former is the MAPK-ERK pathway in which RAS mutations are acquired during early steps, promoting tumor invasion and metastasis.[35-37] On the other hand, tumors harboring BRAF mutation or MMR deficiency are triggered by epigenetic alterations mainly CpG islands hypermethylation. This phenomenon could explain why MMR-deficient tumors are less likely to harbor RAS mutations.[38-40] Furthermore, Shen et al identified three distinct CRC subgroups based on genetic and epigenetic profiling, which were designated as CpG island methylator phenotype (CIMP) 1, CIMP2, and CIMP negative.40 CIMP1 is characterized by a high frequency of MMR deficiency and BRAF mutation, but low KRAS and

Table 4. Association between loss of MLH1 or MSH2 status and patient tumor characteristics.

		MLH1 and MSH2 conserved, n (%)	Loss of expression of MLH1 or MSH2, n (%)	*P* value
Male sex		193 (57.4)	46 (52.9)	.44[b]
Age at diagnosis: mean (standard deviation)		63 (14)	60 (15)	.07[a]
Size of the tumor at diagnosis (in cm)		4.4 (1.9)	4.8 (1.9)	.09[a]
Tumor localization	Right ascending colon	77 (23.3)	29 (34.1)	.04[b]
	Transverse colon	14 (4.2)	6 (6.9)	
	Left descending colon	50 (15.2)	16 (18.4)	
	Sigmoid colon/high rectum	95 (28.8)	13 (14.2)	
	Middle/lower rectum	94 (28.5)	23 (26.4)	
Tumor stage at diagnosis	T1	20 (6.7)	3 (3.6)	.32[b]
	T2	43 (14.3)	12 (14.3)	
	T3	161 (53.7)	40 (47.6)	
	T4	76 (25.3)	29 (34.5)	
Node stage at diagnosis	N0	171 (55.9)	41 (48.2)	.37[b]
	N1	84 (27.5)	25 (29.4)	
	N2	51 (16.7)	19 (22.4)	
Number of positive lymph nodes		2 (4)	3 (5)	.08[b]
Lymph node ratio		0.2 (0.3)	Not available	Not applicable
Stage IV at diagnosis	No	232 (78.6)	74 (90.2)	.02[b]
	Yes	63 (21.4)	8 (9.8)	

[a]Independent samples *t* test; [b]Chi-square test.

P53 mutation rates. CIMP2 frequently harbors RAS mutations, but rarely BRAF, p53 mutations and MMR deficiency.[40] Therefore, CIMP2 might occur at the same rate between different populations whereas CIMP1 is mostly affected by genetic and environmental variations. This is only a hypothesis since epidemiological studies relating geographical variants, ethnicity and lifestyle to RAS, BRAF mutations and MMR deficiency reported inconsistent results.[25,41,42]

Besides, large studies in the Western world have suggested a slightly higher prevalence of RAS mutation in females as well as in patients with right-side CRC,[10,25,29] which is not the case in our study probably because of sample size. Concerning the MMR-deficient CRC, we confirmed what have been published in the literature concerning its low aggressiveness and the right-side predominance.[32] Some reports associated female gender with MMR deficiency,[43] which is not the case in our study.

The prognostic value of RAS mutations in non-metastatic CRC is not well established. This study included patients with localized and metastatic CRC at diagnosis and showed a non-statistically significant trend toward poorer survival among patients with mutated

Table 5. Cox regression model for overall survival among the wild-type RAS group.

		B	*P*	Exp (B)	95.0% CI for Exp (B)	
					Lower	Upper
Age at diagnosis		0.01	.60	1.01	0.98	1.03
Sex		0.75	.04	2.12	1.03	4.36
AJCC staging at diagnosis	T	0.15	.65	1.17	0.60	2.28
	N	0.42	.11	1.52	0.91	2.53
	M	0.68	.09	1.96	0.90	4.29
Primary tumor localization (right versus left)		-0.37	.37	0.69	0.31	1.55

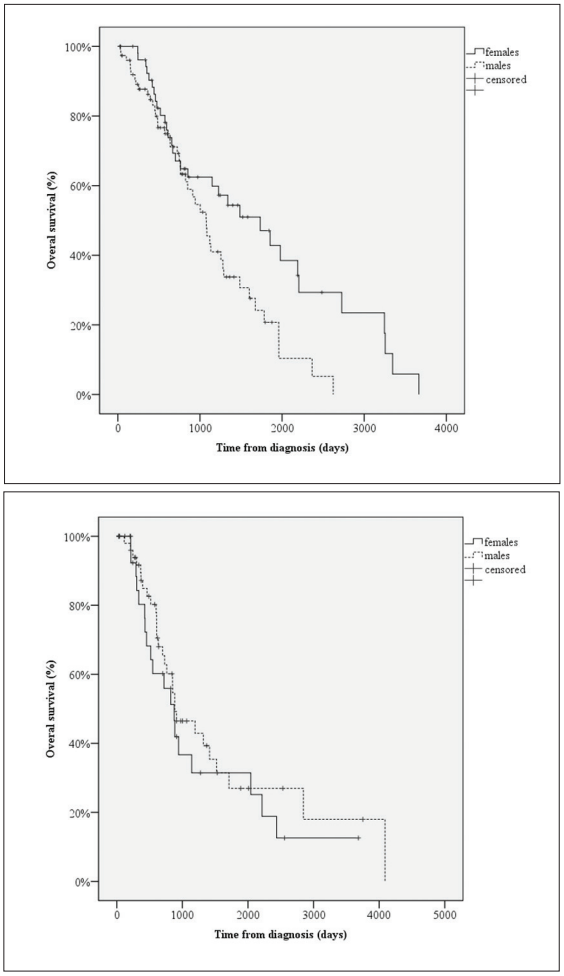

Figure 1. Survival curves from diagnosis until death comparing female with males in RAS wild (Panel A) and mutated groups (Panel B).

RAS. In a study by Phipps et al patients with invasive CRC harboring KRAS mutations had poorer disease-specific survival.[44] Yet, a systematic review and meta-analysis that included stage II and III tumors did not show a significant difference in survival.[45] One potential explanation could be derived from the expanded analysis of the RASCAL collaborative study, which reported that G12V was the only codon 12 mutation associated with worse prognosis in patients with node-positive disease.[46]

The most interesting finding in our study was the higher survival rate of females compared to males in the RAS wild-type group, even after adjustment for age, stage and primary tumor site at diagnosis. To our knowledge, no study had focused on the prognostic role of gender in patients with wild-type RAS. Controversial results relating gender to CRC survival have been reported.[47] In a German population-based study which included 164 966 cases, young females with localized CRC survived better than males from the same age group.[48] In another study, females aged less than 50 years fared better than males from the same age group, whereas the opposite was true in older patients.[49] Finally, Kooh et al reported a higher rate of sporadic right side MSI-H cancer among women in a systematic review, but there was no difference in post-adjuvant chemotherapy survival or postradiotherapy.[43] We found no study reporting a potential difference in survival between women and men who were treated with anti-EGFR therapies for mCRC.[4] Hormonal status might affect tumor growth and responsiveness to chemotherapy and should be further elucidated. Thus, the finding that females fared better than males in the RAS wild-type group deserves to be investigated even though results should be interpreted with caution.

Expression of MSH6 and PMS2 were not analysed in this study, which could be considered a limitation. However, the contribution of MSH6 and PMS2 to MSI is much less important than MLH1 and MSH2. In addition, loss of expression MSH6 and PMS2 are related to loss of MSH2 and MLH1 respectively, and would not affect the rate of MMR deficiency.[50] The sample size is another limitation of this study compared to the large studies carried out in developed countries. However, taking into account the size of the Lebanese population and the incidence of CRC of nearly 400 patients per year,[51] the size of the study sample is relatively satisfactory. Furthermore, this study is among the largest ones that have been performed in the Middle East and Arab world.[13-16,18-20] Finally, this study is mostly limited by being retrospective. The results should be interpreted with caution especially since the biomarkers were not analysed completely in all the patients, which introduced the possibility of selection bias. However, the rationale behind this study was the lack of data in the literature in Middle Eastern populations, especially in Lebanon. Therefore, its goal was to describe the frequency of molecular alterations in Lebanese CRC patients who have benefited from an analysis of these biomarkers in a real-life setting. To our knowledge, this is the first report describing the frequency and the prognostic effect of RAS and MMR deficiency in a Lebanese Middle Eastern CRC population.

In conclusion, the present study evaluated the incidence of RAS and BRAF mutations as well as MMR deficiency by IHC and found similar rates compared to Western countries. No factor was associated with mutated-RAS status, whereas MMR deficient tumors were mainly localized in the right ascending colon and were less aggressive. We also found an independent relationship between sex and survival in the RAS wild-type group. Finally, the prognostic effect of BRAF, PI3K, PTEN and EGFR mutations could not be elucidated in this study and requires a larger sample.

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