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Molecular analysis and clinicopathologic features of advanced colorectal cancer in Algerian patients

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ABSTRACT

Aims: This retrospective study aims to analyze tumors hot spot mutations frequency in KRAS, BRAF and microsatellite instability (MSI) status of tumors in Algerian patients with advanced colorectal cancer (CRC) which can predict prognosis and contribute to decisions on treatment strategies. Methods: KRAS exon 2, BRAF exon 15 were analyzed by direct sequencing of amplified PCR products in 102 tumors patients with advanced CRC cancer. The MSI was determined using a panel of five mononucleotide markers (BAT25, BAT26, NR21, NR22 and NR24). Results: BRAF and KRAS mutations were detected in 4.9% and 31.3% of the tumors patients respectively. Activating mutations in codon 12 and 13 in KRAS was located in the right colon 40.6% versus 25% in the left colon. (62.5%) with KRAS mutations are well or moderately differentiated. The aminoacid changes are more frequently observed in codon 12 (29/32) than in codon 13 (3/32) and G12D (43.8%) is the most frequent mutation. BRAF v600E mutation is observed in proximal colon in 3 of 5 tumors (60%) in patients with older age > 50 years. (53.1%). BRAF wild type tumors (79%) were associated with MSI-H. Conclusion: The results of KRAS and BRAF mutation analysis could be used in the selection of Algerian patients with CRC for anti epidermal growth factor receptor (anti-EGFR) therapy and MSI-H status associated with BRAF wild type (Wt) may be suggesting the possible presence of Hereditary non polyposis colorectal cancer (HNPCC) syndrome.

Keywords: Algeria, BRAF and KRAS mutations, Microsatellite instability (MSI), Colorectal cancer (CRC), Non polyposis colorectal cancer

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INTRODUCTION

Colorectal cancer (CRC) is a heterogeneous disease and the third most common cancer in the western countries [1]. Its incidence rate is lower in North Africa [2] but has significantly increased these last two decades. In Algeria, CRC is the second most common cancer after lung cancer in men and breast cancer in women [3]. More than half of the patients were staged III and IV at the diagnosis and are younger than the patients in the western countries [4]. The possible causes of this include genetic, environmental and lifestyle factors [5]. Diet, obesity and comorbidities such as diabetes increase the risk of developing a cancer. Several factors such as socioeconomic status, screening, diagnosis and differences in treatment can explain the different health outcomes among patients [6]. Despite progress in recent years in the treatment of CRC with the use of the anti-EGFR and anti-vascular epidermal growth factor (anti-VEGF) agents which have significantly improved the survival of CRC patients [7], mortality remains high in Algeria. Molecular biomarkers that are clinically used have become important and may provide treatment with anti-EGFR agents [8]. It has been reported that patients with mutated RAS, do not benefit from anti-EGFR therapy [9, 10]. The aim of the current study was to detect some molecular alterations MSI, BRAF, KRAS which proved to be significant prognosis and /or predictive markers in the daily clinical practice and which may help define a better management of the Algerian patients with CRC. The development of platforms for detection of molecular alterations including BRAF and RAS hot spot mutations in tumors will facilitate the prescription of target therapies in Algeria.

PATIENTS AND METHODS

Clinical data was collected from 102 Algerian patients with advanced CRC histopathologically proven and who were being treated at the medical oncology department in Pierre and Mary Curie Center, a specialized University hospital in Algiers, Algeria, between 2006–2009 and after radical surgical resection. All participants signed an informed consent and the study was approved by the ethical committee of our institution. Clinicopathological information including age at diagnosis, sex, tumor location, stage, pathological tumor staging system (p TNM/UICC), were available for all patients.

TISSUE SELECTION

Primary tumors paraffin included were cut in 4 µm sections and stained with Hematoxylin and Eosin (H&E) for histopathological examination. Formalin fixed and paraffin embedded (FFPE) tumor sections were reviewed by pathologist to confirm diagnosis and define tumor areas containing 50 to 70% tumor cellularity and areas of adjacent normal tissue (25 to 30%) which were immersed in xylene and ethanol. When necessary, the proportion of tumoral cells was maximed by macrodissection.

DNA ISOLATION

Genomic DNA was extracted using the Qiamp miniKit Qiagen following the manufacturer's recommendations (Qiagen courtaboeuf, France) and quantified by spectrophotometry with nanodrop (Thermo Fisher scientific).

MOLECULAR ANALYSIS

Microsatellite instability (MSI) status

Microsatellite markers (Bat25, Bat 26, NR 21, NR 22 and NR24) a pentaplex of mononucleotide repeat were used to evaluate MSI status by polymerase chain reaction (PCR). Instability at only one of the five markers tested was labeled Microsatellite Low (MSL). Instability for two for more was MSI-High (MSI-H) and no instability at any of the five markers tested was labeled Micro-satellite Stable (MSS). In this study MSI-L and MSS are combined into one group which is non MSI-H.

KRAS and BRAF status: Genomic DNA was analyzed by direct sequencing of amplified PCR products. (Applied Biosystem).

STATISTICAL ANALYSIS

Different variables were compared using chi-square test and Fisher student test. P-value < 0.05 was considered statistically significant.

RESULTS

In this study, we analyzed 102 advanced CRC tumors from Algerian patients. Clinicopathological features are summarized in (Table 1). The gender distribution was 58 males (56.8%) and 44 females (43.2%) (p = 830) with 48 patients (47.1%) < 50 years and 54 > 50 years (52.9%) (p = 0.830) range (18–83 years). Tumors location was distributed to the proximal (28.4%), distal (36.3%) and rectum (35.3%) (p = 0.130). Histological analysis demonstrated 41 (40.2%) well differentiated 39 (38.2%) moderately differentiated and 22 (21.6%) poorly differentiated adenocarcinomas (p = 0.130). Of the 102 tumors analyzed, KRAS mutations in exon 2 codons 12–13 were detected in 32/102 (31. 4%). The most prevalent mutation observed in codon 12 was G12D (43.8%), G12A (25%), followed by G12V (9.3%), G12C and G12S (6.3%) respectively and in codon 13 we found G13D (9.3%)

(Table 2). Analysis of KRAS mutations showed 40.6%, in the proximal colon, 25% in the distal colon and 34.4% in the rectum. Correlation of KRAS mutations with gender showed that KRAS mutations were more frequently observed in women (62.5%) than in men (37.5%), and no significant difference was found in other variables. MSI and BRAF status were determined of all colorectal tumors cases and MSI-High (MSI-H) was detected in 19 of 102 (18.6%) tumors patients analyzed. 17 of 19 (89.5%) tumors showed instability in all 5 markers used in this study. BRAF mutation was observed in 5 cases (4.9%) analyzed with a wild type (Wt) profile in KRAS. The majority of tumors MSI-H, 15/19 (79%) were BRAF Wild Type (Wt). Molecular markers included MSI status, KRAS and BRAF mutations in 102 tumors of Algerian patients analyzed are regrouped in (Table 3). As shown in (Table 4) correlation of tumor location and molecular markers shown KRAS mutation at the right colon 40.6% (13/32) versuss 25% (8/32) in the left colon and 34.4% (11/32) in the rectum. 3 of 5 tumors (60%) with v600E BRAF mutation were located significantly in proximal colon and found in patients older of 50 years.

DISCUSSION

The results of this preliminary study show that 70/102 (68.6%) Algerian patients with CRC could benefit from anti-EGFR therapy. EGFR has become an important target for treatment decisions making of CRC. So it has become important to multiply the platforms for determination of molecular alterations in tumors in Algeria, which would facilitate the prescription of target therapy in the country. Activating KRAS mutations in codons 12 and 13 have a clinical impact on patients with CRC [11] and predict resistance to anti-EGFR antibodies, cetuximab a human-mouse chimeric IgG1 and panitumumab a human IgG2 monoclonal antibodies which have been entered in the personalized treatment in patients with CRC [12]. KRAS is part of the EGFR signaling pathway downstream to EGFR. Activation of the pathway leads to the modulation of angiogenesis, cell migration, proliferation and metastasis formation. In this study, we identified 31.4% of tumors with KRAS codons 12, 13 mutations and a higher frequency in women, 20/32, (62.5%) versus 12/32 (37.5%) in males. The most prevalent mutations were observed in codon 12 with (90.7 %) than in codon 13 (9.3%). We found that the most frequent location was in the proximal colon with 40.6% versus 25% in the distal colon. No significant age difference was found in our study between patients with KRAS mutated tumors and Wt KRAS tumors and no association has been found between KRAS mutations and MSI phenotype, this is in accordance with results of other research studies [13, 14]. Recently, prospective and retrospective analyses demonstrated that patients with tumors KRAS and

Table 1: Correlation of KRAS, BRAF mutations with clinicopathological parameters in advanced and metastatic colorectal cancer of primary tumor

	KRAS Wt		KRAS Mut		TOTAL		BRAF Wt		BRAF Mut		TOTAL	
	N	%	N	%	N	%	N	%	N	%	N	%
Number of cases	70	(68, 6)	32	(31, 4)	102		97	95.1	5	(4.9)	102	
Age at diagnosis												
< 50 years	33	(47, 1)	15	(46, 9)	48	(47, 1)	41	(42.2)	2	(40)	43	(42.2)
>50 years	37	(52, 9)	17	(53, 1)	54	(52, 9)	56	(57.8)	3	(60)	59	(57.8)
Sex												
Male	46	(65, 7)	12	(37.5)	58	(56.8)	43	(44.3)	2	(40)	45	(44.1)
Female	24	(34, 3)	20	(62.5)	44	(43, 2)	54	(55.7)	3	(60)	57	(55.9)
Location												
Proximal	16	(22, 9)	13	(40.6)	29	(28.4)	26	(26.8)	3	(60)	29	(28.4)
Distal	29	(41, 4)	08	(25)	37	(36.3)	36	(37.1)	1	(20)	37	(36.3)
Rectum	25	(35, 7)	11	(34.4)	36	(35.3)	35	(36.1)	1	(20)	36	(35.3)
Differentiation												
Well	28	(40)	13	(40.6)	41	(40.2)	45	(46.4)	2	(40)	47	(46)
Moderate	32	(45.7)	07	(21.9)	39	(38.2)	39	(40.2)	2	(40)	41	(40.2)
Poor	10	(14.3)	12	(37.5)	22	(21.6)	13	(13.4)	1	(20)	14	(13.8)
Stage at III	31	(44.3)	14	(43.8)	45	(44.1)	44	(45.4)	2	(40)	46	(45)
Diagnosis IV	39	(55.7)	18	(56.2)	57	(55.9)	53	(54.6)	3	(60)	56	(55)

Proximal colon includes cecum, ascending colon, hepatic flexure, transverse colon; distal colon includes descending colon and sigmoid); Mut: Mutated- Wt: Wild type, N: Number.

Table 2: Number and type of mutations in exon 2 codons 12, 13 and corresponding aminoacids of the KRAS gene. In advanced CRC of primary tumors.

KRAS	Nucleotide acide Change	Amino acide Change	Nucleotides	Cases n = 32/102 (31, 4) N %
Codon 12	c35 G> A	p G 12 D	GGT > GAT	14 43, 8
	c35 G> C	p G 12 A	GGT > GCT	8 25
	c35 G> T	p G 12 V	GGT > GTT	3 9, 3
	c34 G > T	p G 12 C	GGT > TGT	2 6, 3
	c34 G > A	p G 12 S	GGT > AGT	2 6, 3
Codon 13	c38 G> A	p G 13 D	GGC > GAC	3 9, 3

A:Alanine, C: Cysteine, D:Aspartate, S:Serine, V: Valine.

Table 3: MSI status, mutations in exon 2, codons 12, 13 of the KRAS gene and exon 15 v600E BRAF gene in advanced CRC of primary tumors.

MSI STATUS		N = 102	
MSI-H		19	(18.6%)
NON MSI-H		83	(81.4%)
KRAS MUTATION	**CODONS 12-13**	**N = 102**	
KRAS Mut		32	(31.4%)
KRAS Wt		70	(68.6%)
SPECIFIC KRAS MUTATION		**N = 32**	
CODON 12		29	(90.6%)
CODON 13		3	(09.4%)
BRAF MUTATION V600E 1799 T > A		**N = 102**	
BRAF Mut		5	(04, 9%)
BRAF Wt		97	(95, 1%)
MSI – H		**N = 19 of 102**	
KRAS Mut		6	(31.6%)
KRAS Wt		13	(68.4)
BRAF Mut		04	(21%)
BRAF Wt		15	(79%)
NON MSI-H		**N = 83 of 102**	
KRAS Mut		26	(31.3%)
KRAS Wt		57	(68.7%)
BRAF Mut		01	(01.2%)
BRAF Wt		82	(98.8%)

MSI-H high frequency of microsatellite instability, NON MSI-H regrouped MSI-S microsatellite-stable and MSI- L microsatellite low frequency, Mut: Mutated, Wt: Wild type, N: number.

Table 4: Correlation of tumor location and molecular markers in advanced and metastatic CRC of primary tumors.

	Proximal		Distal		Rectum		Total
	N	%	N	%	N	%	
	29	(28.4)	37	(36.3)	36	(35.3)	102
MSI STATUS							
MSI-H 19 of 102	12	(63.2)	3	(15, 8)	4	(21)	19
NON MSI-H 83 of 102	17	(20.5)	34	(41)	32	(38, 5)	83
KRAS							
KRAS Mut 32 of 102	13	(40.6)	8	(25)	11	(34.4)	32
KRAS Wt 70 of 102	16	(22.9)	29	(41.4)	25	(35.7)	70
BRAF							
BRAF Mut 5 of 102	3	(60)	1	(20)	1	(20)	5
BRAF Wt 97 of 102	26	(26.8)	36	(37, 1)	35	(36, 1)	97

MSI-H high frequency of microsatellite instability, NON MSI-H regrouped MSI-S microsatellite-stable and MSI- L microsatellite low frequency, Mut: Mutated, Wt: Wild type, N: number.

NRAS Wt in exons 2, 3 and 4 predict benefit from anti-EGFR therapy associated with chemotherapy [15, 16]. The KRAS mutations are also detected in lung [17], pancreatic and cervical cancers and the anti-EGFR therapies have shown to be effective in patients with CRC. The prognostic role of KRAS mutations is more debated, and has been associated with a worse prognosis in some studies [14]. The BRAF Wt is also required for response to cetuximab or panitumumab [18] suggesting that BRAF analysis should be used with KRAS for the selection of the patients [19]. We observed that activating mutation of BRAF was (5%) in this study whereas it is about 10–20% in the majority of studies performed on sporadic CRC in western countries [20]. This mutation was found to be more frequent in the right colon and old age at presentation 3/5 (60%). However, no activating mutation has been observed at codon 600 of BRAF in 88 cases analyzed in western Africa (Ghana) with a high frequency of MSI-H [21] and the highest frequency is reported in the United States (21%). A low incidence was observed in Taiwan 1% [22], in Morocco 1.6% [23] and 5.4% [24], in Tunisia 2% [25], in Saudi Arabia 2.5% [26], in China, 3.8% [27], Japan, 6.5% [28], Korea and 9.6% [29]. These variations could be attributed to ethnic differences and the effect of other environmental and genetic factors. The implication of genetic factors in a population where the overall incidence of CRC is low, would suggest a greater proportion of familial versus sporadic cases. More studies are needed to confirm these differences. The BRAF mutation detection could have been also influenced by the mutation analysis methodology. Variety of methods including Sanger sequencing, pyrosequencing, high resolution melting, allele -specific PCR and new generation sequencing have been used and may have contributed to the wide variations in the prevalence of those mutations. The BRAF mutation was proposed as a marker to discriminate between sporadic cancer and HNPCC labeled also Lynch syndrome [30]. Its incidence which is 3–5% of CRC cases in western countries is higher in Algeria (7–10%) [4]. BRAF mutation is associated with MSI-H through its relationship to high-level CpG island methylator phénotype (CIMP) and with worse prognosis [31, 32]. The prognostic value of MSI is influenced by the BRAF status which is a genetic consequence of a MisMatch Repair genes (MMR) defect [33]. Other studies suggested that the prognostic effects of BRAF mutations depended on the MSI status [34, 35]. A negative prognostic effect of BRAF mutations was reported only for MSS patients but not for patients with MSI [36] and a predictive effect of BRAF for response to anti-EGFR therapy in metastatic colorectal cancer is not required for treatment decision but it may be useful as a prognostic factor and could be used for better management of patients with CRC because of its implication on microsatellite instability [14]. Our results showed approximately 80% tumors MSI-H / BRAF Wt and suggest the possible presence of HNPCC syndrome. MSI-H tumors in CRCs may be sporadic or associated with Lynch syndrome and germline mutation analysis is required for tumors MSI-H that are BRAF wild type because mutations in the BRAF gene was found in sporadic MSI-H tumors but not in HNPCC syndrome [37, 38].

CONCLUSION

In conclusion, we have found that the clinicopathologic characteristics in Algerian patients with CRC are similar

to those reported in other studies. As a result, a total of 70% Algerian patients could benefit from anti-EGFR therapy. RAS testing had an impact on therapeutic strategy and must be realized in all oncology departments in Algeria. In order to reduce the time of the process and to prescribe targeted therapy for the patients with CRC in the daily clinical practice. A limitation of this study is the absence of data of KRAS exons 3–4 and NRAS exons 2-3-4 which have been established as predictive markers of the response to EGFR-targeted therapy. Our study suggests also the possibility of the presence of the HNPCC syndrome and use of BRAF molecular analysis is only a step before germline genetic testing. Screening program should be set up to determine the real incidence rate of the HNPCC which tends to be more frequent in Algeria than in western countries. Further studies including a large number of patients are needed to confirm our results.

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Author Contributions
Kenza Boudida-Berkane – Substantial contributions to conception and design, Acquisition of data, Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Hind Benchaa – Analysis and interpretation of data, Revising it critically for important intellectual content, Final approval of the version to be published
Sonia Ait Younes – Analysis and interpretation of data, Revising it critically for important intellectual content, Final approval of the version to be published
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Guarantor
The corresponding author is the guarantor of submission.

Conflict of Interest
Authors declare no conflict of interest.

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