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RAS Mutations in Colorectal Cancer in Tunisian Population

Ghada Sahraoui[1,2]*, Rahma Yaïche[1], Hayet Douik[1,2], Lamia Charfi[1,2], Houyem Khiari[3], Montassar Ghalleb[4], Feriel Ksentini[5], Karima Mrad[1,2] and Raoudha Doghri[1,2]

[1]*Pathology Department, Salah Azaiez Institute, Tunisia*

[2]*Research Laboratory LR21SP01, Salah Azaiez Institute, Tunisia*

[3]*Preventive Medecine Department, Salah Azaiez Institute, Tunisia*

[4]*Surgical Oncology Department, Salah Azaiez Institute, Tunisia*

[5]*Medical Oncology Department, Salah Azaiez Institute, Tunisia*

***Corresponding author:** Ghada Sahraoui, Pathology Department, Salah Azaiez Institute, Tunis, Tunisia

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ABSTRACT

Background: Colorectal cancer (CRC) is the third most common cancer worldwide. Anti-EGFR therapy is currently one of the targeted therapies for metastatic CRC. Mutations in the RAS gene are predictive of poor response to anti-EGFR. Approximately 50-60% of CRCs have a RAS mutation with KRAS being the most frequently mutated gene. We aimed to analyse RAS mutations, using Idylla KRAS and NRAS mutation test, in CRC to determine the group of patients potentially candidates for anti-EGFR therapy. Besides, we tried to identify statistically significant associations between some clinicopathological parameters and the different types of KRAS mutations detected.

Methods: A series of 119 cases of CRC was enrolled the Pathology department of Salah Azaiez Institute of Tunis from 02 April 2021 to 01 November 2021. Idylla KRAS and NRAS mutation test detected 21 mutations on the KRAS gene (exons 2, 3 and 4) and 18 mutations on the NRAS gene (exons 2, 3 and 4).

Results: RAS gene mutations were found in 54% of cases, mainly of KRAS type (98.4%). For KRAS mutations, exon 2 mutations were found in 87.3%, followed by exon 4 (8%) and exon 3 (4.7%). Mutations in exon 2 involved codon 12 (56% G12D, 23.2% G12V, 9.3% G12C, 4.6% G12A, 4.6% G12S, 2.3% G12R) in 68% and codon 13 (G13D then G13V) in 32%. Mutations in exon 3 concerned codon 61 (the Q61H mutation followed by the Q61RL mutation). Mutations in exon 4 affected codon 146 (the A146P/T/V mutation). The only NRAS mutation found was a G12D mutation. Our work revealed that statistically, the presence of KRAS mutations was significantly associated to male patients (p=0.03) on one hand and to the presence of distant metastasis (p=0.03) on the other hand.

Conclusion: Ras gene mutations seem to play a pivotal role in the resistance to anti-EGFR monoclonal antibodies in CRC treatments. Thus, testing for these mutations has become essential to select eligible candidates to benefit from this targeted therapy without unwarranted toxicity and expenses.

Keywords: Colorectal Cancer; Mutation; KRAS; NRAS; Molecular Biology

Introduction

Colorectal cancer (CRC) ranks among the third most common cancer worldwide, with more than one million patients diagnosed each year, 50% of whom will go on to develop metastatic disease [1,2]. The treatment of metastatic colorectal cancer has evolved significantly over these recent years with the development of targeted therapies including anti-EGFR treatment. These latter inhibit the activation of the epidermal growth factor receptor (EGFR) and thereby of the downstream signal transduction cascades (RAS/RAF/MAPK and PI3K/AKT/mTOR), known to be responsible for cell proliferation, resistance to apoptosis, tumor invasion and metastatic progression [3]. RAS proteins are part of the GTPases family. There are 4 isoforms encoded by three different RAS genes: KRAS (Kirsten RAS), HRAS (Harvey RAS) and NRAS (Neuroblastoma RAS). These oncogenic RAS proteins play a central role as molecular transmettors of extracellular signals to the nucleus from activated membrane receptors, in particular EGF-Rs (Epidermal Growth Factor Receptors) [3,4]. Any genetic alteration of intracellular effectors of the EGFR pathway could be involved in the response to this therapy. Therefore, RAS gene mutations are predictive of a poor response to anti-EGFR therapy, thus representing a poor prognostic factor in the treatment of metastatic CRC. Approximately 50-60% of CRC patients have a RAS mutation [5]: the KRAS gene is the most frequently mutated (40-50% of cases), NRAS gene mutations are found in about 5-8% of cases and no mutation of the HRAS gene has been described.

Since only patients with RAS wild type tumors can effectively benefit from targeted anti-EGFR drugs (Cetuximab or Panitumumab), RAS gene mutation testing has therefore become an essential criterion in choosing the optimal treatment for metastatic colorectal cancer. This search for mutations is based on molecular biology techniques from tumor samples on biopsies or abdomino-pelvic amputation or colectomy specimens [6]. Through this work, we sought to study the mutational profile of RAS genes in metastatic CRC in order to determine the group of patient's candidates for anti-EGFR treatment. Moreover, we attempted to identify statistically significant associations between certain clinicopathological parameters such as age, gender, tumor site, histological grade, tumor infiltration (pT), lymph node invasion (pN), presence of distant metastasis, and the different types of KRAS mutations detected.

Materials and Methods

Patients

From April 2021 to November 2021, 119 formalin-fixed paraffin-embedded (FFPE) samples of primary tumor or metastasis from mCRC were prospectively gathered from different Tunisian centers of pathology, from both public and private sectors. All samples were assessed for KRAS, NRAS and BRAF mutations in the routine management of their cancer. Clinicopathological and epidemiological including the following features: age, sex, tumor location, histological type, degree of differentiation, depth of invasion, TNM stage were collected for each patient referring to their pathological records. All patients gave their consent for the detection of tumor mutations of KRAS, NRAS and BRAF genes. All data were anonymized prior to analysis for this study. This study has been approved by the ethical and scientific board of Salah Azaiez Institute of Oncology.

Inclusion Criteria

All patients presenting a CRC of Lieberkuhnian adenocarcinoma histological type, at the stage of lymph node metastasis (N+) and / or distant metastasis (M+).

Exclusion Criteria

All patients presenting a histological type of CRC other than Lieberkuhnian adenocarcinoma.

Idylla KRAS And NRAS-BRAF Mutation Test

Before performing the mutations screening, a "pre-analytic" slide corresponding to an extra FFPE slide from N+ and/or M+ lieberkhunian colorectal adenocarcinomas was systematically prepared for hematoxylin-eosin stain in order to assess the tumor nuclei content by a pathologist to ensure the presence of tumor cells in the analyzed samples and identify the tumor contingent to be studied molecularly. The technique used is Idylla TM from Biocartis. The Idylla platform is a fully cartridge-based automated molecular PCR platform: multiplex real-time PCR, which uses microfluidics processing with all reagents on-board. It combines 2 technologies: real-time PCR and a new technology called PlexPrimes/PlexZymes. The steps integrated in the cartridge are as follow:

Liquefaction: Homogenization of solid samples using chemical reagents, enzymes, by heating and use of high intensity focused ultrasound (HIFU).

Cell lysis: Destruction of cells containing the nucleic acids to be analysed using chemical reagents, enzymes, by heating and HIFU.

DNA/RNA Extraction: Filtration of the lysed sample then silicate extraction, purification, and concentration of nucleic acids.

Real-Time PCR Amplification and Detection: Real-time amplification in five individually controlled PCR chambers, each containing 25µl of eluate.

a) Primer/probe mixes and enzymes are detected in all PCR chambers, including the primer/probe mix to detect/amplify a sample processing control (for example endogenous control).

b) Detection of 6 different fluorophores per PCR chamber, allowing identification of up to 30 different molecular targets in standard mode in each Cartridge which will be subsequently inserted into the Idylla TM system. We used two different

cartridges for this study. Idylla KRAS mutation test detects 21 mutations on the KRAS gene (exons 2, 3 and 4) and the Idylla NRAS-BRAF mutation test detects 18 mutations on the NRAS gene (exons 2, 3 and 4) and 5 on the BRAF gene (exon 15). Briefly, FFPE tissue section was "sandwiched" in filter papers and introduced in the cartridge according to manufacturer's protocol. The tissue area of the FFPE specimen should minimally be 50 mm2 when 5 µm FFPE tissue sections are used or 25 mm2 when 10 µm FFPE tissue sections are used. If tissue area with one section is less than required, multiple FFPE tissue sections will be employed. All samples in this study have been run in accordance with the manufacturer's recommendations. After 130 min for Idylla KRAS mutation test and 110 min for Idylla NRAS-BRAF mutation test, final reports were directly available on the Idylla console.

3 Kinds of results are possible:

a) "No mutation detected"

b) "Mutation detected in KRAS (or NRAS or BRAF) indicating the codon mutated and the nature of base change.

c) "Invalid result".

For "invalid result" in KRAS test, we made the choice to perform a new test (using another cartridge) for the same sample to avoid any risk of bad manipulation.

Then, for « invalid » or « mutated » results, the process was stopped, and no further test was assessed. For samples with no KRAS mutation detected, an Idylla NRAS-BRAF mutation test was performed following the same conditions.

Statistical Analysis

All statistical analyses were performed using SPSS software, version 20. Associations between KRAS mutation status and clinicopathological parameters were tested with the chi-square (χ^2) test. A probability (p) value of less than 0.05 was considered as statistically significant.

Results

Clinical Characteristics

Among our 119 CRC patients, more than a half were male (59%, 70/119) versus 41% (49/119) of female. The mean age was of 58 years old, ranging from 27 to 83 years old.

Tumor Characteristics

The tumor was located in the rectum in 31 cases (26%), in the left colon in 31 cases (26%), in the right colon in 20 cases (16.8%) and in the colon without specifying which side in 8 cases (6.8%). The site of the primary tumor was not indicated in 29 cases (24.4%). Histologic classification of tumors was based on the international TNM staging system, the 8th edition of the American Joint Committee on Cancer (AJCC; 8th edition) [7,8]. Tumors were mostly low grade in 85 cases (71.4%) and high grade in only 9 cases (7.6%). Grade was not indicated in 25 of the pathology reports though. Tumor infiltration within the colorectal wall (pT) was classified as pT3 in 26 cases (22%) and pT4 in 23.4%. the latter was distributed as follow: pT4a in 15 cases (12.6%), p T4b in 9 cases (7.5%) and pT4 (unspecified) in 4 cases (3.3%). Infiltration was not specified in 65 cases (54.6%). As for regional lymph node involvement, it was classified as pN0 in 13 cases (11%), pN1 in 15 cases (12.7%) among which 3 cases (2.5%) specified as pN1a and 8 cases (6.8%) as pN1b and pN2 in 21 cases (17.6%) among which 9 cases (7.6%) categorized as pN2a and 8 cases (6.8%) as pN2b. Lymph node invasion status not specified in 69 cases (58.5%). Distant metastases were present (M1) in 37 cases (31.4%) and unspecified (Mx) in 81 cases (68.6%).

Mutational Profile of Ras Genes

RAS genes mutation were found in 64/119 cases of CRC patient samples (54%), involving almost entirely KRAS gene in 63/64 cases (98,4%). However, NRAS mutation was only detected once. The distribution of KRAS and NRAS mutations in the 64 CRC patient samples is summarized in Table 1.

Table 1: Distribution of KRAS and NRAS mutations in the 64 RAS gene mutated CRC patient samples.

Gene	Exon	Codon	Numbers of Mutation (% of 64 Ras Mutation)	Type of Mutation
KRAS	2	12	43 (67%)	G12D, G12V, G12C,G12A,G12,G12R

KRAS	2	13	12 (19%)	G13D, G13V
KRAS	3	61	3 (5%)	Q61H, Q61RL
KRAS	4	146	5 (8%)	A146P/T/V
NRAS	2		1 (1%)	G12D

Mutational Profile of KRAS Gene

Among KRAS mutations, exon 2 mutations were the most common (55/63), accounting for 87.3% of KRAS mutations, followed by exon 4 mutations (5/63 cases, i.e. 8%) and finally exon 3 mutations (3/63 cases, i.e. 4.7%).

Exon 2 Mutations: Thus, in a total of 63 cases of KRAS mutations, exon 2 mutations occurred in 55 Cases And Were Distributed as Follows: 43 (68%) were identified in codon 12, and 12 (32%) were detected in codon 13. The most prevalent codon 12 mutations were of type G12D representing 56% (24/43) of all exon 2 codon 12 mutations, followed by G12V at 23.2% (10/43), then G12C at 9.3% (3/43). G12A and G12S mutations accounted for 4.6% (2/43) each and only one mutation of G12R type was observed (2.3% i.e. 1/43). As for codon 13 mutations, the G13D type was predominant, found in 11/12 cases. The G13V mutation was found in only one case.

Exon 3 Mutations: The exon 3 mutations, noted in 3 cases (4.7%) as mentioned above, were located in codon 61 and were of type Q61H in 2 cases and Q61RL in 1 case.

Exon 4 Mutations: Mutations in exon 4, detected in 5 cases (8%) all concerned the codon 146 and were of A146P/T/V type.

Mutational profile of the NRAS gene: The one NRAS mutation identified was on the exon 2 and matched a mutation of G12D type.

Statistical analysis of the associations between The KRAS mutation and clinicopathological parameters of patients: Our work revealed a statistically significant association between the detection of KRAS mutations and the gender of the patients (p=0.03) with a predominance in male patients in one hand, and metastatic CRC (p=0.03) on the other hand. However, there was no significant correlation between KRAS mutation and patients age (p=0.8), tumor site (p=0.4), histological grade (p=0.8), tumor pT stage (p=0.8) and lymph node metastasis (p=1). (Table 2).

Table 2: Correlation between KRAS mutations and clinicopathological parameters.

Clinicopathological Parameters	Number		KRAS Profile	
		Wild type (n=55)	Mutant type (n=64)	P value
Age	110	-	-	P=0.8
Gender	119			
Male	70	35	35	P=0.03
Female	49	20	29	
Tumor location	90			P=0.4
Rectum	31	16	15	
Left colon	31	17	14	
Right colon	20	9	11	
US colon	8	4	4	

	94			
Histological grade				
	85		47	
Low grade	9	38	4	
High grade		5		P=0.8
	55			
pT stage				
	26			
p T3	29	11	15	
p T4	49	11	18	P=1
p N stage				
p N0	13	4	9	
p N1	15	6	9	P=0.8
p N2		9	12	
	21			
distant metastasis (M)				
	119			
M1		14	24	P=0.03
Mx	38	41	40	
	81			

Discussion

According to the literature, the KRAS and NRAS genes are well-known oncogenes in CRC. Their mutations predict a poor response or even resistance to targeted therapy with anti-EGFR drugs [1]. In our series, KRAS mutations were found in 54% of cases. These findings are perfectly consistent with those of the literature where these mutations are reported in 40 to 50% of metastatic CRCs. The KRAS mutation was the most common one, found in 98.4% of mutated cases, which is supported by numerous studies in the literature [2,3]. The different bibliographic works report that KRAS mutations concern up to 89% of exon 2. This was well confirmed in our series where exon 2 mutations reached 87.3% [9]. Some studies have shown that mutations outside exon 2 are associated with shorter overall survival and relapse-free survival with Panitumumab-FOLFOX4-based therapy. These same studies report that, despite their low prevalence, non-exon 2 mutations are considered predictors of poor response to cetuximab and panitumumab-based treatments [10].

Along with previous reports through the literature, the present study demonstrated that the majority of exon 2 KRAS mutations occurred in codon 12 (68%) and codon 13 (32%). Additionally, the investigation for mutations in these sites revealed the presence of two heterozygous mutations: G12D at codon 12 and G13D at codon 13. For these latter mutations, it has already been established that Erlotinib/Gefitinib treatments, which are EGFR-TKIs (tyrosine kinase inhibitor), are not effective in metastatic colorectal cancer [9]. However, treatment with EGFR inhibitors does not seem to have the same prognostic value in these metastatic forms, depending on the type of mutation identified. Indeed, in patients with the G13D mutation, therapy with cetuximab (EGFR inhibitor) combined with chemotherapy resulted in improved overall and relapse-free survival compared to patients harbouring other KRAS mutations, whereas no significant difference was found for patients with wild-type KRAS profile [10]. Previous studies have exhibited a higher frequency of G12A, G12V and G13A mutations. However, the most common mutations in our series were G12D for codon 12 (54%), followed by G12V (23.2%) and G12C (9.3%); while G13D is the most found one on codon 13.

Interestingly, rare KRAS mutations were detected in our investigation: exon 3 (4.7%) and exon 4 (8%) mutations. The prevalence

of these mutations in our study are higher than those reported in several series in the literature where exon 3 mutations were around 0.5% and exon 4 mutations accounted for about 2.5%. This may be attributed to the fact that we included locally advanced and metastatic CRC on one hand, and that we used a more sensitive and specific molecular biology technique (real-time PCR combined with PlexPrimes/PlexZymes) [11]. Unlike the frequency ofKRAS mutations, NRAS mutation is rare. Among our cases, only one NRAS mutation was found (1.6%). These results are close to the literature values (2- 7%). The mutation concerned exon 3 or exon 2. The one we detected was indeed located in exon 2 (G12). Besides, the NRAS mutation can, according to the literature, coexist with the KRAS mutation. This was not confirmed in our series [1]. In our work, we investigated the correlation between RAS mutational status and different clinicopathological features. Indeed, we pinpointed a statistically significant association between the KRAS mutation and the gender of the patients (p=0.03). The mutation seems to arise more in male patients. Moreover, we noted a statistically significant correlation between the KRAS mutated profile and the presence of distant metastasis (p=0.03). KRAS mutations would be more present in metastatic CRC, which aligns with the fact that KRAS mutations are of poor prognosis in CRC [12]. In the literature, it has been shown that mutated KRAS status correlates with female gender, distal colon, moderate degree of differentiation, presence of neoplastic vascular emboli and advanced TNM stage [9,12].

In our investigation, regarding the other clinicopathological parameters studied (age, tumor site, histological grade, p T and pN), our statistical analysis did not reveal significant associations. Besides, our series lacked other histopronostic factors in CRC such as tumor size, neoplastic vascular emboli, perineural sheaths, clearance... Therefore, their correlations with KRAS mutations could not be explored. KRAS mutations correlate with poor survival in a series of colon cancer patients. Patients with a KRAS mutation have a significantly worse prognosis than those without mutations [12].

Conclusion

we highlighted through this work mutational profile of KRAS and NRAS gene in a cohort of 119 CRC Tunisian patients and the potential correlations to various clinicopathological parameters. Our findings revealed both similarities and differences when contrasted to those reported in the literature. The strength of our work relies on the study of the genetic profile of the third world population in general and specifically the Tunisian population, which is poorly known because of the difficulties of access to molecular biology in oncology centers. Hence, our investigation is a draft to mutations study known for their predictive value in cancerology, more precisely in the selection of suitable candidates for specific therapies and preventing the waste of ineffective ones economically and regarding the patient's health. Furthermore, the molecular biology technique we used (real-time PCR combined with PlexPrimes/PlexZymes) seems to be a sensitive and specific tool for the screening of somatic gene mutations which were the focus of our present study. Nevertheless, our main weakness consisted essentially in the numerous missing data involving the patient information provided in the pathology reports which represent prognostic factors that can be correlated to the mutational profile. In the near future, it is likely that other biomarkers will be used in clinical practice, on which further studies could focus on, given the importance of such molecular assessment. In this context, a few avenues are widely open, including the PI3K / AKT pathway, activated by EGF-R, and C-MET, a tyrosine kinase receptor that could be a biomarker of poor prognosis and secondary resistance to anti EGF-R, thus representing a therapeutic target under evaluation, at this date, in advanced CRC.

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Data Availability Statement

Data will be made available on request.

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