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Research Article

A SIMPLE METHOD FOR DETECTION OF *KRAS* AND *BRAF* HOTSPOTS MUTATIONS IN PATIENTS WITH COLORECTAL CANCER

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ABSTRACT

Objective: Accurate mutation detection assays for *KRAS* and *BRAF* genes in colorectal cancer are strongly needed. We describe a simple and reliable technique for determination of *KRAS* and *BRAF* mutational status, and we estimate the *KRAS* and *BRAF* mutations frequency in Moroccan patients with colorectal cancer.

Methods: Forty-seven samples from patients with metastasic colorectal adenocarcinomas were studied for *BRAF* exon 15 and *KRAS* codons 12 and 13 mutations. Tumor tissue was removed by manual macrodissection from formalin-fixed paraffin-embedded tissues specimens. After DNA extraction, conventional PCR was performed and the DNA was analyzed by direct sequencing.

Results: *KRAS* codon 12 or 13 mutations were present in 51% of patients. Gly12Val mutation was present in 21% of all patients, Gly12Asp in 15%, Gly13Asp in 6%, Gly12Arg in 4%, Gly12Cys in 2% and Gly12Ala in 2%. No *BRAF* mutation was detected.

Conclusion: Our data suggest that *KRAS* mutations are more frequent than *BRAF* mutations in Moroccan patients with colorectal carcinomas. To our knowledge, we are the first to report such a high proportion (more than 50%) of potentially non responsive patients for the anti-EGFR treatment in Morocco. These results show that the method we used was accurate, cost-effective and time-efficient.

Keywords: Colorectal cancer, KRAS, BRAF, PCR, Sequencing, Personalized Medicine.

INTRODUCTION

Cetuximab (Erbitux®, ImClone Systems) and panitumumab (Vectibix®, Amgen) are monoclonal antibodies that bind to the epidermal growth factor receptor (EGFR), preventing intrinsic ligand binding and activation of downstream signaling pathways essential for cancer cell proliferation, invasion, metastasis, and stimulation of neovascularization.

KRAS is a G-protein that cycles between active (KRAS-GTP) and inactive (KRAS-GDP) forms, in response to stimulation of the EGFR. This protein acts as a binary switch between the cell surface and the downstream signaling pathway. The *KRAS* gene can harbor oncogenic mutations, most commonly codon 12 and 13 (exon 2) missense mutations, that result in a constitutively active protein, independent of EGFR ligand binding, and making the fixation of antibodies to the upstream EGFR ineffective [1-5]. *KRAS* mutations occur in approximately 30%-50% of colorectal cancer tumors [5,6]. *BRAF* encodes a serine/threonine kinase, which is involved in intracellular signaling and cell growth. It is the principal downstream effector of KRAS. The most frequently reported *BRAF* mutation is a valine-to-glutamic acid V600E substitution (exon 15) [7,8]. *BRAF* V600E mutation occurs in 5-10% of colorectal cancer and appears to be a marker of poor prognosis [8-13]. It has been shown that patients whose tumors express the mutated *KRAS* do not respond to cetuximab or panitumumab [1, 14-16]. Nevertheless, there are still some patients with *KRAS* wild-type tumors that do not respond to these agents, suggesting that other factors, such as alterations in other EGFR effectors could drive resistance to anti-EGFR therapy, therefore, *BRAF* mutations are now increasingly being investigated in metastatic colorectal cancer [9-17]. *KRAS* and *BRAF* mutations are considered to be mutually exclusive [17,18]. There is no standardized method for *KRAS* and *BRAF* mutations testing, sequencing is considered the gold standard for the detection of these mutations. The aim of this study is to develop an accurate molecular process to screen *KRAS* and *BRAF* hotspots in Moroccan patients with colorectal cancer in order to provide reliable results to the oncologists with the shortest delay, contributing to the best care that can be provided to the patients.

MATERIALS AND METHODS

FFPE Specimens

We assessed 47 formalin-fixed paraffin-embedded specimens from metastatic colorectal cancer patients (26 males and 21 females; mean age, 61.6 ± 12 years). All patients had a histologically confirmed metastatic colorectal adenocarcinoma and underwent surgical resection of their primary tumor.

Manual Macrodissection

10µm-thick serial sections were macrodissected manually after hematoxylin–eosin slide qualification by a pathologist who carefully marked the most dense tumor area on the sections, ensuring a minimum of 70% tumor tissue content and avoiding as much as possible necrotic and hemorrhagic areas and extracellular mucous aggregates. Tumor tissues were collected in Eppendorf® vials. The tissue was washed several times in xylene and centrifuged to dissolve the wax.

DNA extraction and evaluation

DNA was extracted manually with the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration (µg/ml) and absorbance (A260/280 ratio) were measured in a UV spectrophotometer NanoDrop 8000 (Thermo Scientific, Wilmington, DE, USA).

Polymerase Chain Reaction (PCR)

ACTB

Amplification of the β-actin gene *ACTB* served as an internal control to validate the PCR reaction. *ACTB* is an unregulated, stable and constitutively expressed gene. It encodes the ubiquitous β-actin protein. Detection of this gene on the gels gives an idea of the amount and quality of the DNA. The primers used to amplify the *ACTB* gene were 5'-TGCGTGACATTAAGGAGAAG-3' and 5'-CTGCATCCTGTCGGCAATG-3'.

The characteristics are shown in the Table 1:

Table 1: Characteristics of the *ACTB* primers

Primer	Sequence	Length (bp)	Tm (°C)	%GC	Size of the PCR product
ACTB-F	5'-TGCGTGACATTAAGGAGAAG-3'	20	52.0	45%	
ACTB-R	5'-CTGCATCCTGTCGGCAATG-3'	19	57.0	58%	316bp

The PCR program for the *ACTB* gene amplification has been established as shown in the Table 2:

Table 2: PCR program for the *ACTB* gene amplification

Step	Temperature	Time	Number Of cycles
Initial denaturation	95°C	4min	1
Denaturation	95°C	30sec	
Annealing	55°C	30sec	35
Extension	72°C	1min	
Final extension	72°C	7min	1
	4°C	∞	

KRAS

The primers used to detect the *KRAS* hotspots mutations were 5'-AAGGCCTGCTGAAAATGACTG-3' and 5'-CAAAGAATGGTCCTGCACCAG-3'. The characteristics of these primers are shown in the Table 3:

Table 3: Characteristics of the *KRAS* primers

Primer	Sequence	Length (bp)	Tm (°C)	%GC	Size of the PCR product
KRAS-F	5'-AAGGCCTGCTGAAAATGACTG-3'	21	55.6	48%	
KRAS-R	5'-CAAAGAATGGTCCTGCACCAG-3'	21	57.6	52.38%	173bp

The PCR program for the *KRAS* sequence amplification has been established as shown in the Table 4:

Table 4: PCR program for the *KRAS* sequence amplification

Step	Temperature	Time	Number Of cycles
Initial denaturation	95°C	4min	1
Denaturation	95°C	30sec	
Annealing	**54°C**	30sec	35
Extension	72°C	1min	
Final extension	72°C	7min	1
	4°C	∞	

BRAF

The primers used to detect the *BRAF* hotspot mutation were 5'-CTCTTCATAATGCTTGCTCTGATAGG-3' and 5'-TAGTAACTCAGCAGCATCTCAGG-3'. The characteristics of these primers are shown in the Table 5:

Table 5: Characteristics of the *BRAF* primers

Primer	Sequence	Length (bp)	Tm (°C)	%GC	Size of the PCR product
BRAF-F	5'-CTCTTCATAATGCTTGCTCTGATAGG-3'	26	58.0	42.31%	
BRAF-R	5'-TAGTAACTCAGCAGCATCTCAGG-3'	23	57.8	47.83%	250bp

The PCR program for the *BRAF* sequence amplification has been established as shown in the Table 6:

Table 6: PCR program for the *BRAF* sequence amplification

Step	Temperature	Time	Number Of cycles
Initial denaturation	95°C	4min	1
Denaturation	95°C	30sec	
Annealing	**52°C**	30sec	35
Extension	72°C	1min	
Final extension	72°C	7min	1
	4°C	∞	

PCR was performed using Veriti™ Thermal Cycler ABI (Applied Biosystems, California, USA). PCR fragments were visualized on agarose gel and archived prior to sequencing.

PCR Products Purification

The ExoSAP-IT protocol is a single-step enzymatic method designed for simple, quick PCR cleanup before sequencing. The exonuclease I removes leftover primers, while the Shrimp Alkaline Phosphatase removes the dNTPs. The enzyme is active at 37°C and inactive at 80°C. In a reaction plate (MicroAmp Optical 96), 5µl of the PCR product and 2µl of ExoSAP-IT were mixed in a final volume of 7µl. The reaction plate was incubated at 37°C for 15 min in the thermal cycler to degrade primers and free nucleotides, then at 80°C for 15 min to inactivate the ExoSAP-IT.

***KRAS* and *BRAF* sequencing**

Sense and antisense sequencing was performed in a 20µl reaction using the Big Dye Teminator kit v.3.1 (Applied

Biosystems, Foster City, USA). Sequences were visualized upon capillary electrophoresis using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Further analysis was performed with the SeqScape Software v2.5 (Applied Biosystems).

RESULTS

Detection of *ACTB*, *KRAS* and *BRAF* sequences by agarose gel electrophoresis

ACTB gene

Bands corresponding to the *ACTB* gene (control gene) were observed in all gels in this study, which confirmed the presence of the DNA in the sample.

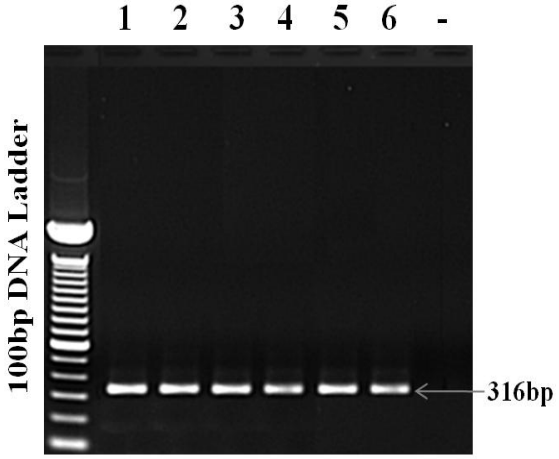

Fig. 1: Gel electrophoresis showing the fragments amplified with the *ACTB* primers. All samples show specific bands for the *ACTB* gene.

KRAS sequence

Amplification of the *KRAS* sequence was observed in all samples.

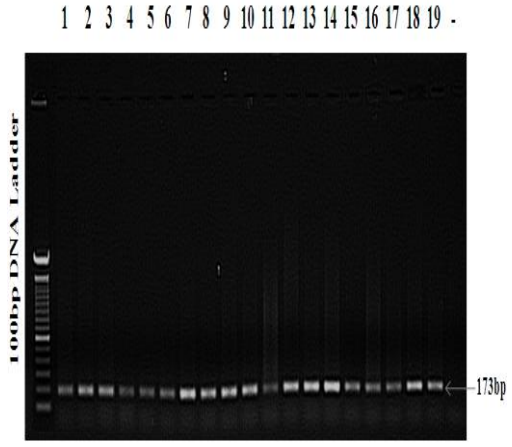

Fig. 2: Gel electrophoresis showing the fragments amplified with the *KRAS* primers. All samples showed amplification of the *KRAS* gene and the bands had the expected size.

BRAF sequence

Amplification of the *BRAF* sequence was observed in all samples.

Quality control of the *KRAS* and *BRAF* sequences

Electropherograms were visualized with Sequence Scanner Software v1.0 (Applied Biosystems). The blue color indicates a base of good quality, yellow indicates a base of medium quality, and red indicates poor quality. All *KRAS* and *BRAF* sequences were of good quality.

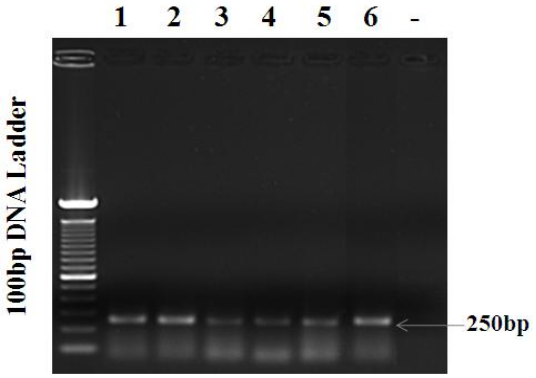

Fig. 3: Gel electrophoresis showing the fragments amplified with the *BRAF* primers. All samples showed amplification of the *BRAF* gene and the bands had the expected size.

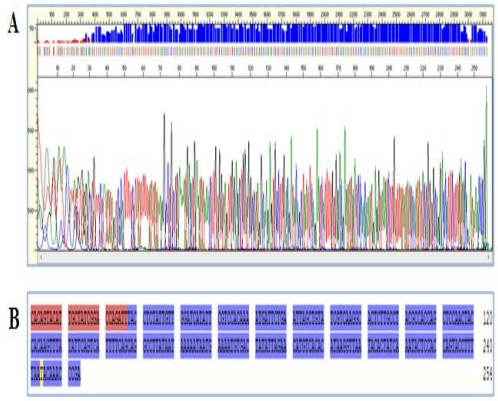

Fig. 4: Example of an electropherogram (A) and a *KRAS* sequence (B) visualized using Sequence Scanner Software v1.0 and showing the good quality of the sequence (blue color).

Detected mutations

KRAS mutations

A total of 23 patients (**49%**) were *KRAS* wild-type (*wt*), 24 patients had activating *KRAS* mutations, which represents a percentage of **51%.**

G35T Mutation (Gly12Val) was present in 10 patients (21%). This mutation affects the codon 12 of the *KRAS* gene. Guanine G at the position 35 is substituted by Thymine T, which induces the replacement of the amino acid glycine by valine.

G35A Mutation (Gly12Asp) was present in 7 patients (15%). This mutation affects the codon 12 of the *KRAS* gene. Guanine G at the position 35 is substituted by Adenine A, which induces the replacement of the amino acid glycine by aspartate.

G38A Mutation (Gly13Asp) was present in 3 patients (6%), This mutation affects the codon 13 of the *KRAS* gene. Guanine G at the position 38 is substituted by Adenine A, which induces the replacement of the amino acid glycine by aspartate.

G34C Mutation (Gly12Arg) was present in two patients (4%). This mutation affects the codon 12 of the *KRAS* gene. Guanine G at the position 34 is substituted by Cytosine C, which induces the replacement of the amino acid glycine by arginine.

G34T Mutation (Gly12Cys) was present in one patient (2%). This mutation affects the codon 12 of the *KRAS* gene. Guanine G at the position 34 is substituted by Thymine T, which induces the replacement of the amino acid glycine by cysteine.

G35C Mutation (Gly12Ala) was present in one patient (2%). This mutation affects the codon 12 of the *KRAS* gene. Guanine G at

position 35 is substituted by Cytosine C, which induces the replacement of the amino acid glycine by alanine.

Fig. 5 shows two examples of the *KRAS* detected mutations: G35A and G35T. The results of the *KRAS* mutational status in the sample studied are presented in Fig. 6.

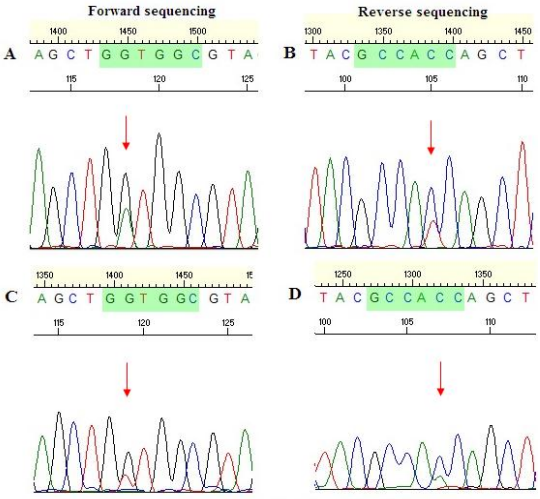

Fig. 5: Two examples of the *KRAS* detected mutations. A: Forward sequencing of the *KRAS* gene showing the G35A mutation, B: Reverse sequencing of the *KRAS* gene showing the G35A mutation. C: Forward sequencing of the *KRAS* gene showing the G35T mutation, D: Reverse sequencing of the *KRAS* gene showing the G35T mutation.

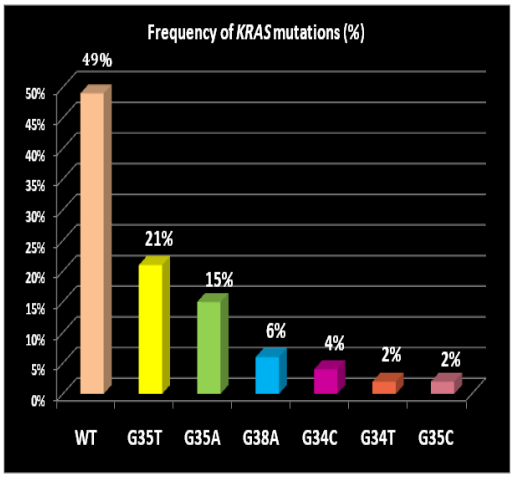

Fig. 6: Results of the *KRAS* sequencing.

BRAF mutations

The sequencing of the 47 samples amplified with the *BRAF* primers showed no V600E mutation in this gene. The 47 patients were all *BRAF wt.*

DISCUSSION

The EGFR is overexpressed in many types of cancers, especially colorectal cancer [19-21]. Not all patients show clinical benefit from treatment with EGFR-targeted monoclonal antibodies: the efficacy of cetuximab and panitumumab is limited to patients whose tumors carry a wild type *KRAS* gene [22-24]. Activating *KRAS* mutations are almost exclusively detected in codons 12 and 13 of the exon 2 of the *KRAS* gene. Other mutations, as in codons 61, 146 or 154 have been described in 1% of colorectal cancer [25]. *BRAF* V600E mutation occurs in 5-10% of colorectal cancer, it is usually exclusive with *KRAS* mutations.

Routine *KRAS/BRAF* screening of pathological specimens is required to promote the appropriate clinical use of anti-EGFR monoclonal antibodies. The detection of *KRAS* and *BRAF* mutations can be challenging because of high testing volume and frequent low tumor content. To address these issues, we evaluated a rapid, reliable and accurate assay to screen mutant *KRAS* and *BRAF* genes.

Molecular testing in colorectal cancer is usually performed on formol-fixed paraffin embedded samples, it is essential to avoid fixatives containing picric acid, which generally prohibit molecular testing. The sensitivity of the direct sequencing depends on the percentage of tumor cells in the analyzed sample. Manual macrodissection is a key step: it is performed to increase the tumor purity. Indeed, when the sample is contaminated with normal cells, it leads to false negative results.

Several molecular techniques are used to detect the *KRAS* point mutations. In this study we used dideoxy sequencing, since it is a highly specific technique and considered the gold standard for the detection of mutations. We performed duplicate sequencing in both forward and reverse directions.

In the present study, we found that the most frequent *KRAS* mutation is the G35T mutation. This mutation presents 41% of all mutations found. The G35A mutation comes in second place with a frequency of 29% of all mutations, then the G38A mutation with a frequency of 12%. G34C, G35C and G34T mutations were present as minor fractions in the sample.

In our study, we looked for the *BRAF* V600E mutation not only in *KRAS wt* patients but also in the patients with *KRAS* mutations. No V600E *BRAF* mutation was detected, neither in *KRAS wt* patients, nor in patients with *KRAS* mutations. This result is logical, since V600E *BRAF* mutation occurs in only 5% to 10% of colorectal cancer, and since this mutation is usually exclusive with the *KRAS* mutations, a sample of 47 patients with a percentage of 51% of *KRAS* mutations does not reveal this rare mutation. In their study, *Lièvre* and *Laurent-Puig* searched for *KRAS* and *BRAF* mutations in 30 patients with colorectal cancer: 43% of patients had *KRAS* mutations and no *BRAF* mutation was detected [26].

CONCLUSION

There is no standardized method for *KRAS* and *BRAF* mutation testing in colorectal cancer and there is currently an intense interest in rapid, reliable, and accurate methods of *KRAS* and *BRAF* mutation screening. The procedure used in this study proved to be an adequate and less time consuming method to detect *KRAS* and *BRAF* hotspots mutations which influence response to anti-EGFR monoclonal antibodies. Our data suggest that *KRAS* mutations are more frequent than *BRAF* mutations in Moroccan patients with colorectal carcinomas. To our knowledge, we are the first to report such a high proportion (more than 50%) of potentially non responsive patients for the anti-EGFR treatment in Morocco. These results show that, without doubt, the method we used is accurate, cost-effective and time-efficient.

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