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PIK3CA mutations in *KRAS* and *BRAF* wild type colorectal cancer patients. A study of Spanish population

Marta Herreros-Villanueva · Noemí Gomez-Manero · Pilar Muñiz · Carlos García-Girón · Maria Jesús Coma del Corral

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Abstract The objective of the work was to study *PIK3CA* mutations in wild type *KRAS* and *BRAF* colorectal cancer. Clinicopathological data and paraffin-embedded specimens were collected on 73 patients who underwent colorectal resections at General Yagüe Hospital in Burgos. *KRAS*, *BRAF* and *PIK3CA* status were analyzed by real-time PCR in all patients. *PIK3CA* mutations were present in 8.22% of wild type *KRAS* and *BRAF* colorectal cancers. The most frequent mutation is E545K/D in exon 9 which represents 83.3% of all mutations. By contrast, we did not found any tumour harbouring H1047R mutation in exon 20. Among the patients who undergo a curative resection of colorectal cancer, *PIK3CA* mutation is present in an important percentage of *KRAS* and *BRAF* wild type tumours. *PIK3CA* mutation may be considered as it could be a hypothetic reason to be not responder to anti-EGFR antibodies.

Keywords *PIK3CA* · *BRAF* · *KRAS* · Cetuximab · Colorectal cancer

M. Herreros-Villanueva (✉) · M. J. Coma del Corral
Unidad de Investigación, Hospital General Yagüe, Avenida del Cid 96, Burgos, Spain
e-mail: mhv@hgy.es

N. Gomez-Manero
Servicio de Medicina Interna, Hospital General Yagüe, Burgos, Spain

P. Muñiz
Departamento de Bioquímica, Universidad de Burgos, Burgos, Spain

C. García-Girón
Servicio de Oncología, Hospital General Yagüe, Burgos, Spain

Abbreviations

BRAF	V-raf murine sarcoma viral oncogene homolog B1
KRAS	Human homolog of the Kirsten rat sarcoma-2 virus oncogene
EGFR	Epidermal grown factor receptor
CRC	Colorectal cancer
PIK3	Phosphatidylinositol 3-kinase

Introduction

Colorectal cancer is the second commonest cause of cancer-related death in the United Sates and Western World. In the last few years its incidence has increased while the age at diagnosis is decreasing [1]. New therapeutic options have increased the overall survival rate of advanced disease from 10 to 18–24 months during the past decade [2]. Advances in surgery, chemotherapy and adjuvant therapy are effective in prolonging time to disease progression and survival in patients with advanced colorectal cancer [3]. Changes in adjuvant therapy include treatment with oxaliplatin combined with 5-FU/leucovorin or capecitabine, with the use concomitant of targeted agents such as cetuximab and bevacizumab.

Epidermal growth factor receptors (EGFRs) have been validated as a therapeutic target in colorectal cancer (CRC) [4, 5]. Ligand occupancy of the EGFR activates the RAS/RAF/MAPK, STAT, and PIK3/AKT signalling pathways, which together modulate cellular proliferation, adhesion, angiogenesis, migration, and survival [6, 7].

KRAS and *BRAF* can harbour oncogenic mutations that yield a constitutively active protein [8–10]. Such mutations are found in approximately 30–50% [11, 12] and 10–15%

[12, 13] of CRC tumours, respectively. Several studies have indicated that the presence of mutant *KRAS* in CRC tumours correlates with poor prognosis [12, 13] and is associated with lack of response to EGFR inhibitors [14–17].

In several studies wild type *KRAS* and *BRAF* status was shown to be required but not sufficient to confer sensitivity to anti-EGFR antibodies as cetuximab and panitumumab. The mechanisms of resistance to these drugs in patients with wild type *KRAS* and *BRA*F tumours are unknown. One potential mechanism making the patients without mutations in *KRAS* and *BRAF* fails to respond to anti-EGFR therapy could be mutations in *PIK3*.

Activation of the Phosphatidylinositol 3-kinase PIK3/AKT pathway is thought to play a critical role in the development of a variety of human malignancies [18–20]. The *PIK3CA* gene encodes the catalytic subunit p110α of PIK3. Mutant *PIK3CA* stimulates the AKT pathway and promotes cell growth in several cancers, including colorectal cancer being associated in these cases with poor prognosis [21].

Elucidating mechanisms of resistance to these antibodies will prove to be important for the selection of therapeutic combinations in order to maximize clinical benefit.

In addition to ascertaining resistance mechanisms, the study of biomarkers such *PIK3CA* mutations will be useful to further refine the responder population and would allow to offer to patients other alternative treatments.

In colorectal cancer there are only a small number of studies analyzing that and numerous discrepancies in the *PIK3CA* mutations frequencies reported ranking from 10 to 30% [22–24]. Thus the percentage and distribution of *PIK3CA* mutations in colorectal cancer still remains uncertain and this retrospective study which is the first in Spain, to our knowledge will help to clarify.

The aim of this study was to evaluate a cohort of 73 wild type *KRAS* and *BRAF* CRC patients in order to investigate the percentage of them which harbours *PIK3CA* mutations and it could be a hypothetic reason for being non-responder to anti-EGFR therapies.

Materials and methods

Patients

This retrospective study included 73 *KRAS* and *BRAF* wild type patients with histological confirmed colorectal primary tumour at the Pathology Department in General Yagüe Hospital (Burgos, Spain). Clinical data were obtained from the tumour registry and from hospital charts. These data included patient age at diagnosis, gender, Dukes Stage and Histological grade (see Table 1).

Table 1 Patient demographics and baseline characteristics

		%
Gender		
Male	52	71.2
Female	21	28.8
Age (years)		
Median	69.9 years	
Range	(range 31–88)	
Dukes stage		
A	3	4.1
B	4	5.6
C	66	90.3
D	0	0
Histological grade		
Well	3	4.1
Moderate	9	12.3
Poor	61	83.6

Assays methods

Mutational analysis of KRAS, BRAF and PIK3CA in tumor samples

Formalin-fixed, paraffin-embedded tumour sections were deparaffinized and air dried, and DNA was isolated using proteinase K and QIAamp® DNA FFPE Tissue kit (Qiagen) according to the manufacturer's protocol.

KRAS

Mutant *KRAS* in exon 2 was detected using a validated *KRAS* mutation kit (DxS Ltd, Manchester, United Kingdom) and following manufacturer's instructions. This analysis identifies seven somatic mutations located in codons 12 and 13 (Gly12Asp, Gly12Ala, Gly12Val, Gly12Ser, Gly12Arg, Gly12Cys, and Gly13Asp) using allele-specific real-time polymerase chain reaction [25–27]. The analysis was performed in an ABI Prism 7500 instrument (Applied Biosystems).

BRAF

BRAF mutation in exon 15 and codon 600 (V600E) were evaluated modifing a method previously reported by Benlloch et al. [28]. The primers were designed to avoid amplification of a pseudogen located in chromosome X. Briefly, we used the set of primers and probes as follow: BRaf-F (forward) 5′CTACTGTTTTCCTTTACTTACTACACCTCAGA-3′: BRaf-R (reverse) 5′ATCCAGACAACTGTTCAAACTGATG-3′, Wild type probe 5′VICCTAGCTACAGtGAAATC-3′ and Mutant probe 5′-FAM-TAGCTA

CAGaGAAATC-3′. The primers and probes were tested with controls of DNA from the HT29 cell line (ATCC) which harbours the V600E B-Raf heterozygotic mutation and DNA from the SW480 cell line (ATCC) which is BRAF wild type and *KRAS* mutant. Real-time PCR was performed in a final reaction volume of 20 µl containing 10 µl of 2× Genotyping PCR Master Mix (Applied Biosystems), 900 nmol/l of each primer, 250 nmol/l of each probe, and 5 µl of DNA solution. PCR was performed in MicroAmp optical 96-well plates with optical adhesive covers (Applied Biosystems). Amplification and detection were performed with an ABI prism 7500 sequence detection system (Applied Biosystems). The amplification conditions were 2 min at 50°C for AmpErase uracil-*N*-glycosylase activity and 10 min at 95°C for AmpliTaq Gold activation, followed by 50 cycles of 15 s at 92°C for denaturation and 1.5 min at 60°C for annealing and extension. The fluorescence data were analyzed with the allelic discrimination software of the ABI Prism 7500 instrument.

PIK3CA

PIK3CA mutations in exons 9 and 20 were detected using a validated *PIK3CA* mutation kit (DxS Ltd, Manchester, United Kingdom) that identifies four somatic mutations (H1047R, E542K, E545D and E545K) using Real-Time Polymerase Chain Reaction based in ARMS® and Scorpion® technology. This method is highly selective and can detect approximately 1–2% mutants in a background of genomic DNA [29]. The analysis was performed in an ABI Prism 7500 instrument (Applied Biosystems).

Statistical analysis methods

Statistical analysis was performed using SPSS version 17.0. A comparison has been made among the variables. We used Chi-square distribution on categorical variables and the non parametrical U-Mann–Whitney distribution on continuous variables and in both cases $P < 0.05$ was considered to be statistically significant.

Results and discussion

PIK3CA mutational profile

We analyzed 73 patients (71.3% male and 28.7% female) aged 31 to 88 years old, medium age 69.9. Six of 73 successfully analysed samples (8.22%) carried a *PIK3CA* mutation. The missense mutations were found in the hot spots located in exon 9 of the *PIK3CA* gene (E542D, E545D, E545K) (Table 2) while we did not found any mutation in exon 20 (H1047R).

We observed one tumour with E542K mutation and five with E545K/D mutation. In our population, the most frequent mutation is E545K/D which represents 83.3% of all mutations. By contrast, we did not found any tumour harbouring H1047R mutation in exon 20.

Making a comparison between the age and the *PIK3CA* mutations using Mann–Whitney test we found that there is significant difference between age and *PIK3CA* mutations (signification of 0.026 and sample power 47%). Comparing sex and *PIK3CA* mutations using Chi-square test we found no significant difference between male and female (signification of 0.999 and sample power 9%).

In a Spanish population we found a distribution of specific *PIK3CA* mutations similar to Perrone's data [24] where mutations in exon 9 were the most frequent and no mutation in exon 20 was found. We speculate that the specific distribution of *PIK3CA* mutations could be homogeneous in different populations as the Spanish cohort is similar to the European series published although both published series have a relatively small sample size and future studies are required to confirm these data.

There are numerous discrepancies in the *PIK3CA* gene mutation frequencies reported from a small number of studies and previous data reported different percentages ranking from 10 to 30% (including *KRAS* and *BRAF* wild type and mutant) [22–24, 30].

This retrospective study, analyze *PIK3CA* mutation in wild type *KRAS* and *BRAF* patients, has shown that there is an important percentage of these tumours which have PIK3CA/AKT deregulation.

We found an 8.22% of patients who present *PIK3CA* mutations. Our results are similar to Velho et al. [31] who determined that the prevalence of *PIK3CA* mutations in 51 CRC patients was 7.1.

These results are important because corroborate the data presented by Ogino et al. and Baldus et al. [32] describing that in *KRAS* wild type tumours, the presence of *PIK3CA* mutations was associated with both a bad prognosis and with an increase in colorectal cancer-specific mortality.

We have demonstrated compelling evidence supporting the *PIK3CA* mutation in colorectal carcinoma. Although we have not provided data about patients' response, our data attend to speculate that futures studies are necessary to prove that some patients who present *PIK3CA* could be non-responders to anti-EGFR therapies as cetuximab.

Therefore, as we have demonstrated that *PIK3CA* gene is mutated in an important percentage of *KRAS* and *BRAF* wild type patients, we thought that this could be a potential target for developing drugs to treat colorectal cancer.

Table 2 Distribution of *PIK3CA* mutational status

Case No.	Sex	Localization	Dukes stage	Histol. grade	Age	PI3K
1	M	Colon	C	Moder	76	WT
2	M	Colon	C	Moder	77	WT
3	F	Recto	A	Moder	81	WT
4	M	Colon	B	Moder	77	WT
5	F	Recto	B	Moder	72	WT
6	M	Colon	B	Moder	75	WT
7	M	Colon	C	Poor	65	WT
8	F	Colon	C	Good	64	WT
9	M	Colon	C	Moder	83	WT
10	M	Colon	C	Moder	62	MutE542K
11	F	Colon	C	Moder	78	WT
12	M	Colon	C	Moder	65	MutE545K/D
13	M	Colon	C	Moder	84	WT
14	M	Colon	C	Moder	81	WT
15	M	Recto	C	Moder	69	WT
16	M	Colon	C	Moder	51	WT
17	M	Colon	C	Moder	63	WT
18	M	Colon	C	Moder	56	WT
19	F	Colon	C	Poor	71	WT
20	F	Colon	B	Moder	83	WT
21	M	Colon	C	Poor	61	WT
22	M	Colon	C	Poor	69	WT
23	F	Colon	C	Moder	50	WT
24	M	Colon	C	Moder	54	MutE545K/D
25	F	Recto	C	Moder	54	WT
26	M	Recto	C	Moder	50	WT
27	M	Colon	C	Moder	76	WT
28	M	Colon	C	Moder	50	WT
29	M	Colon	A	Good	68	WT
30	M	Colon	C	Moder	61	WT
31	F	Colon	C	Moder	82	WT
32	F	Colon	C	Moder	72	WT
33	F	Colon	A	Moder	39	MutE545K/D
34	F	Colon	C	Moder	66	WT
35	M	Colon	C	Moder	76	WT
36	M	Colon	C	Moder	69	WT
37	M	Colon	C	Poor	31	WT
38	M	Colon	C	Moder	76	WT
39	M	Colon	C	Poor	86	WT
40	M	Colon	C	Moder	82	WT
41	F	Colon	C	Good	68	WT
42	M	Colon	C	Poor	45	WT
43	M	Colon	C	Poor	72	WT
44	M	Colon	C	Moder	73	WT
45	M	Colon	C	Moder	70	WT
46	M	Colon	C	Moder	44	WT
47	M	Colon	C	Moder	69	WT
48	M	Colon	C	Moder	72	WT
49	M	Recto	C	Moder	48	WT
50	M	Colon	C	Poor	68	WT
51	M	Colon	C	Moder	81	WT
52	M	Recto	C	Moder	88	WT
53	M	Recto	C	Moder	67	WT
54	F	Colon	C	Moder	77	WT
55	F	Colon	C	Moder	61	MutE545K/D
56	F	Colon	C	Moder	72	WT
57	M	Colon	C	Moder	64	MutE545K/D
58	M	Colon	C	Moder	55	WT
59	M	Colon	C	Moder	47	WT
60	F	Colon	C	Moder	62	WT
61	F	Colon	C	Moder	72	WT
62	M	Colon	C	Moder	56	WT
63	M	Colon	C	Moder	31	WT
64	F	Colon	C	Moder	54	WT
65	M	Colon	C	Moder	81	WT
66	F	Colon	C	Moder	72	WT
67	M	Colon	C	Moder	66	WT
68	M	Colon	C	Moder	76	WT
69	M	Colon	C	Moder	76	WT
70	M	Colon	C	Moder	79	WT
71	F	Colon	C	Moder	77	WT
72	M	Colon	C	Moder	79	WT
73	M	Colon	C	Moder	59	WT

M male, *F* female, *WT* wild type, *Mut* specific mutation

Conclusion

PIK3CA mutations may be one of the genes that could predict the lack of efficacy of EGFR inhibitors in *KRAS* and *BRAF* patients.

PIK3CA mutation, observed in more than 8% of the *KRAS* and *BRAF* wild type patients, could predict escape from cetuximab treatment. These results also suggest that *PIK3CA* could be a promising target for adjuvant therapy in colorectal cancer patients.

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Conflict of interest statement The authors declare that they have no competing interests.

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