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Rev Invest Clin. 2020;72(6):337-43 BRIEF COMMUNICATIONS

Complete Screening of Exons 2, 3, and 4 of *KRAS* and *NRAS* Genes Reveals a Higher Number of Clinically Relevant Mutations than Food and Drug Administration Quantitative Polymerase Chain Reaction-Based Commercial Kits

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ABSTRACT

Background: The presence of clinically relevant mutations in *KRAS* and *NRAS* genes determines the response of anti-epidermal growth factor receptor antibody therapy for metastatic colorectal cancer (mCRC). The only quantitative polymerase chain reaction (qPCR)-based diagnostic tests approved by the Food and Drug Administration (FDA) screen merely for mutations in codons 12 and 13 of *KRAS*. **Objective:** The objective of the study was to study the frequency of clinically relevant mutations in *KRAS* and *NRAS* genes that are not included in FDA-approved qPCR tests. **Methods:** Formalin-fixed paraffin-embedded tumor specimens from 1113 mCRC Mexican patients from different health institutions across the country were analyzed by Sanger sequencing for *KRAS* mutations in exons 2, 3, and 4. Furthermore, 83 were analyzed in exons 2, 3, and 4 of *NRAS*. **Results:** From the specimens tested for *KRAS*, 33.69% harbored a mutation. From these, 71.77% were in codon 12 and 27.69% in codon 13 (both located in exon 2). Codons 59 (exon 3) and 146 (exon 4) accounted for the remaining 0.54%. From the 83 specimens, in which *NRAS* was analyzed, three mutations were found in codon 12 (3.61%). Approximately 6% of *RAS* mutated specimens would have been falsely reported as *RAS* wild type if an FDA-approved qPCR diagnostic test had been used. **Conclusions:** While these kits based on qPCR can be very practical and highly sensitive, their mutation coverage ignores mutations from poorly genetically characterized populations. (REV INVEST CLIN. 2020;72(6):337-43)

Key words: Colorectal cancer. Molecular diagnostics. RAS genes. Real-time polymerase chain reaction. Sanger sequencing.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common neoplasia in men and the second in women (10.9% and 9.5% of all cancers, respectively) worldwide. In North America, CRC is the fourth most common cancer with an estimated incidence in 2018 of 49.9 for every 100,000 individuals[1]. Precursor lesions of the colonic mucosa known as adenomatous polyps (adenomas) may develop into colorectal tumors; however, < 10% of colorectal adenomas develop into invasive cancers. The progression from adenoma to adenocarcinoma has been associated with many different molecular mechanisms, including epigenetic events, DNA mismatch repair defects, chromosomal rearrangements, and mutations in key oncogenes and tumor suppressor genes[2,3].

Colon cancer is one of the main diseases treated by targeted therapy as there is extensive information about the genes affected by cancer-causing mutations, their normal functions, and their carcinogenic effects when mutated[2,4]. Epidermal growth factor receptor (EGFR) triggers a signaling pathway related to the proliferation of tumor cells. The EGFR signaling cascade promotes cell proliferation by activation of Ras-GTPase and Erk/Map kinase. Because of this, medical treatment consists of blocking EGFR with monoclonal antibodies (mAbs), such as cetuximab and panitumumab[5,6]. However, mutational screening is necessary to identify resistant patients and is mandatory as a companion diagnostic test[7]. Even though EFGR is commonly overexpressed in all human cancers, its abnormality fails to predict the success of anti-EGFR therapy. On the other hand, the *RAS* gene family has become widely accepted as a biomarker for the response to treatment of several types of cancer, such as metastatic CRC (mCRC). Correspondingly, a patient with mCRC not harboring a *KRAS* or *NRAS* mutation could benefit from the treatment with anti-EGFR mAbs[8,9] and vice versa.

RAS is one of the most studied and best characterized mCRC-associated gene families. Oncogenic mutations of three of its members, *H, K,* and *NRAS*, were among the first genetic changes identified in human tumors. These three members encode membrane-associated guanine nucleotide-binding proteins (p21ras) and are closely related, having 85% amino acid sequence identity and very similar functions[10]. At the molecular level, the above-mentioned *KRAS* and *NRAS* mutations result in reduced intrinsic GTPase activity, which, in turn, leads to permanent activation of KRAS or NRAS itself and of its downstream signaling pathways, thereby mediating malignant transformation. *KRAS* gene is in the short arm of chromosome 12, in p12.1, and is composed of 46,148 nucleotides divided into six exons. In contrast, the *NRAS* gene is in 1p13.2 of chromosome 1 and consists of seven exons spread over 12,431 nucleotides. Of the three human *RAS* isoforms, *KRAS* (Kirsten ras or Ki-ras) is the most frequently mutated oncogene in mCRC[11,12], from which codons 12 and 13 of the second exon and 61 of the third exon have been found to harbor mutations in roughly 40-50% of tumors, with approximately 85-90% of mutations occurring in codons 12 and 13[4]. Mutations involving codon 61 in the third exon and codon 146 in the fourth exon of the *KRAS* gene have also been described, with frequencies ranging from 1 to 6.6%[13]. An approximately 10% of the remaining *KRAS* negative tumors have been found mutated, mostly in *NRAS* codon 61[14].

Around the world, several research groups are studying and publishing data on the relation of *RAS* genes with mCRC[15], revealing that mutations in these genes might be associated with an increase of tumor aggressiveness and poor survival (prognostic value), as well as with resistance to gene-targeted therapies (predictive value)[16].

A number of methods to detect *KRAS* mutations are available, including real-time or quantitative polymerase chain reaction (qPCR) with or without melting curve analysis, pyrosequencing, and Sanger sequencing (SS), the latter being the "gold standard" in clinical mutation testing[17]. Sanger sequencing can detect essentially all base substitutions, small insertions, and deletions; however, its modest limit of detection (≥ 10% mutated DNA in a wild-type background) could be highly variable depending on the DNA extraction method and the laboratory performing the test[18].

The only qPCR-based companion diagnostic tests approved by the U.S. Food and Drug Administration (FDA) are the Cobas® *KRAS* Mutation Test from Roche (Pleasanton, CA, USA) and the therascreen *KRAS* RGQ PCR Kit from QIAGEN (Manchester, UK), which screen only for mutations in codon 12 and 13 of *KRAS*. This study aims to state the frequency of mutations that

are not included in FDA-approved qPCR-based commercial companion diagnostic devices.

METHODS

Tumor samples

Tumor samples consisted of formalin-fixed paraffin-embedded (FFPE) tissue of primary tumor lesions from 1030 consecutive mCRC patients received in our laboratory for routine KRAS gene mutation screening from April 2010 to December 2013. This study also included 83 consecutive mCRC patient tumor specimens received from 2013 to 2016 that were routinely analyzed for both, *KRAS* and *NRAS* genes. The patients, 646 men and 447 women, were residents from Mexico and had Stage IV carcinoma. Patients signed informed consent before the performance of tests where it is agreed that their results may be used for research purposes. The study was approved by the Ethics and Research Committee from Hospital La Misión private hospital (17CI19039096). The specimens came from High Specialty Regional Hospitals of Bajío (HRAEB), Oaxaca (HRAEO), Yucatan's peninsula (HRAEPY), Chiapas (HRAEC), Ciudad Victoria (HRAECV), and Ixtapaluca (HRAEI), as well as from Doctor's Hospital, Opción Oncología, ONCARE, OCA Hospital, Hospital San José, Hospital Zambrano Hellion, and Hospital La Misión, Monterrey, NL, Mexico. Information on previous or current treatments received was not available.

Biological samples

In most cases, the FFPE blocks were accompanied with their respective slide stained with hematoxylin and eosin; the tumor area was bounded by the pathologist of the institution where the sample came from. For FFPE blocks that had < 20% of tumoral tissue, the tumoral area was microdissected until reaching an area of approximately 20 mm^2 of tumoral tissue since the limit of detection of SS mutation screening is equal or greater than 10% of mutated cells in a background of wild-type cells. When only blocks were received, one section was obtained and stained with hematoxylin and eosin; a certified pathologist was on hand to identify the tumor region of interest. Additional histological information about the tumors was not available.

DNA extraction and mutation analysis

DNA extraction from scrapped tumor areas performed with the QIAamp DNA FFPE Tissue Kit (QIAGEN, Mexico City, Mexico) was used as a template to amplify the exons of interest of *RAS* genes by PCR followed by SS using an automated capillary electrophoresis sequencer from Applied Biosystems (Mexico City, Mexico) as described in our previous study[9]. Primer pairs for amplification of exons 2, 3, and 4 of *KRAS* and *NRAS* genes are described in table S1.

Commercial kits

Conceptual analysis of the sequencing revealed mutations was performed based on the information in the fact sheets from Cobas *KRAS* Mutation Test and the therascreen *KRAS* RGQ PCR Kit describing the mutations that can be detected by them, as well as the reported limits of detection.

Public and patient involvement statement

Patients signed informed consent before the performance of tests where it is agreed that their results may be used for research purposes. This study was carried out as a result of the finding of uncommon mutations in routine tests. This is a retrospective study; patients were not specifically recruited to perform this analysis. The information obtained will not be notified to patients unless it represents a risk to their health.

RESULTS

In our laboratory, of the 1113 samples tested for *KRAS*, 375 (~33.69%) harbored a mutation. From these, 267 were in codon 12 (~72%) and 103 in codon 13 (~28%) (exon 2). Codons 59 and 146 (exon 3 and 4, respectively) accounted for the remaining ~0.1%, with incidences of one case for each codon. In *KRAS*, we found c.36T > A and c.39C > T mutations in codons 12 and 13, respectively, which are synonymous mutations not available for screening by the FDA-approved qPCR kits.

These devices can detect all non-synonymous mutations in codon 12 (Table 1). However, they cannot

Table 1. Comparison of Sanger sequencing versus commercial kits for detecting RAS mutations

Mutation[a]	COSMIC ID	Type of mutation[b]	Sanger sequencing			Commercial kits mutation coverage[e]	
			Incidence[c]	%[d]	% p/codon	1	2
***KRAS* Codon 12 (n = 1113)**							
c.35G > A p.(G12D)	55497369	NS	129	11.59	48.31	✓	✓
c.35G > T p.(G12V)	55497419	NS	66	5.93	24.72	✓	✓
c.34G > T p.(G12C)	55497469	NS	28	2.52	10.49	✓	✓
c.35G > C p.(G12A)	55497479	NS	25	2.25	9.36	✓	✓
c.34G > A p.(G12S)	55497461	NS	12	1.08	4.49	✓	✓
c.34G > C p.(G12R)	55497582	NS	5	0.45	1.87	✓	✓
c.36T > A p.(G12=)	55510792	S	2	0.18	0.75		
			267	23.99	100.00		
***KRAS* Codon 13 (n = 1113)**							
c.38G > A p.(G13D)	55497388	NS	80	7.19	77.67	✓	✓
c.39C > T p.(G13=)	55756103	S	8	0.72	7.77		
c.37G > T p.(G13C)	55497378	NS	9	0.81	8.74		
c.37G > A p.(G13S)	55509530	NS	4	0.36	3.88		
c.37G > C p.(G13R)	55502117	NS	1	0.09	0.97		
c.38G > C p.(G13A)	55497357	NS	1	0.09	0.97		
			103	9.25	100.00		
***KRAS* Codon 61 (n = 1113)**							
c.183A > C p.(Q61H)	55498802	NS	1	0.09	100.00		
			1	0.09	100.00		
***KRAS* Codon 146 (n = 1113)**							
c.436G > A p.(A146T)	55501778	NS	1	1.20	100.00		
			1	0.09	100.00		
***NRAS* Codon 12 (n = 83)**							
c.34G > T p.(G12C)	54736487	NS	1	1.20	33.33		
c.35G > A p.(G12D)	54736383	NS	1	1.20	33.33		
c.35G > C p.(G12A)	54736555	NS	1	1.20	33.33		
			3	3.61	100.00		
Total			375	33.69		94.0%	94.0%

[a]Mutations found by Sanger sequencing.
[b]Synonymous (S) or non-synonymous (NS).
[c]Total number of patients harboring each mutation.
[d]Percentage of each mutation over total samples analyzed by SS.
[e]Commercial kits numbered as follows: 1, Cobas® *KRAS* Mutation Test; 2, TheraScreen®: *KRAS* Mutation Kit. Percentages on bottom represent the proportion of mutations covered by each kit, calculated as the sum of the incidence of all covered mutations divided by the total of mutations in codons 12 and 13.

detect the mutations c.37G > A (p.G13S) and c.38G > C (p.G13A), which account for 0.45% of false negatives according to our results. In addition, we found unique cases of the mutations c.176C > T (p.A59V) and c.183A > C (p.Q61H) in exon 3 and c.436G > A (p.A146T) in exon 4.

Of the 83 tumors analyzed for *NRAS*, the mutations c.34G > T, c.35G > A, and c.35G > C in codon 12 (~4%) were found. None of the kits included in this study include mutation screening in the *NRAS* gene; the commercial kits included would have reported 6% of false negatives.

There were no significant differences in the frequency of mutations by age or sex.

DISCUSSION

Our findings agree with the previous studies, in which 35-40% incidence has been reported for KRAS mutations[4,8,14,19]. Nonetheless, recent studies have unveiled the importance and potential risk of synonymous mutations in disease causation, suggesting that these mutations could affect the predictive value of different malignancies[20,21]. Physiological effects of synonymous mutations could be related to cancer through diverse mechanisms, such as altering splicing, RNA structure and stability, the translational rate, and other unknown mechanisms[21]. Moreover, according to a study based on a data set of 292,405 missense mutations and 123,193 synonymous mutations identified in the exomes of 3851 cancer samples, synonymous mutations tend to be enriched in oncogenes (but not in tumor suppressor genes) in a cancer type-specific manner and often affect splicing motifs[22]. This suggests a greater relevance for synonymous mutations in cancer than previously thought, hence highlighting the importance of detecting and reporting these "silent" mutations. The *KRAS* c.36T > A (p.G12=) and c.39C > T (p.G13=) synonymous mutations found in this study are predicted to be pathogenic by Functional Analysis through Hidden Markov Models (FATHMM) (COSMIC mutation IDs COSV55573999 and COSV55510792, respectively). The first one has been reported as a somatic mutation in the biliary tract, large intestine, and pancreas carcinomas, while the latter has been found in the large intestine, hematopoietic and lymphoid tissue, pancreas, endometrium, and biliary tract carcinomas[23].

Mutations c.37G > A (p.G13S) and c.38G > C (p.G13A), despite being in codon 13, are not covered by FDA-approved qPCR-based kits but were found in our study. These are known predictive biomarkers to determine anti-EGFR therapies. The first has been found distributed in the large intestine, thyroid, stomach, pancreas, and lung carcinomas, while the latter, in the large intestine, lung, ovary, pancreas, and thyroid carcinomas (COSMIC mutation IDs COSV55509530 and COSV55497357, respectively).

In exon 3 of *KRAS*, we found the c.176C > T (p.A59V) mutation that has not been reported in mCRC, but in the breast (accession RCV000119371.1 in NCBI ClinVar database) and thyroid cancers (COSMIC mutation ID COSV55904854). In contrast, the c.183A > C (pQ61H) mutation (also in exon 3 of *KRAS*) has been found distributed in the large intestine, hematopoietic and lymphoid tissue, lung, and biliary tract cancer lesions (COSMIC mutation ID COSV55498802). Both mutations are predicted to be pathogenic by FATHMM. We also found the c.436G > A (p.A146T) mutation in exon 4 of *KRAS*, which is predicted as pathogenic by FATHMM and has been reported to be present in the large intestine, hematopoietic and lymphoid tissue, stomach, biliary tract, and pancreas carcinomas. Further research should explore the effect of these mutations on mCRC proliferation and treatment, and whether they imply a clinical risk or interfere with anti-EGFR antibody treatments.

The mutations found in codon 12 of *NRAS* in this study have been well documented to predict resistance to anti-EGFR therapy[24].

It is worth mentioning that all specimens in this study corresponded to Stage IV carcinomas. The previous studies have shown that the frequency of mutations varies according to the stages[25], and for a wider analysis, further studies should include samples from all stages.

Despite 6% of false negatives that would be reported if FDA-approved companion diagnostic tests were used, SS has important constraints when compared to these kits: the processing time is much longer in SS due to the additional steps required (amplification,

cycle sequencing, purification of products, and capillary electrophoresis) and the time needed to analyze results. In addition, some problems can arise in the process due to degradation of DNA and the presence of inhibitors as a result of formalin fixation and dewaxing, respectively[26]. Furthermore, the limit of detection of SS is considerably lower than that of kits: a mutation can be detected by SS in a specimen with at least 20% mutant DNA in a background of wild-type DNA, which means ~20-40% heterozygous mutant cells[27]. On the other hand, the commercial kits here discussed have reported sensitivities of <5% of mutated DNA in a wild-type background. Despite its modest limit of detection and relative complexity, SS is currently one of the most reliable techniques for mutation discovery and validation, especially helpful in molecular genetic studies of genetically poorly characterized populations[18]. Studies have reported an overall concordance of 97.5% between SS and qPCR for *KRAS* mutation detection[28]. Nevertheless, the drawbacks must be considered in the selection of the technique to be used for a certain application. A notable limitation of SS is that large deletion/duplications could not be detected. An alternative for the detection of low-frequency mutations that are not covered by commercial kits could be next-generation sequencing, which can cover the whole sequence of target genes with an even higher sensitivity than SS[29,30], although its cost still prevents it from competing with SS in a commercial laboratory.

Similar studies analyzing additional mutations to those found in KRAS codons 12 and 13 in American mCRC patients using SS have reported that 17% of specimens harbor potentially relevant RAS mutations that are missed with tests limited to codons 12 and 13 of *KRAS*[31].

In conclusion, commercial kits based on technologies such as qPCR and STA-FA can be very practical for routine molecular diagnosis given their high sensitivity and efficiency, their mutation coverage leaves out rare mutations from poorly genetically characterized populations. However, kits could be used for clinical investigation or codon-specific research but not for the identification of low-frequency mutations, tracing of unreported mutations, or analyses in new populations. For those purposes, SS or other sequencing techniques are more suitable, especially considering that the life of mCRC patients could depend on the results obtained.

It should be noted that NRAS should also be included in every companion diagnostic for mCRC, at least in the Mexican population, as we found that ~4% of the 83 specimens analyzed for this gene contained a mutation. Given the non-neglectable frequency of KRAS mutations in mCRC revealed here, to understand their contribution to tumor proliferation, further studies should explore their possible association with the outcome of anti-EGFR therapies.

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SUPPLEMENTARY DATA

Supplementary data are available at Revista de Investigación Clínica online (www.clinicalandtranslationalinvestigation.com). These data are provided by the corresponding author and published online for the benefit of the reader. The contents of supplementary data are the sole responsibility of the authors.

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