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Original article

The KRAS StripAssay for detection of KRAS mutation in Egyptian patients with colorectal cancer (CRC): A pilot study

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Abstract *Background:* Epidermal growth factor receptor (EGFR) and its downstream factors KRAS and BRAF are mutated in several types of cancer, affecting the clinical response to EGFR inhibitors. Mutations in the EGFR kinase domain predict sensitivity to the tyrosine kinase inhibitors gefitinib and erlotinib in lung adenocarcinoma, while activating point mutations in KRAS and BRAF confer resistance to the anti-EGFR monoclonal antibody cetuximab in colorectal cancer. The development of new generation methods for systematic mutation screening of these genes will allow more appropriate therapeutic choices.
Purpose: Detection of KRAS mutation in Egyptian colorectal cancer (CRC) patients by the KRAS StripAssay.

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Methods: Examination of 20 colorectal cancer (CRC) patients is done to detect KRAS mutations by KRAS StripAssay. For the StripAssay, a mutant-enriched PCR was followed by hybridization to KRAS-specific probes bound to a nitrocellulose strip.
Results: Among 20 patients, KRAS mutations were identified in 80% of patients by the KRAS StripAssay.
Conclusions: Our preliminary results suggest that KRAS StripAssay is an alternative to protocols currently in use for KRAS mutation detection.

Introduction

The epidermal growth factor receptor (EGFR) plays a key role as a receptor tyrosine kinase (TK), controlling several signaling pathways that stimulate cell growth, proliferation and survival. Mutations involving the EGFR axis can cause its constant activation, leading to uncontrolled cell proliferation. Not surprisingly, EGFR mutations have been identified in several types of cancer and it is a target of many anticancer therapies, including small-molecule TK inhibitors (e.g., gefitinib and erlotinib for lung cancer) and monoclonal antibodies (e.g., cetuximab and panitumumab for colon cancer) [1]. Several reports indicate that an increased gene copy number of EGFR or mutations of genes responsible for downstream signaling, especially KRAS, are important determinants of response or resistance to anti-EGR antibodies [2].

The KRAS gene is located on the short (p) arm of chromosome 12 at position 12.1. KRAS is part of a group of three homologous oncogenes and encodes a small 21 kDa protein (p21Ras) involved in the transduction of external stimuli to effector molecules across plasma membranes, downstream from the EGFR. This protein has intrinsic guanosine triphosphatase (GTPase) activity, allowing inactivation after signal transduction in the normal cellular environment [3]. Mutations in the KRAS gene occur early in the development of many cancers and are found in more than 90% of pancreatic adenocarcinomas, 40% of colorectal cancers (CRC) and 33% of non-small cell lung carcinomas (NSCLC) [4].

Somatic point mutations of KRAS occurring early in CRC tumorigenesis are thought to abolish GTPase activity, leading to a constitutive activation of KRAS and inevitably to increased and unregulated cellular proliferation and malignant transformation [4,5]. Oncogenic mutations of the KRAS gene are observed in ~40% of sporadic CRC, and up to 90% of these mutations are detected in codons 12 and 13 and less frequently also in codons 61 and 63 [2].

Several retrospective analyses of tumor samples in CRC patients receiving anti-EGFR antibody treatment have shown that patients with mutated KRAS did not benefit from anti-EGFR therapy [6,7]. Based on systematic reviews of the relevant literature, the American Society of Clinical Oncology suggested, in a Provisional Clinical Opinion in 2009, that when KRAS mutations in codon 12 or 13 were detected in patients with mCRC, such patients should not receive anti-EGFR antibody therapy as part of their treatment [8]. The European Medicines Agency has also recognized these findings, and indeed also restricts the use of anti-EGFR antibody therapy only to CRC patients with wild-type (wt) KRAS tumors [9].

Accurate mutation detection depends on several factors, including available tissue, DNA quality, DNA input, and tumor cell percentage. When choosing an assay for routine diagnostics, additional factors such as workload, time to results, hands-on time, dedicated equipment, costs, assay flexibility, and robustness of a technique need to be addressed as well [13]. In most of the pathology laboratories, direct sequencing, i.e., PCR followed by dideoxy sequencing, is considered the gold standard for KRAS mutation detection. However, this technique is not only laborious and time consuming, but sensitivity plays an important role. To reliably test a sample, at least 20–30% of tumor cells are needed. To date, there are several alternative assays available for (KRAS) mutation detection, including "homebrew" assays, such as high-resolution melting curve analysis (HRM) [14], pyrosequencing [15], single nucleotide primer extension assay [16], and allele-specific real-time PCR [17], and commercially available assays, such as reverse hybridization test KRAS StripAssay (Vienna Labs, Vienna, Austria) [18] and real-time PCR-based Thera-Screen (Roche Diagnostics, Almere, the Netherlands); all these assays greatly differ in sensitivity, specificity, DNA input, time to results, hands-on time, flexibility, workload, and costs.

Therefore, in this study, we aimed to use the reverse hybridization StripAssay as an easy method for KRAS mutation detection in fresh CRC samples in a small Egyptian population.

Patients and methods

Study population

The current study was carried out on 20 newly diagnosed Egyptian patients with colorectal carcinoma (CRC). Patients were chosen during the period between October 2011 and May 2012 among cases referred to the National Cancer Institute (NCI), Cairo University, after taking their informed consents. The research was approved by the IRB of the NCI. They were 6 (30%) males and 14 (70%) females. Their ages ranged between 47 years and 72 years with a median of 56.5 years. Exclusion criteria included pediatric age group, non-Egyptians and patients with double malignancy. Diagnosis of colorectal carcinoma (CRC) was based on excision biopsy from the affected sites. Histopathological studies were done to confirm the diagnosis (Table 1). According to the Dukes classification, 18 patients have stage A, one has stage B and one has stage C. Only one patient has metastatic deposits in pericolic and paraaortic LNs.

Table 1 Clinicopathologic features of the 20 cases with colorectal adenocarcinoma.

Sample id	Gender	Age (years)	Site	Grade	Dukes stage	Lymph nodes
A	F	72	Colonic	Low	A	Not affected
B	F	56	Rectosigmoid	Low	A	Not affected
C	F	58	Sigmoid colon and mass 50 cm from anal verge	Low	B	Not affected
D	F	59	Colorectal	Low	A	Not affected
E	F	60	Colonic	High	C	Pericolic LNs and para-aortic LNs showed metastatic deposits
F	M	62	Colonic	Low	A	Not affected
G	M	49	Colonic	Low	A	Not affected
H	F	47	Colonic	Low	A	Not affected
I	F	62	Colonic	Low	A	Not affected
J	M	57	Colonic	Low	A	Not affected
K	F	47	Colonic	Low	A	Not affected
L	F	70	Rectal	Low	A	Not affected
M	F	56	Colonic	Low	A	Not affected
N	F	59	Colonic	Low	A	Not affected
O	F	50	Colonic	Low	A	Not affected
P	M	52	Colonic	Low	A	Not affected
Q	M	49	Colonic	Low	A	Not affected
R	F	47	Colonic	Low	A	Not affected
S	F	62	Colorectal	Low	A	Not affected
T	M	53	Colonic	Low	A	Not affected

F = female and M = male.

Samples and DNA extraction

Fresh surgical specimens were obtained. DNA was extracted from tissue by (QIAamp DNA Mini Kit) Cat. No. 51304 according to the manufacturer's instructions. DNA concentration and purity was defined using the Nanodrop 1000 (Isogen, Sint-Pieters-Leeuw, Belgium) with a range of 55–110 ng/μl. All genomic DNAs were stored at −80 °C until further research.

KRAS StripAssay

The KRAS StripAssay kit (Lot. 5-590) was kindly provided by the Vienna Lab of Austria. All procedures were conducted according to the manufacturer's instructions in sterile area (laminar flow) to avoid false positive results due to contamination. Briefly, for each PCR reaction a total volume of 25 μL was used. PCR products were amplified in a tube containing 15 μL of amplification mix, 5 μL of diluted Taq DNA polymerase and 5 μL of DNA template. The cycling conditions were 94 °C for 2 min, 35 cycles of 94 °C for 60 s, 70 °C for 50 s, 56 °C for 50 s and 60 °C for 60 s, with final extension at 60 °C for 3 min. Following hybridization (45 °C, shaking water bath), stringent washing (45 °C, shaking water bath) and color development (room temperature), the results were interpreted using the enclosed Collector sheet.

Statistical analysis

Quantitative data were presented as minimum, maximum, mean, median and standard deviation (SD) values. Qualitative data were presented as frequencies and percentages. The Chi-square (×2) test was used for studying the comparisons between different qualitative variables.

Table 2 Molecular genetic alterations.

Sample ID (No. of patients = 20)	KRAS mutation genotype
A	Codon 12 Ala + 12 Val
B	Codon 12 Asp + 12 Ser
C	Codon 12 Asp + 12 Cys + 12 Ser + 12 Val
D	Codon 12 Ser + 12 Val + 13 Asp
E	Codon 12 Asp + 12 Ser
F	Codon 12 Val
G	No mutation
H	Codon 12 Ala + 12 Asp
I	Codon 12 Val
J	No mutation
K	Codon 12 Val
L	Codon 12 Asp + 12 Ser
M	Codon 12 Val
N	Codon 12 Ala + 12 Asp
O	Codon 12 Asp + 12 Cys + 12 Ser + 12 Val
P	No mutation
Q	Codon 12 Ser + 12 Val + 13 Asp
R	Codon 12 Ala + 12 Val
S	No mutation
T	Codon 12 Asp + 12 Ser

Ala, alanine; Asp, aspartic acid; Cys, cysteine; Ser, serine; Val, valine.

Results

The study included 6 males and 14 females with a median age of 56.5 years (range from 47 years to 72 years). The primary locations of tumors were the colon (n = 16) and the rectum (n = 4). Sixteen patients had KRAS mutations representing (16/20) 80% of the study sample. Females showed a statistically significantly higher prevalence of mutation than males

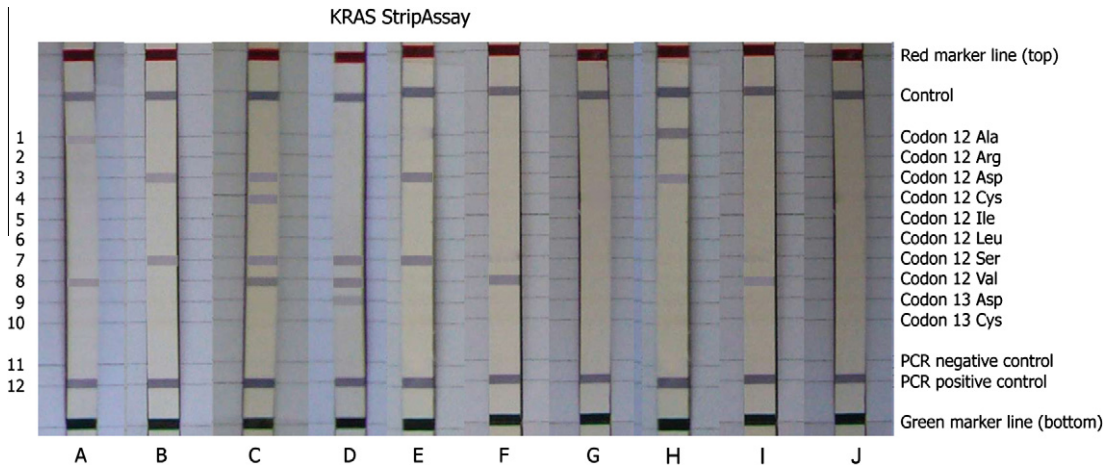

Figure 1 KRAS mutations present on StripAssay. (A) Positive lines at positions 1 and 8. (B) Positive lines at positions 3 and 7. (C) Positive lines at positions 3, 4, 7 and 8. (D) Positive lines at positions 7, 8 and 9. (E) Positive lines at positions 3 and 7. (F) Positive lines at position 8. (G) No mutation. (H) Positive lines at positions 1 and 3. (I) Positive lines at position 8. (J) No mutation.

as the KRAS mutations were detected in 2/6 (33.3%) of males and 14/14 (100%) of females. Fourteen patients 14/20 (70%) had a single mutation site at codon 12, two patients 2/20 (10%) in codons 12 and 13 and 4 patients 4/20 (20%) showed no mutations (Table 2 and Fig. 1).

Discussion

The recent advice from the American Society of Clinical Oncology (ASCO) and a European expert panel to perform KRAS mutation detection before therapy with cetuximab in metastatic colorectal cancer [8] and in stage II and III colon cancer [10] respectively, has made the need urgent for a sensitive, flexible, and fast assay that is easy to implement in daily practice. Therefore, the commercially available StripAssay was used in this study to detect KRAS mutations.

Sixteen of the 20 examined patients with CRC harbored a mutated KRAS gene. The most striking observation is that almost all mutations occurred in codon 12 of the KRAS gene as it was found in 14 cases and only two patients had the mutation in both codons 12 and 13. In 4 cases KRAS gene mutations could not be detected in the tumor DNA, although histologically these tumors did not differ from the tumors with a mutated KRAS gene.

The StripAssay is based on mutant-enriched PCR followed by reverse hybridization. The mutant-enriched PCR is based on the clamping of the wild-type sequence by PNA nucleotides; therefore, only the mutant DNA template is amplified. Such a sensitive technique could detect small subpopulations of tumor cells carrying mutant alleles within a majority of wild-type tumor cells. Although KRAS mutation is generally accepted as an early event in colon carcinogenesis [11], tumor heterogeneity is a known feature [12]. Baldus et al. [12] have recently reported that mutations are differentially present in different areas of the tumor as well as in positive lymph nodes and metastases. The clinical relevance of this finding is not fully understood, but it could greatly contribute to difficulty in therapy decision making. Mutated clones could be preferentially detected with the StripAssay, while the remaining undetectable with standard techniques such as direct sequencing and SNaPshot, even when sufficient tumor cells are present.

For colon cancer diagnostics, in which sensitivity is generally not an issue, and when capillary electrophoresis facilities are already available, SNaPshot can be as valuable as direct sequencing. Workflow, time to results, hands-on time, and costs do not vary much between both techniques. However, the multiplex possibilities of the SNaPshot can reduce DNA input, costs, and workload. Thus, SNaPshot is a good alternative for direct sequencing for KRAS mutation detection in colon cancer patients in daily diagnostic practice. However, when sensitivity is an important issue, such as in the case of lung cytology samples, or for small laboratories without dedicated equipment, highly sensitive techniques such as the StripAssay should be considered due to its high sensitivity, rapidity, and ease to perform. Nevertheless, one should be aware of the false-positivity risks of such a technique and perform assays in duplicate to avoid false positives [13]. Thus, the high sensitivity of the StripAssay could be its biggest caveat, and one should be very cautious when carrying out such a sensitive assay. It might well be that even more expertise, more restricted laboratory discipline, and special additional precautions are necessary to circumvent false positivity due to sample contamination [13].

The workflow of the StripAssay is easy and time to results after DNA isolation is half a working day. This assay also does not require any dedicated equipment. Thus, results can be easily obtained.

This research warrants further, larger scale studies.

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