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Detecting K-*ras* mutations in stool from fecal occult blood test cards in multiphasic screening for colorectal cancer

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Abstract

Fecal occult blood testing (FOBT) is proven an efficient way of reducing mortality from colorectal cancer but has a relatively low positive predictive value (PPV). This study evaluated the ability to detect K-*ras* mutations in stool DNA from FOBT cards and to improve the PPV of the screening process. Two hundred and five consecutive positive FOBT cards and an arbitrary sample of 38 negative cards from a population-based screening program were included. K-*ras* mutations in FOBT card stool were sought using allele-specific hybridization. DNA was successfully amplified from 87.2% of cards. In 130 cases with positive FOBT and amplifiable DNA 23 malignancies and 25 adenomas were detected. In 34.8% of the malignancies, a mutation in K-*ras* was detected. The PPV for malignancies increased from 17.7% (all positive cards) to 60.0% if cards with four or more fields were positive and K-*ras* was positive (RR = 2.66, 95% CI: 1.2–6.1). Testing for K-*ras* mutations in DNA extracted from stool from positive FOBT cards is feasible. Sequential detection of cancer-associated genetic markers from FOBT-based stool samples may potentially help separate true from false positives in a FOBT-based screening process.

Keywords: Colorectal cancer; Screening; Molecular testing; Fecal occult blood tests (FOBT); K-*ras*; Positive predictive value (PPV)

1. Introduction

Three randomized controlled trials have been conducted over the last 30 years to evaluate the contribution of periodic screening with fecal occult blood tests (FOBT) to the reduction in mortality of colorectal cancer in the studied population [1–5]. The results of these studies demonstrate signifi-

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cant reduction in mortality with biennial as well as annual testing after 8–18 years of follow-up. Yet, despite the cost effectiveness of this screening tool, these tests carry a high false positive rate resulting in the need for many unnecessary colonoscopies.

To improve upon FOBT, stool-based tests detecting common colorectal neoplasia-associated DNA mutations were developed by Sidransky et al. [6], refined by Jen et al. [7] and subsequently validated in a prospective screening clinical trial [8]. The DNA-based diagnostic panel enhanced diagnostic sensitivity for colorectal adenocarcinoma but not for colorectal adenoma [8]. If applied sequentially, stool DNA testing might improve the differentiation of true positive from false positive FOBT results, thus increasing the positive predictive value of the FOB test.

This study was designed to test the feasibility of extracting, of amplifying, and of detecting normal and mutated K-*ras* DNA from stool samples that were submitted on guaiac-positive and guaiac-negative FOBT cards.

2. Materials and methods

2.1. Study population

The National FOBT Screening Program for Colorectal Cancer of Clalit Health Services in Israel (CHS, a non-profit HMO-type organization covering more than 70% of the Israeli older population), served as the source of FOBT cards for this study. The Screening Program invites all women and men, aged 50–74, members of CHS to have an annual, free-of-charge, FOBT. Of 5000 cards screened, 205 consecutive positive (traces or any positive field) FOBT cards and 38 arbitrarily assigned negative FOBT cards were included in this study. The study was approved by the IRB Committee of Carmel Medical Center.

2.2. FOBT screening process

Following suggested diet and medication restriction, subjects applied feces to two windows on a Hemoccult Sensa test card (Beckman Coulter Inc.) on each of three consecutive days for a total of six test windows). The card was then mailed to the CHS National Cancer Control Center laboratories in a pre-paid, pre-addressed envelope supplied as part of the FOBT kit to each subject. In order to eliminate peroxidases from dietary vegetables and fruits known to be responsible for false positive test results [9], the cards were not processed earlier than a week from first stool application. The test was analyzed by applying the supplied developing solution (a stabilized mixture of less than 4.2% hydrogen peroxide and 80% denatured ethyl alcohol and enhancer in an aqueous solution) in a single laboratory. A test was called positive if at least one of the six tested fields showed the typical color change following the guaiac reaction.

2.3. Laboratory methods

2.3.1. DNA extraction

After development for the guaiac reaction, FOBT cards were kept dry at −20 °C until DNA isolation. Stool samples were then excised from all six windows of the FOBT cards and agitated overnight in 5 ml preservation solution containing 1.2 M guanidium thiocyanate, 100 mM sodium acetate, pH 5.2, 0.5% Triton X-100. The next morning, the samples in the preservation solution were extracted briefly with 3 ml of chloroform:isopropanol (94/6, v/v) and then centrifuged at 8000*g* for 10 min at room temperature. The top 4 ml were then diluted with an equal amount of 6 M guanidium hydrochloride. DNA purification resin (Promega) was added and the mix was applied onto a vacuum column. The resin was washed twice with total 6 ml Wash-1 solution (40% isopropanol, 3 M guanidium hydrochloride, 50 mM NaOAc, pH 5.2) and then twice with a total of 6 ml Wash-2 solution (60% ethanol, 100 mM sodium acetate, pH 5.2). The DNA was eluted with 100 μl of 10 mM Tris–hydrochloride, pH 7.6. Approximately 200 mg of stool was normally available from the six card-windows of stool, with a varying yield between 100 and 1000 ng of apparently mixed bacterial and human DNA.

2.3.2. K-ras mutation analysis

After extracting DNA from the stool samples the first exon of K-*ras* was amplified by PCR. Primers used were: Ki-*ras* 11 primer aggaattcatgactgaatataaacttgt; Ki-*ras* 12 primer atcgaattcctctattgttggatcatatcc. Five to ten microliters of DNA purified from feces (10–100 ng) was added to 50 μl of PCR mix containing PCR-buffer, 200 μM dNTP, 1 μM of Ki-*ras* 11 and Ki-*ras* 12 primers. The mix was heated up to 80 °C for 1 min. One and half units of *Taq* polymerase was added. The tubes were sealed and reaction continued for 60 cycles as follows: 56 °C for 30 s, 72 °C for 15 s, 94 °C for 15 s. Samples were spotted on nylon membrane (Hybond-N, Amersham or Gene Screen, NEN). Thirty microliters PCR product was diluted with 150 μl of 0.15 N sodium hydroxide and allowed to melt for 20 min at room temperature. Two hundred microliters of 0.5 M phosphate buffer (pH 7) was added into each well of a dot apparatus, and 12–15 μl of a DNA sample in alkali were transferred into each of the wells before the vacuum was applied. DNA was fixed with UV cross-linking (1200 mW/cm^2).

Probe labeling for point mutation detection were labeled by a primer extension approach. A short template was designed that complements the nine last nucleotides of the 3′-end of the allele-specific oligodendronucleotide

(ASO) probe detailed below, plus three extending thymidine triphosphate nucleotides at its 5′-end, 12 nucleotides in total. After annealing, Klenow DNA polymerase is added in the presence of [^{32}P]-deoxyadenine triphosphate, and the ASO probe is labeled by extension upon the template. In this way the final product is the ASO probe containing three labeled dAs at its 3′-end. Dotted nylon sheets had been incubated in 20–30 ml of hybridization solution containing 100 mg/ml of herring sperm DNA and 200 pmol of normal sequence competitor oligo for 1 h before the labeled probe was added. Blots were routinely hybridized overnight at 56–59 °C. Hybridized filters were briefly washed 2–3 times with a washing solution containing 3× SSC and 0.2% SDS at room temperature, and washed 3–4 times (each time for 15 min) with a washing solution pre-warmed to 50 °C. The washed filters were briefly air-dried and exposed to the Phosphoimager plate for 1–3 h.

Mutations were detected by ASO hybridization. Two copies of each membrane were prepared: one hybridized with a general probe from outside the mutated region detecting any amplified K-*ras* DNA. The second membrane was hybridized with an ASO probe. Since most of the mutations in codons 12 and 13 in the Israeli population are found in four out of 12 possible mutations [10], we used only four different ASO probes, representing 86% of the available mutations, as follows: codon12, AGT cctacgccactagctcca; codon12, GAT cctacgccatcag ctcca; codon12, GTT cctacgccaacagctcca; codon13, GAC cctacgtcaccagctcca; competitor oligo, cctacgccaccag ctcca; tailer, ttttggagctgg.

2.3.3. Signal evaluation

Due to some background expected with normal sequence, a strong signal may be obtained from either the presence of mutated sequences or the presence of high amount of normal sequences. Also, the efficiency of the different probes was variable. Accordingly, the signals on a given membrane were divided by eye into three levels of intensity: background (the majority of signals), weak (slightly above background), and strong (significantly above weak). A signal was considered positive if it was in the strong class of signals, and if it was not highly strong with the general probe.

2.4. Follow-up

All screened positive cases were referred to primary care physicians and appropriate subspecialists. Recommended evaluation of a positive case was either a full colonoscopy or a flexible sigmoidoscopy with a double contrast barium enema. Post-evaluation findings were coded as: (a) no neoplasia (e.g. hemorrhoid, diverticulosis, inflammatory bowel disease, gastritis), (b) hyperplastic polyp (non-neoplastic), (c) adenomas, of all types (tubular, tubulo-villous, villous), (d) invasive neoplasm. The rest of the cards were summarized as no finding (or false positives). Evaluation of the upper GI tract was not mandatory in cases with positive occult blood and no findings from colonoscopy.

2.5. Statistical analysis

Positive predictive value (and 95% confidence intervals) of the addition of K-*ras* results to a positive FOBT was calculated by dividing the number of cases with a positive FOBT result in which a pathological finding was found in follow-up by the total number of positive FOBT. This was done separately for each type of pathologic finding in follow-up. The analysis was performed separately for (a) any FOBT positivity and (b) FOB finding of four or more positive fields (out of six). The later was shown to carry a much higher probability of cancer [11]. No measures of sensitivity or specificity were calculated because this pilot study evaluated only K-*ras* performance as a sequential adjunct to the guaiac test results. The K-*ras* outcome is not intended to be interpreted independently.

3. Results

3.1. Feasibility

Of the 243 FOBT cards included in this study, 205 (84.4%) tested positive and 38 (15.6%) tested negative. Two hundred and twelve of the 243 (180 positive and 32 negative) samples (87.2%) were successfully amplified. Most of the unamplified samples had insufficient stool mass resulting in insufficient DNA recovery for amplification. Amplification failures were not dependent upon the FOBT reaction (non-amplified, negative FOBT cards (6/38, 15.8%); non-amplified positive FOBT cards (25/205, 12.2%) $p = 0.6$ (Fig. 1). Mutations in K-*ras* were found in 48 amplified samples; 39 among 180 positive FOBT amplified samples (21.7%) and 9 among 32 negative FOBT amplified samples (28.1%) $p = 0.42$.

3.2. Follow-up evaluation

Follow-up on cases with positive FOBT was complete in 130 of the 180 positive amplified samples (72.2%). Among these, 23 malignancies in the colon and rectum, 25 adenomatous polyps and 14 hyperplastic polyps were detected. Among the 50 amplified samples with positive FOBT who did not have an evaluation procedure, 31 cases (62.0%) had one positive FOBT field and 11 cases (22.0%) had two positive fields. The main reasons for incomplete follow-up were, in equal proportions, patient refusal, and primary care physician decision not to proceed. This was most commonly the case when only one positive field was found. After a median of 6 years follow-up, no colorectal cancer cases were detected among

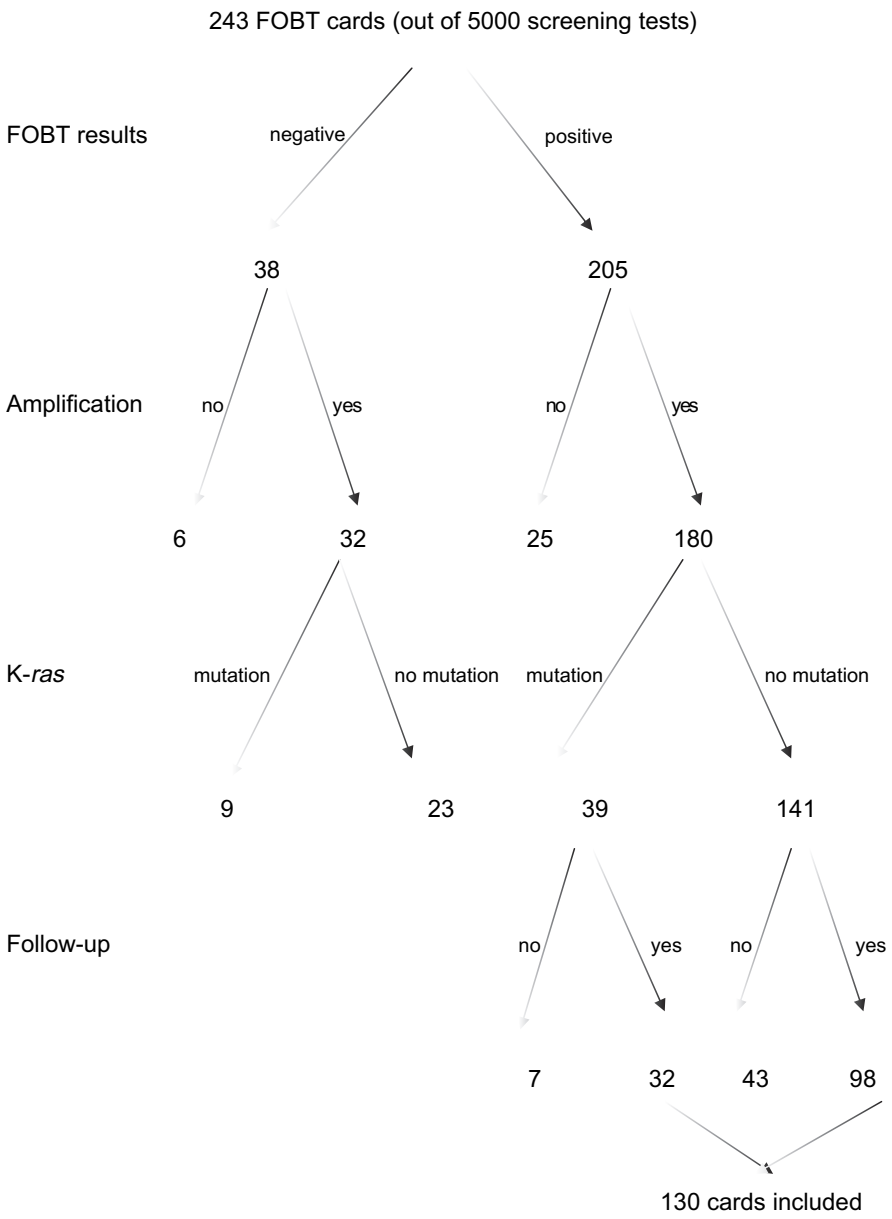

Fig. 1. K-*ras* in stool from FOBT cards study flow chart.

hemoccult-negative subjects. Four more cancers were detected 3–5 years after the index-positive FOBT in cases with either no findings or adenomas only in colonoscopy.

The proportion of K-*ras* positive tests differed between cases with positive and negative FOBT, and by the type of finding in follow up ($p = 0.07$) (Table 1). No differences were noted in the distribution of the specific K-*ras* mutations between the different diagnostic groups. AGT was the most commonly identified K-*ras* mutation and was noted in 50% of the mutated stools of cases with or without tumor findings.

3.3. Impact of adding K-ras detection upon FOBT detection of colorectal neoplasia

The positive predictive value for cancer diagnosis of the combined test (FOBT + K-*ras*) approximately doubled that of a single FOBT measure. For adenomas, K-*ras* improved FOBT's positive predictive value when the FOBT tested positive in less then four of the six test slides. Such a situation is common because adenomas usually bleed less. The positive predictive value of FOBT in four or more fields reached 60% (positive/negative cards

Table 1
Proportion of K-*ras* positivity in stool from positive FOBT cards with amplified DNA, by type of finding in the colon

Finding in colon	Number of cases with positive FOBT and DNA amplification	Number of cases with K-*ras* mutation	Proportion of K-*ras* positivity (%)
Carcinoma + adenoma	48	16	**33.0**
Adenocarcinoma	23	8	34.8
Adenoma	25	8	32.0
All other[a]	82	16	**19.5**
No follow-up	50	7	**14.0**
Total positive FOBT	180	39	**21.7**

[a] Includes non-adenomatous polyps, diverticuli, IBD, hemorrhoids, and upper GI findings.

Table 2
Positive predictive values of FOBT alone or in combination with stool K-*ras* results, for the detection of malignancies, adenomas, any tumors or benign non-neoplastic findings in the colon

Findings in colon	Any FOBT+	FOBT+ >4 fields	Any K-*ras*+ In FOBT+	FOBT >4 + K-*ras*+
N	130	41	32	10
Adenocarcinoma	23	13	8	6
PPV	17.7% (2.1–33.3)	31.7% (12.7–50.7)	25.0% (7.3–42.7)	60.0% (40.0–80.0)
Adenoma	25	9	8	2
PPV	19.2% (3.8–34.7)	22.0% (5.7–38.2)	25.0% (8.0–42.0)	20.0% (4.3–35.7)
All neoplasms[a]	62	25	17	8
PPV	47.7% (35.3–60.1)	61.0% (48.8–73.1)	53.1% (40.7–65.5)	80.0% (70.0–90.0)
No-neoplastic GI disease[b]	46	13	9	1
PPV	35.4% (21.6–49.2)	31.7% (18.3–45.2)	28.1% (15.1–41.1)	10.0% (1.3–18.7)
Normal colonoscopy[c]	22	3	6	1
PPV	16.9%	7.3%	18.8%	10.0%

[a] Includes adenocarcinomas, adenomas, and polyps NOS.
[b] Includes diverticulosis, hemorrhoids, and IBD.
[c] Does not preclude upper GI findings.

ratio = 2.66, 95% CI: 1.2–6.1). K-*ras* enhanced the positive predictive value of FOBT in four or more fields to 80% for all neoplastic lesions; cancer + adenoma (ratio = 1.46, 95% CI: 0.9–2.3). K-*ras* reduced the FOBT positive predictive value (from 35.4% to 10.0%) for non-neoplastic conditions (e.g. diverticulosis, hemmorhoids, ulcers) (Table 2).

4. Discussion

This study demonstrated that DNA extracted from a Hemoccult Sensa card is of sufficient quality and quantity to detect common mutations associated with colorectal carcinogenesis progression. K-*ras* was chosen as the test gene for this new method of DNA isolation and amplification from stool guaiac cards due to its high mutational frequency in the earlier phases of the colonic epithelial transformation process [12]. Among the histologicall verified malignancies, K-*ras* was found in 32%. While we did not perform a concordance validation with tissue samples from the sample patients' stool samples we amplified, the percentage of positive K-*ras* tests in the subjects who had subsequent neoplastic disease proven corresponds well with the available literature [12–15].

K-*ras* mutations occurred in 28% of the stool samples of negative FOBT cards. Other groups have also demonstrated a similar rate of "false positive" results for K-*ras* in urine [16] and in stool [14]. Ito et al. [14] reported that three out of six stools found positive for K-*ras* not to have K-*ras* in the corresponding tumor tissue. Jen et al. [17] showed 25% of hyperplastic polyps to be K-*ras*-positive. Others, such as Puig et al. [18] did not find any false positive samples for K-*ras*. The source of these positive tests without known tissue-based mutation is unclear [14,16,18].

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