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Circulating free DNA in a screening program for early colorectal cancer detection

Federica Perrone[1], Andrea Lampis[1], Claudia Bertan[1], Paolo Verderio[2], Chiara M Ciniselli[2], Sara Pizzamiglio[2], Milo Frattini[3], Martina Nucifora[3], Francesca Molinari[3], Gianfranco Gallino[4], Manuela Gariboldi[5,6], Emanuele Meroni[7], Ermanno Leo[8], Marco A Pierotti[5,9], and Silvana Pilotti[1]

[1]Laboratory of Experimental Molecular Pathology, Department of Pathology, [2]Unit of Medical Statistics, Biometry and Bioinformatics, [4]Melanoma and Sarcoma Unit, [5]Department of Experimental Oncology and Molecular Medicine, [7]Division of Diagnostic Endoscopy and Endoscopic Surgery, Department of Surgery, [8]Colorectal Surgery Unit, and [9]Scientific Directorate, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; [3]Laboratory of Molecular Diagnostics, Institute of Pathology, Locarno, Switzerland; [6]Molecular Genetics of Cancer, Fondazione Istituto FIRC di Oncologia Molecolare, Milan, Italy

ABSTRACT

Aims and background. The quantification and molecular characterization of circulating free DNA (cfDNA) have attracted much interest as new and promising, noninvasive means of detecting and monitoring the presence of surgical resectable colorectal cancer (CRC). Instead, the role of cfDNA in the early detection of malignant and premalignant colorectal lesions is still unclear. The aim of this study was to evaluate the predictive power of the quantification and *KRAS* status of cfDNA in detecting early colorectal lesions in plasma from healthy high-risk subjects.

Methods. The study population consisted of 170 consecutive healthy high-risk subjects aged >50 years who participated in the screening program promoted by the Local Health Service (ASL-Milano) for early CRC detection and who underwent endoscopic examination after being found positive at fecal occult blood test (FOBT). Thirty-four participants had malignant lesions consisting of 12 adenocarcinomas (at an early stage in half of the cases) and 22 instances of high-grade intraepithelial neoplasia (HGIN) in adenomas; 73 participants had premalignant lesions (adenomas and hyperplasia), and 63 participants had no lesions. Plasma cfDNA was quantified by quantitative real-time PCR and analyzed for *KRAS* mutations by a mutant-enriched PCR. *KRAS* status was assessed also in matched adenocarcinoma and HGIN tissues. The distribution of cfDNA concentrations among FOBT-positive subjects with diagnosed lesion (cases) was compared with that of FOBT-positive subjects without lesions (controls) and its predictive capability (AUC) was assessed.

Results. The predictive capability of cfDNA levels was satisfactory in predicting adenocarcinomas (AUC 0.709; 95% CI, 0.508-0.909) but not HGIN and premalignant lesions. The rate of *KRAS* mutations in plasma was low (5/170 = 3%) compared with the rate observed in the matched adenocarcinoma and HGIN tissues (45%).

Conclusions. The use of cfDNA quantification to predict adenocarcinoma at an early stage in high-risk (aged >50 years and FOBT positive) subjects seems to be promising but needs more sensitive methods to improve cfDNA detection.

Key words: colorectal cancer, plasma, circulating free DNA, early diagnosis.

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Correspondence to: Silvana Pilotti, Department of Pathology, Fondazione IRCCS Istituto Nazionale dei Tumori, Via Venezian 1, 20133 Milan, Italy.
Tel +39-02-23902293;
fax +39-02-23902877;
email silvana.pilotti@istitutotumori.mi.it

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Introduction

It has been reported that the circulating free DNA (cfDNA) detected in human plasma samples may be used to diagnose various types of cancer. Although little is known about its origin, form or rate of release, it is believed that cfDNA is a result of increased and abnormal activation of apoptotic pathways in cancerous lesions leading to DNA fragmentation[1,2].

As plasma cfDNA from cancer patients can oncogenically transform cultured cells[3], it has been suggested that the cell-free nucleic acids in plasma participate in tumorigenesis and the development of metastases by means of their transfection-like uptake by susceptible cells, a phenomenon known as "genometastasis".

Over the last few years, the quantification of cfDNA in patients with colorectal cancer (CRC) has attracted much interest as a new and promising noninvasive means of detecting the presence of CRC. In line with the findings of others[4-6], we have previously demonstrated that plasma cfDNA levels in CRC patients are significantly higher at the time of surgery than those found in healthy subjects[7,8]. We have also shown that tumor cfDNA levels in plasma gradually decrease during the post-surgical follow-up, but rapidly increase when a recurrence or metastasis develops[7,8]. In addition to being a useful means of monitoring CRC patients during follow-up, cfDNA is also a reliable surrogate for monitoring CRC therapy[9-11]. In addition to cfDNA quantification, it has been suggested that CRC-related genetic alterations in plasma could be used to improve the diagnostic and prognostic accuracy, and monitoring recurrences and therapeutic responses. These qualitative analyses encompass various types of DNA alterations, including *KRAS*, *APC* and *TP53* mutations, DNA hypermethylation, microsatellite instability and the loss of heterozygosity, all of which have been detected in the cfDNA of patients with CRC[12]. We have previously found *KRAS* mutations and $p16^{INK4a}$ methylation in plasma samples taken from relapsed patients, and so they might help in detecting recurrences during follow-up[7,8]. The same results have been obtained in serum by Wang *et al.*[13] Furthermore, it has recently been found that, using the BEAMing approach, mutant *KRAS* alleles can be detected with high sensitivity and before the radiographic documentation of disease progression in plasma of patients with wild-type *KRAS* CRC who developed resistance to cetuximab[14]. However, this qualitative approach requires previous knowledge of the molecular alterations of a given tumor and thus is not applicable in the context of early diagnosis.

In light of these considerations it seems reasonable to assume that quantitative levels of cfDNA might be a useful means of detecting CRC early, but the role of cfDNA in this setting is still unclear. The aim of this study was to evaluate the predictive capability of plasma cfDNA quantification in detecting malignant (adenocarcinomas) and early malignant lesions (adenomas, high-grade intraepithelial neoplasia, etc.) in fecal occult blood test (FOBT)-positive individuals who took part in the population-based Lombardy Region Screening Program (LRSP) for the early detection of CRC. In addition, since no data are currently available on *KRAS* status in the context of early diagnosis, we complemented this analysis with the qualitative evaluation of *KRAS* mutations which, although they are not specific as they are shared by various malignancies and may be present in both premalignant and malignant colorectal tissue lesions, might improve the diagnostic sensitivity.

Material and methods

Patients

The study population consisted of 170 FOBT-positive subjects (94 men and 76 women; median age 62 years; range 49-74 years) who participated in the screening program promoted by the Local Health Service (ASL-Milano) between October 2008 and March 2010, and who agreed to undergo endoscopic examination at the Fondazione IRCCS Istituto Nazionale Tumori of Milan.

The LRSP, in which our institute is a working unit, is based on FOBT[15]. Participants who are FOBT negative are invited to repeat the test after 2 years, while FOBT-positive individuals who agree to undergo colonoscopy are submitted to endoscopic examination in one of the hospitals participating in the screening program. If colonoscopy reveals the presence of malignant lesions, the patient will receive cancer treatment (surgical or endoscopic tumor removal) whereas in case of negative colonoscopy the FOBT test will be repeated 5 years later.

Before sampling, each patient signed an informed consent form approving the use of his/her biological samples for research purposes. The study was approved by the ethics committee of our institute.

Samples

Peripheral blood samples (16 mL) were collected in BD Vacutainer® EDTA tubes at the time of endoscopic examination, stored at 4 °C and processed within 3-4 hours, as recommended for obtaining a good DNA yield[16]. Each blood sample was divided into two 15-mL tubes and centrifuged at 3000 rpm for 10 minutes at room temperature. The obtained plasma sample (an average of 8 mL) was stored at -80 °C. One milliliter of plasma was used to extract total DNA by means of a QIamp DNA Blood Extraction Kit (Qiagen) as previously described[7]; the DNA was eluted in 50 µL of double-distilled water (ddH_2O) and stored at -20 °C until use.

Genomic DNA was extracted by means of micro-dissection from 7-µm formalin-fixed, paraffin-embedded (FFPE) serial sections of the adenocarcinoma and adenoma biopsy specimens taken endoscopically during colonoscopy using a QIAamp DNA FFPE Tissue Kit (Qiagen).

Plasma cfDNA quantification by means of real-time polymerase chain reaction (PCR)

Plasma cfDNA was quantified by means of quantitative real-time (qRT) PCR using TaqMan probe and primers specific for the ubiquitous single-copy gene human telomerase reverse transcriptase (*hTERT*), and

the previously described PCR thermal profile[17]. We selected *hTERT* because it is usually a single-copy gene in CRC and we previously quantified it in lung cancer[17]. Each qRT PCR was performed in triplicate in a final volume of 20 µL using a reaction mixture consisting of 5 µL DNA solution, 1X Universal Master Mix (Applied Biosystems), 0.2 µM hTERT fluorogenic probe, 0.9 µM hTERT forward and reverse primers, and 4.37 µL sterile ddH_2O. Amplification was carried out in 96-well plates using the standard protocol for the 7900HT Fast Real-Time PCR system (Applied Biosystems). Each sample was analyzed in triplicate on the same plate.

To quantify the amount of DNA in the experimental sample, a 6-point calibration curve was constructed on each plate using a commercially available human genomic leukocyte DNA (Roche) with appropriate serial dilutions (1:5): 25000 pg, 5000 pg, 1000 pg, 200 pg, 40 pg and 8 pg.

The results were evaluated by means of Sequence Detection System software (Applied Biosystems).

KRAS analysis

Genomic DNA extracted from plasma (4 µL) and tissue (100 ng) was analyzed for *KRAS* mutations. *KRAS* exon 2 status was assessed by means of a mutant-enriched PCR consisting of 2 (semi-nested) amplification steps in which artificial restriction sites (*Bst*NI for codon 12 and *Bgl*I for codon 13) are introduced during the first step using mismatched primers. The wild-type amplicons were digested by restriction enzymes, whereas the mutant products were enriched during the second round of amplification. The primers and PCR conditions have been previously described[18].

Statistical analysis

The distribution of cfDNA concentrations among the FOBT-positive subjects with newly diagnosed lesion (cases) was compared with that of FOBT-positive subjects without neoplastic lesions (controls) using the Kolmogorov-Smirnov test[19]. This approach was also used to compare the distribution of cfDNA concentrations within each type of lesion with that of the controls. To assess the predictive capability (i.e. diagnostic performance) of cfDNA, the area under the receiver operating characteristic (ROC) curve (AUC) was generated[20] using a logistic regression model[21] for each comparison. In this model, cfDNA was included as a continuous variable, and its relationship with the endoscopic outcome was investigated using a regression model based on restricted cubic splines[22]. Bonferroni's correction was used to adjust for multiple comparisons[23].

All of the statistical analyses were made using the SAS software (version 9.2, SAS Institute Inc., Cary, NC, USA) by adopting a significance level of alpha = 0.05.

Results

Study population

Among the 170 FOBT-positive study participants, 34 (20%) were found to have malignant lesions including 12 adenocarcinomas and 22 cases of high-grade intraepithelial neoplasia (HGIN) in adenomas; 73 subjects had premalignant lesions consisting of adenomas (1-6 per subject) without evidence of any grade of dysplasia (54 cases) or hyperplasia (19 cases); the remaining 63 subjects had no lesions (Table 1). Three of the 12 adenocarcinomas were pT1 lesions detected in endoscopically resected polyps.

The 12 patients with adenocarcinomas (7 men and 5 women) had a median age of 63 years (range 49-72) and all underwent major surgery. Pathological staging classified 3 cases as pT0NxMx (corresponding to the 3 previously resected polyps), 1 as pT4N2Mx, 1 as pT3N1Mx, 3 as T3N0Mx, and 4 as pT2N0Mx. Three of the adenocarcinomas were located in the colon, 1 in the cecum, 6 in the sigmoid colon, and 2 in the rectum.

The 22 patients with HGIN (10 men and 12 women) had a median age of 63 years (range 51-73). HGIN was observed in 24 endoscopically resected polyps, all with clear margins, distributed in tubular and tubular-villous adenomas (12 each). Eight were located in the colon, 2 in the cecum, 12 in the sigmoid colon, and 2 in the rectum.

The 54 patients with adenomas (38 men and 16 women) had a median age of 62 years (range 51-74). Endoscopy revealed 2 or more (up to 6) adenomas in 12 patients. A total of 82 adenomas were identified and classified as tubular (74), villous-tubular (6) or serrated adenomas (2). Fifty adenomas were located in the colon, 8 in the cecum, 20 in the sigmoid colon, and 4 in the rectum.

The 19 patients with hyperplasia (10 men and 9 women) had a median age of 63 years (range 51-72), and the 63 FOBT-positive but lesion-free subjects (29 men and 34 women) had a median age of 62 years (range 49-72).

Table 1 - Patient characteristics

Characteristics	No. (%)
Lesions	
Adenocarcinoma	12 (7.06)
HGIN	22 (12.94)
Adenoma	54 (31.76)
Hyperplasia	19 (11.18)
No lesion	63 (37.06)
Gender	
Male	94 (44.71)
Female	76 (55.29)
Median age: 62 years (range 49-74)	

HGIN, high-grade intraepithelial neoplasia.

cfDNA quantification

The box plots in Figures 1 and 2 show the distribution of cfDNA levels in the cases as a whole (individuals with lesions) and controls (individuals with no lesions), and their distribution in patients with each type of lesion. Table 2 shows some descriptive statistics relating to these distributions. The Kolmogorov-Smirnov test showed that the difference in the distribution of cfDNA levels between cases and controls as well as the difference in distribution between each type of lesion and controls was not statistically significant. It is worth noting that only the difference between the adenocarcinoma group and the controls showed borderline significance ($P = 0.07$).

A linear relationship between the log odds and cfDNA values (using a logarithmic scale) was found to be appropriate for all of the fitted logistic models. The capability of cfDNA to predict at least 1 lesion or a specific lesion was assessed by calculating the AUC and, as shown in Table 3, this was satisfactory only in the case of adenocarcinomas (AUC 0.709; 95% confidence interval [CI], 0.508-0.909) (Figure 3).

KRAS mutations

The *KRAS* status of all of the plasma samples was qualitatively analyzed. Mutant-enriched PCR revealed the missense activating mutation G12C in 1 (8%) of the 12 samples taken from the adenocarcinoma patients. Moreover, 3 (16%) of the 19 samples taken from HGIN patients harbored *KRAS* mutations (G12D; G12V and G12S in 1 case each), and the same was true for 1 (2%) of the 54 samples taken from adenoma patients (G13D). No mutations were detected in the samples taken from the hyperplasia patients or those in the no-lesion group. *KRAS* mutations were therefore found in 5 (3%) of the 170 plasma samples.

To verify the low rate of *KRAS* mutations in plasma, we compared *KRAS* status in FFPE tumor tissue and plasma in the 12 adenocarcinomas and 19 HGINs that were surgically resected. *KRAS* mutations were found in 5 adenocarcinomas (42%) and 9 HGINs (47%). Overall, *KRAS* mutations were observed in the tumor tissue of 14 patients (45%), only 2 of whom (6%) also showed *KRAS* mutations in their plasma (G12V and G12S). Both these patients belonged to the HGIN group.

Discussion

We have previously found that both the quantitative and qualitative characterization of cfDNA may be able to confirm the presence of CRC and monitor disease relapse after surgery[7,8], but the quantification of cfDNA as an informative biomarker in cancer screening has recently been questioned[24].

In this study we investigated the possible role of cfDNA concentration as a tool for detecting early lesions in a series of 170 FOBT-positive subjects who participated in a regional population-based screening program carried out by ASL-Milano. We analyzed plasma cfDNA from FOBT-positive subjects who underwent colonoscopy at our institute. We chose to work with plasma because it is less likely to be contaminated by leukocytes

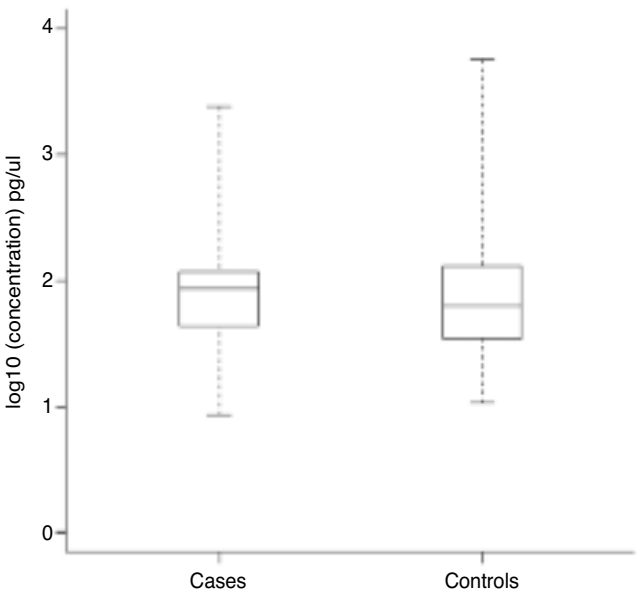

Figure 1 - Box plots of the distribution of cfDNA concentrations among cases (subjects with all types of newly diagnosed lesions) and controls (subjects with no neoplastic lesions). Each box indicates the 25th and 75th percentiles. The horizontal line inside the box indicates the median and the whiskers the extreme measured values.

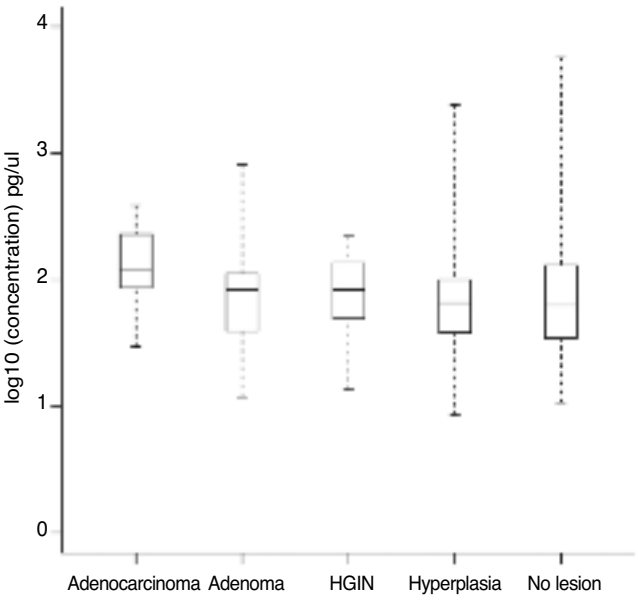

Figure 2 - Box plots of the distribution cfDNA concentrations among cases (subjects with all types of newly diagnosed lesions) and controls (subjects with no neoplastic lesions). Each box indicates the 25th and 75th percentiles. The horizontal line inside the box indicates the median and the whiskers the extreme measured values. HGIN, high-grade intraepithelial neoplasia.

Table 2 - cfDNA values (pg/µL): descriptive statistics

	No.	Minimum	25th Percentile	Median	75th Percentile	Maximum	IQR	*P* value†
No lesion	63	1.029	1.536	1.798	2.114	3.755	0.578	-
Adenocarcinoma	12	1.469	1.936	2.071	2.357	2.584	0.421	0.07*
HGIN	22	1.131	1.693	1.922	2.135	2.344	0.442	1.00*
Adenoma	54	1.074	1.594	1.921	2.048	2.911	0.454	1.00*
Hyperplasia	19	0.932	1.541	1.818	2.012	3.382	0.471	1.00*
All lesions	107	0.932	1.638	1.935	2.074	3.382	0.436	0.14

IQR, interquartile range; HGIN, high-grade intraepithelial neoplasia.
†P value from Kolmogorov-Smirnov test by comparing the distribution within each lesion type with no lesions.
*Bonferroni's adjustment.

Table 3 - Predictive capability of cfDNA - AUC

	AUC	AUC 95% CI
Newly diagnosed lesion (cases) *versus* no lesion (controls)	0.555	0.463-0.646
Adenocarcinoma *versus* no lesion	0.709	0.509-0.909†
HGIN *versus* no lesion	0.573	0.402-0.744†
Adenoma *versus* no lesion	0.535	0.400-0.670†
Hyperplasia *versus* no lesion	0.509	0.324-0.694†

AUC, area under the curve; CI, confidence interval; HGIN: high-grade intraepithelial neoplasia.
†Bonferroni's adjustment.

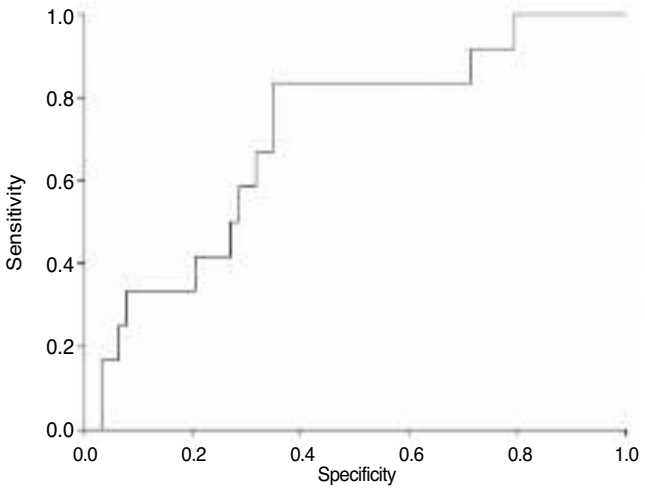

Figure 3 - ROC curve showing the capability of cfDNA to predict adenocarcinoma.

than serum[25]. On the basis of the endoscopic and histopathological findings, the study participants were divided into 5 groups: those with adenocarcinomas, HGIN, adenomas, or hyperplasia, and those with no lesions (the last group was used as the control group).

Although our findings show an overall inability of cfDNA to discriminate subjects with premalignant lesions from those without endoscopic lesions, we observed a satisfactory capability of the cfDNA concentration to predict adenocarcinomas (AUC: 0.709, 95% CI 0.508-0.909). The distribution of cfDNA in subjects with adenocarcinomas was consistently higher than that observed in subjects without lesions, although the difference was not statistically significant. Remarkably, more than half of the detected adenocarcinomas (7 out of 12) were at an early stage (pT1 or pT2), confirming the possible usefulness of cfDNA in improving CRC detection at a resectable stage by means of a simple blood test, and its corroborative role in identifying patients needing colonoscopy.

To our knowledge, no data on this topic are available from other studies, so we cannot compare or validate our findings. The only exception is the study by Mead *et al.*[26] where, in a population-based study, a potential role of cfDNA as a marker for both adenocarcinoma and precancerous polyps was observed. To explain the discrepancy between our findings and those of the above study, it should be taken into account that Mead *et al.* analyzed plasma samples from 26 patients carrying 29 polyps (11 of which harbored HGIN), 24 patients with CRC chosen among subjects with known inflammatory conditions, and 35 selected normal controls, whereas we analyzed plasma obtained from a single cohort of 170 unselected and consecutively assessed FOBT- positive subjects who were found to carry 106 adenomas (24 harboring HGIN), 12 carcinomas, 19 hyperplastic/reactive lesions, and 63 with no endoscopic changes.

It can also be speculated that the discordant results may be due to the different molecular targets analyzed by Mead *et al.*[26], who used qRT-PCR to quantify cfDNA of the repetitive line 1 79 bp DNA sequence and Alu fragments, and we cannot exclude that such combined quantification of several sequences/fragments may be more sensitive than our single hTERT. Consistent with this hypothesis, we found lower mean values of cfDNA in the series as a whole than those reported by Mead *et al.*[26]

An additional diagnostic benefit may come from testing for *KRAS* mutations, the detection of which has been used to screen for somatic mutations in a population at high risk of CRC[27]. As *KRAS* mutations can be found in a number of malignancies and are common to both premalignant and malignant lesions in CRC patients, they are not very specific, but correlating those found in tis-

sue and plasma samples may improve the methodological sensitivity. We found activating *KRAS* mutations in plasma samples taken from patients with adenocarcinomas (8%), HGIN (16%) and adenomas (2%) but not in those taken from subjects in the hyperplasia and no-lesion groups. *KRAS* mutations were also found in the plasma samples of 3 patients with HGIN and 1 with adenoma who were not distinguished by the cfDNA quantification procedure.

Furthermore, comparison of the paired FFPE tissue and plasma samples of 31 patients with adenocarcinomas/HGIN showed that 14 patients (45%) had *KRAS* mutations in the tissue, but only 2 of them (6%) also had mutations in plasma. Our results are in line with those of Wang *et al.*[13] who, in a study of an unselected cohort of patients who had undergone surgical resection for colorectal adenocarcinoma, found *KRAS* mutations in 45.2% and 15.4% of tumor tissue and serum samples, respectively, taken from patients at all disease stages except those at Dukes stage A (tumor confined to the bowel wall), none of whom had *KRAS* mutations in their serum samples. As 24 of our lesions (in 22 patients) were not invasive (HGIN) and 3 of the 12 adenocarcinomas were pT1 (early invasive), 27 out of 36 lesions (75%) fell into Dukes stage A; this means that the *KRAS* mutations observed in 13% of our HGIN patients might be considered acceptable in a cohort of surgical patients but would be unsatisfactory for a screening program intended to detect early CRC.

These findings also suggest that plasma samples from healthy subjects may require more sensitive molecular methods for *KRAS* mutation assessment than mutant-enriched PCR, such as the BEAMing assay[28] or next-generation sequencing technology, which have potential advantages over traditional approaches[29]. The results of our nested PCR-based exploration of p16 promoter methylation are in line with this assumption, as we did not find any evidence of methylation in any of the plasma samples from the adenocarcinoma or adenoma patients (data not shown).

Two patients with HGIN had *KRAS* mutations in their plasma but not tissue specimens, and additional diagnostic evaluations failed to demonstrate any abnormalities at any other sites that would have justified the mutant DNA. We can reasonably exclude false positive results related to sequence errors introduced by DNA polymerase during PCR because we repeated the *KRAS* mutation analysis 3 times. We also rejected the hypothesis of possible genetic heterogeneity between different tumor areas because many areas within the same HGIN sample were analyzed, and all of them were wild type. Finally, the detected mutations may have come from aberrant crypt foci with an endoscopically normal appearance[30], but it is more likely that the discordance between the tissue and plasma samples is related to the still unexplored questions concerning the origin, function and significance of cfDNA. This lack of knowledge could also explain a number of other contradictory results involving different types of tumors indicating *KRAS* mutations in cancer-free subjects[31] or more *KRAS* mutations in serum than in the primary tumor[32], as suggested by the oncogenic Ras-mediated cell suicide hypothesis in tumors characterized by a low occurrence of *KRAS* mutations[33].

In conclusion, the use of cfDNA quantification to predict malignancies in FOBT-positive subjects seems to be promising but requires further investigation. Our quantitative and qualitative cfDNA analyses of both malignant and premalignant lesions suggest it may be worthwhile to use more sensitive methods for cfDNA quantification or, alternatively, attempt to discover other blood-based diagnostic markers. For this reason, we are currently extending the screening program analyses and planning to analyze quantitative differences in the expression of miRNAs, which we have found to be significantly altered in CRC patients[34].

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