International Journal of Surgery (2013) 11(S1), S54–S57

Contents lists available at ScienceDirect

International Journal of Surgery

journal homepage: www.journal-surgery.net

ORIGINAL ARTICLE

Free circulating DNA as a biomarker of colorectal cancer

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ARTICLE INFO

Keywords:
Colorectal cancer
Biomarker
Free circulating DNA

ABSTRACT

Objectives: The purpose of this study is to evaluate the sensitivity and specificity of free circulating DNA (FCDNA) as a biomarker in patients suffering from colorectal cancer (CRC), investigating both its prognostic value correlated with stage of disease and its potential role in early recurrence diagnosis.
Methods: The quantification of plasma DNA was achieved through the use of real time quantitative polymerase chain reaction (PCR) amplification of the RNAse P gene. The study enrolled patients undergoing surgery for primary CRC, at different stages of disease; samples were collected before surgery and during follow-up examinations every 3 months after surgery. Data were statistically analyzed using Software Packages SPSS® for Windows.
Results: FCDNA was detectable in all pre-operative samples and the mean value was 47.8 ng/mL. FCDNA values increased progressively related to UICC stage of disease, although statistical significance was demonstrated only when comparing patients by pT stage. The analysis of postoperative samples showed a significant decrease of FCDNA quantity after radical surgery and in specific cases a rise preceding disease recurrence.
Conclusions: This study shows that absolute quantification of FCDNA in CRC patients could have a prognostic value, being related to stage of disease, and could be used as potential tool for early detection of recurrences.

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1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of death from cancer. [1,2] Outcomes have improved with advances in surgical technique and adjuvant therapy. Mortality and survival rates in CRC are highly influenced by the stage of the disease at the moment of diagnosis: 5-year survival is excellent for disease localized to the colon (95% for stage I and 82% for stage II) but decreases to 61% for patients with regional spread to the lymph nodes (stage III) and to only 8% for patients with distant metastases (stage IV). [3,4] These data confirm that early diagnosis of the primary tumor as well as the recurrences is crucial to improve overall and disease free survival.

The most common tumor marker in colorectal cancer is serum carcinoembryonic antigen (CEA), which is helpful for the detection of cancer recurrence developing after primary tumor resection; however, it has relatively poor sensitivity (being elevated in only about 40% of CRC patients) and specificity. [5–7]

Therefore, during the last few years, there has been an increasing interest in molecular biomarkers as non-invasive diagnostic tools for identifying at-risk population and early cancer detection. [8] In addition, tumor-specific markers such as *KRAS* and microsatellite instability (MSI) are useful for both prognosis and directing chemotherapy. [9]

Several studies have already reported the presence of FCDNA in cancer patients' plasma, showing the same characteristics of primary tumour DNA such as oncogene expressions, tumour-suppressor gene mutations, microsatellite and epigenetic alterations. [10] There are different hypotheses concerning the origin of FCDNA. It may emerge from tumor necrosis, apoptosis or even active secretion, but the exact mechanism is unknown. [11] The purpose of this study is to evaluate FCDNA as a biomarker in patients suffering from colorectal cancer, investigating both its prognostic value correlated with stage of disease and its potential role in early diagnosis of recurrence.

2. Patients and Methods

All patients who underwent surgical resection of primary colorectal cancer at the Department of Surgery of the University of Insubria in Varese were enrolled in the study.

A 3 mL sample of peripheral blood was collected in vials containing EDTA on the day before surgery. Plasma was immediately separated from the cellular fraction by centrifugation at 3000 rpm for 20 min at 4°C and then stored at −20°C.

300 μL of plasma were digested with 100 μL of a Proteinase K solution 20 ng/ml in the thermomixer at 58°C. After that, DNA extraction was performed using BloodPrep™ Chemistry – DNA Isolation from Fresh and Frozen Whole Blood (Applied Biosystems, Foster City, CA,

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Table 1
Variables examined and correlation with FCDNA values

	No. of patients	% of patients	C_T (mean±SD)	p	CFDNA, ng/ml (mean±SD)	p
Gender						
Female	101	45.3	5.2±3.3	0.793	33.6±64.4	0.254
Male	122	54.7	5.2±3.4		58.8±149.9	
Location of tumor						
Colon	178	79.8	5.3±3.3	0.003	47.9±111.0	0.027
Rectum	45	20.2	3.8±3.1		78.9±193.8	
pT stage						
1	19	8.5	5.7±3.6	0.162	15.6±31.4	0.032
2	38	17.1	5.1±3.1		61.8±196.3	
3	147	65.9	5.0±3.4		44.8±87.3	
4	19	8.5	4.5±3.3		123.1±217.9	
pN stage						
0	120	53.8	5.1±3.0	0.093	39.3±81.2	0.146
1	65	29.1	5.1±3.9		55.5±158.7	
2	38	17.1	4.1±3.0		87.8±173.3	
M stage						
0	193	86.5	5.3±3.3	0.220	43.0±106.6	0.568
1	30	13.5	4.4±3.4		84.7±197.9	
UICC stage						
I	43	19.3	5.5±3.3	0.064	25.4±51.8	0.146
II	74	33.2	5.1±3.0		46.5±93.8	
III	75	33.6	4.8±3.7		59.4±147.0	
IV	31	13.9	4.4±3.4		84.7±197.9	

USA) with the ABI PRISM 6100 Nucleic Acid Prepstation (Applied Biosystems), according to the manufacturer's instruction, in eight steps of purification, obtaining a final elution of 180 μL. The DNA was then stored at −20°C until further analysis.

Quantification of circulating free DNA in plasma was performed by using a real time quantitative PCR approach with ABI Prism® Applied Biosystems™ 7300 Real-Time PCR System, based on the 5' nucleotide method. This methodology is based on continuous monitoring of a progressive fluorogenic PCR by an optical system. We used primers and fluorogenic probes to specifically amplify the RNAse P gene.

Each PCR reaction mixture was composed of 10 μL of DNA solution, 12.5 μL of TaqMan® Universal PCR Master Mix (2x), No AmpErase® UNG (Applied Biosystems™), 1.25 μL of Primer TaqMan® RNase P and 1.25 μL of sterile water. Thermal cycling started with a first incubation step of 50°C for 2 min and then a denaturation step of 95°C for 10 min. The thermal profile for the PCR amplification was 95°C for 15 seconds and 60°C for 1 minute. Data obtained during 45 cycles of amplification were analyzed using the Sequence Detection System software (SDS, Applied Biosystems™) to compare the amplification cycle threshold (CT) of the DNA control sample to the CT of the unknown experimental samples, obtaining an absolute quantification value. At least three independent quantification assays were performed for each plasma sample.

Data were evaluated with Student's t-test when comparing two median values, while ANOVA univariate analysis was performed to compare more than two groups. In both cases the level of significance was set at 5% ($p < 0.05$). Data were statistically analyzed using Software Packages SPSS® for Windows.

3. Results

3.1. Absolute quantification analysis of free circulating plasma DNA in CRC patients: correlation with stage of disease

Between November 2009 and June 2012, 223 patients admitted to our department and undergoing colorectal resection for cancer (122 males, 101 females; mean age 66 years) were enrolled in the study.

FCDNA was detectable in all pre-operative samples; the mean value was 47.8 ng/mL (SD 120.6; range: 0–1096.6; median 11.7).

All variables examinated and their correlation with FCDNA values in ng/mL are reported in Table 1.

The mean FCDNA values for rectal cancer were significantly higher than those for cancer located in the colon (78.9±193.8 vs 47.9±111.0; $p = 0.027$, Fig. 1).

Patients were then divided into 4 groups according to UICC stage of disease (43 patients stage I, 74 patients stage II, 75 patients stage III and 31 patients stage IV). The mean absolute quantities of CFDNA of these groups were compared (Fig. 2).

FCDNA quantity increased progressively from stage I to stage IV with a mean value of 25.4 ng/mL (±51.8) for stage I patients, of 46.5 ng/mL (±93.8) for stage II patients, of 59.4 ng/mL (±147) for stage III and of 89.7 ng/mL (±197.9) for stage IV patients, although this did not reach statistical significance.

In univariate analysis the pT stage variable was significantly related to increasing CFDNA values (Fig. 3); looking at the pN stage variable, although FCDNA values increase from pN0 to pN2, statistical analysis showed significance only considering the pN0 versus the pN+ group (Fig. 4).

3.2. Absolute quantification analysis of free circulating plasma DNA in CRC patients: postoperative values and recurrence diagnosis

The mean value of preoperative FCDNA was compared to the value obtained from the analysis of samples collected 3 months after surgery, showing a significant decrease: 47.8±120.6 ng/ml vs 12.8±52.4 ng/ml; $p = 0.019$ (Fig. 5).

Furthermore we observed that during follow-up FCDNA quantity increased in selected patients who developed recurrence, while it remained low (close to 0 ng/ml) in disease-free patients (Figs. 6, 7, 8, 9, 10). In all cases of recurrence FCDNA increased before there was clinical evidence of disease.

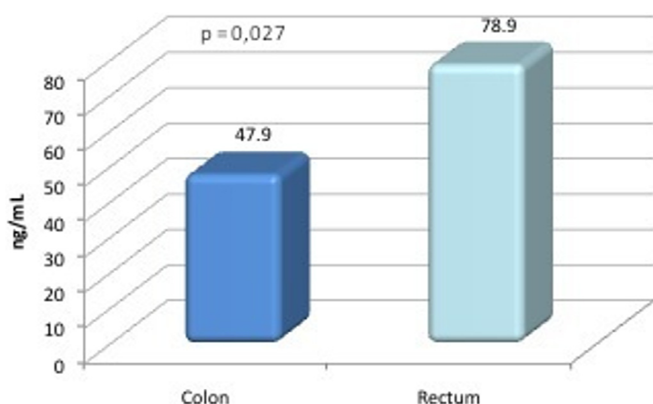

Fig. 1. Median FCDNA values for rectal vs colon cancer.

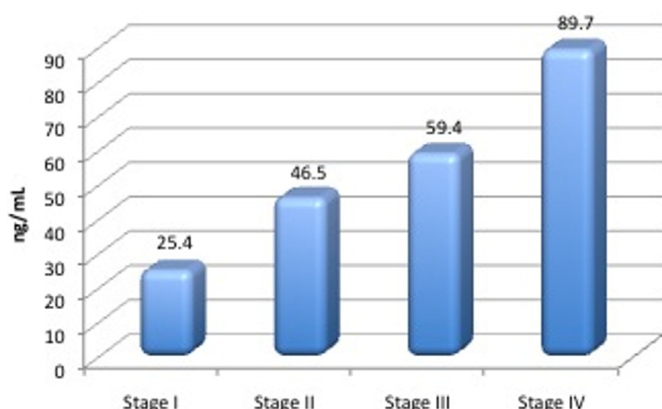

Fig. 2. FCDNA values at different UICC stages of disease.

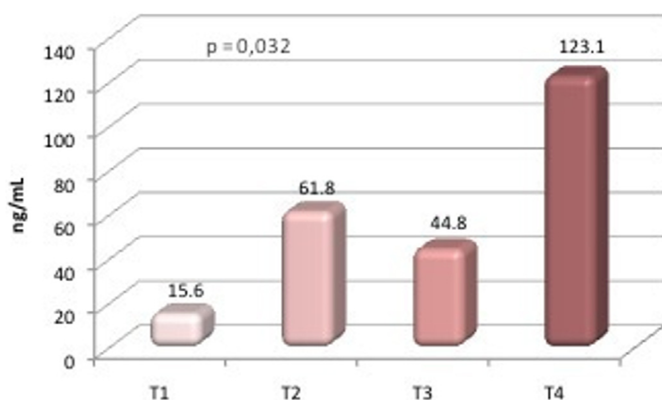

Fig. 3. FCDNA correlated to pT stage of disease.

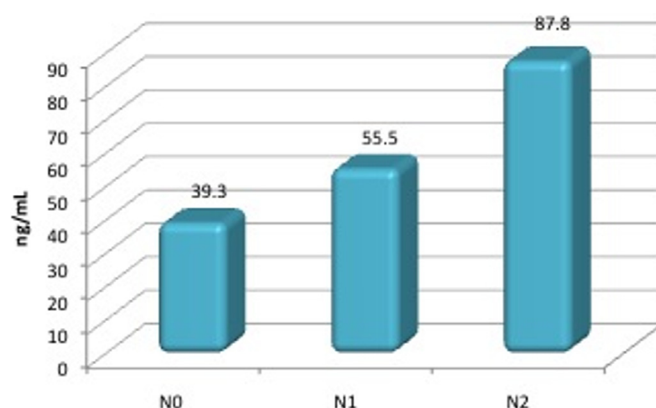

Fig. 4. FCDNA correlated to pN stage of disease.

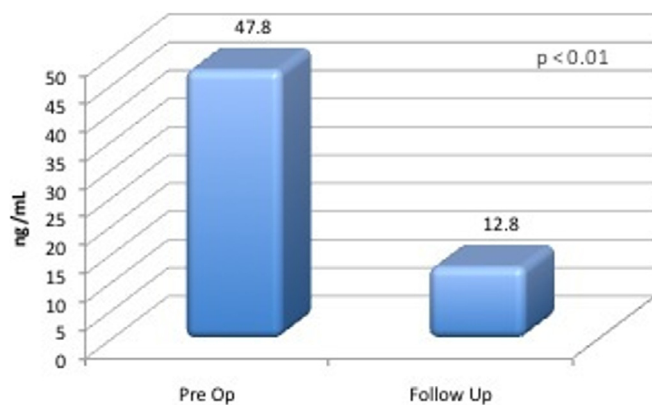

Fig. 5. FCDNA preoperative vs follow-up values.

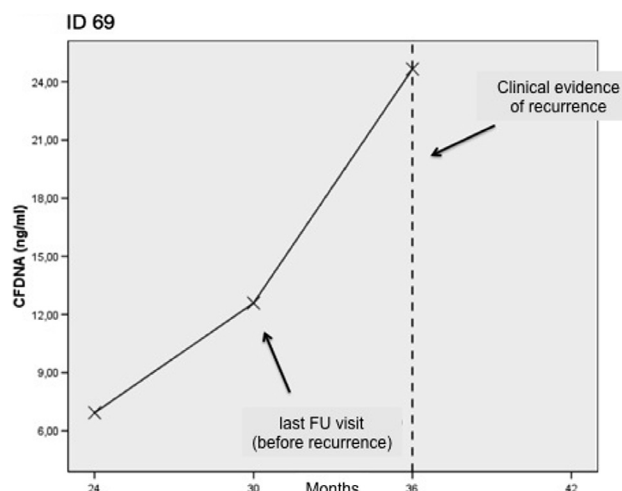

Fig. 6. FCDNA increasing in disease recurrence.

4. Discussion

The presence of high levels of CFDNA in cancer patients' plasma was initially demonstrated by Leon et al. in 1977; this was the first study exploring the clinical potential of circulating nucleic acids as molecular marker for cancer. [12]

FCDNA as a biomarker has several advantages such as the possibility to be measured by non-invasive techniques, its stability (it appears to be stable for several years in stored samples of plasma) and a relatively easy detection.

Nevertheless, the use of CFDNA as a biomarker in colorectal cancer is not yet ready for widespread clinical use, as there are large numbers of genes that can be mutated, and while several methods have been reported for FCDNA quantification, none have been evaluated in terms of reproducibility; for these reasons results from different studies are not comparable. [13,14]

Several studies confirmed that quantification of FCDNA could be a promising biomarker in various cancers, including CRC. [13,14] However, the different methodologies and the lack of standardization in technique of analysis have hampered the implementation of these tumour markers in clinical practice.

We previously reported the presence of FCDNA in patients suffering from CRC, while it was almost undetectable in a cohort of healthy controls (volunteers with negative colonoscopy). [15] In the present study, we demonstrated that FCDNA in plasma is related to location of tumor (higher values in rectal cancer compared to

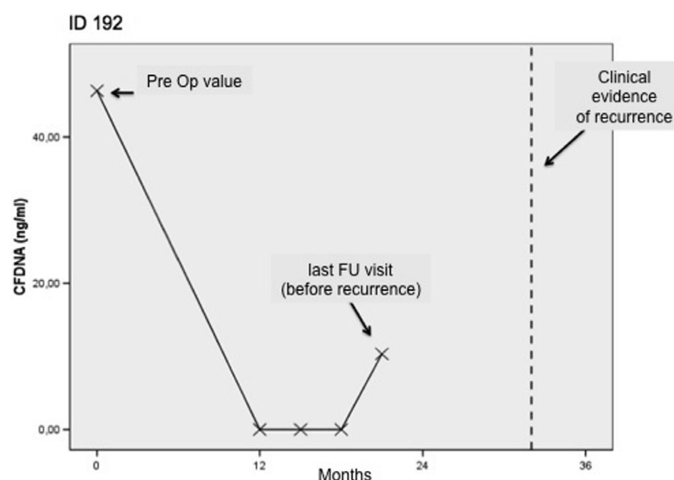

Fig. 7. FCDNA increasing in disease recurrence.

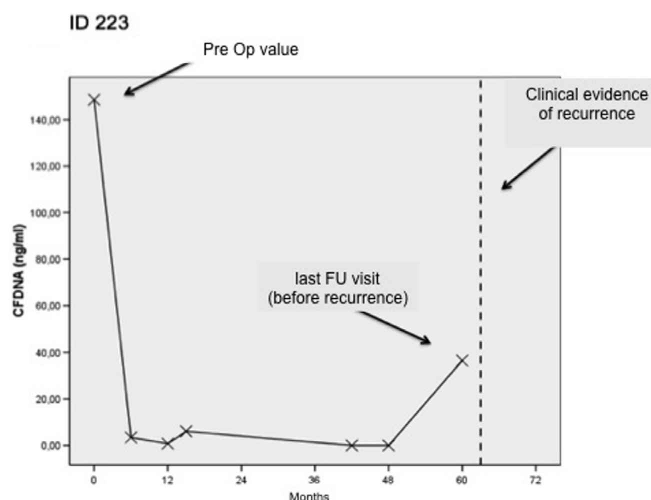

Fig. 8. FCDNA increasing in disease recurrence.

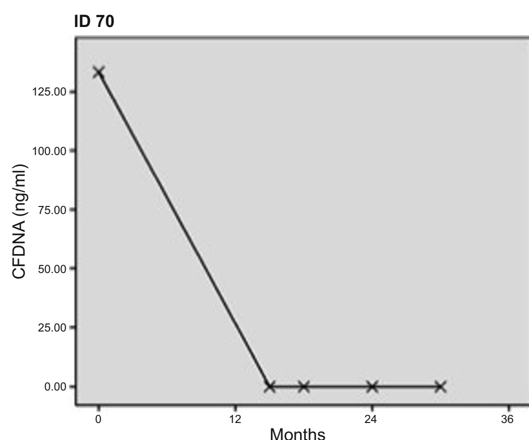

Fig. 9. FCDNA during follow up of a disease-free patient.

colonic localization) and to stage of disease (both to depth of tissue infiltration, pT, and to lymph node invasion, pN).

5. Conclusions

Our data show that pre-operative measurement of FCDNA in CRC patients contributes to better estimate prognosis because of its

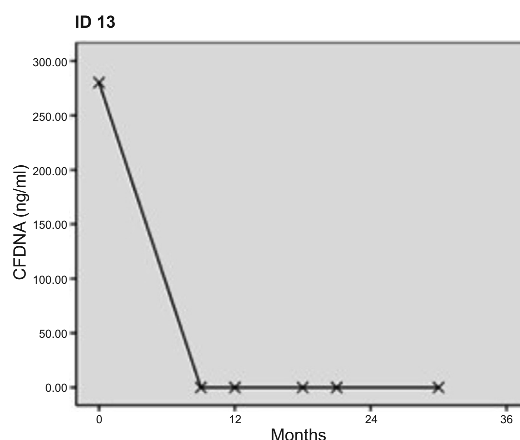

Fig. 10. FCDNA during follow up of adisease-free patient.

significant correlation with stage of disease; postoperative FCDNA determination appears to be a potential tool for early detection of recurrences and might be used as a biomarker in addition to the clinical/instrumental examination.

Funding
None.

Disclosure statement
The authors have no conflicts of interest to declare.

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