Ann Surg Oncol (2011) 18:2461–2468
DOI 10.1245/s10434-011-1638-y

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – COLORECTAL CANCER

# Circulating Cell-Free DNA: A Promising Marker of Pathologic Tumor Response in Rectal Cancer Patients Receiving Preoperative Chemoradiotherapy

**Marco Agostini, PhD[1], Salvatore Pucciarelli, MD[1], Maria Vittoria Enzo, PhD[1], Paola Del Bianco, PhD[2], Marta Briarava, PhD[1], Chiara Bedin, PhD[1], Isacco Maretto, MD[1], Maria Luisa Friso, MD[3], Sara Lonardi, MD[4], Claudia Mescoli, MD[5], Paola Toppan, MD[1], Emanuele Urso, MD, PhD[1], and Donato Nitti, MD[1]**

[1]Department of Oncological and Surgical Sciences, 2nd Surgical Clinic, University of Padova, Padova, Italy; [2]Clinical Trials and Biostatistics Unit, Veneto Institute of Oncology IOV – IRCCS, Padova, Italy; [3]Department of Radiotherapy and Nuclear Medicine, Veneto Institute of Oncology IOV – IRCCS, Padova, Italy; [4]Department of Medical Oncology, Unit 1st, Veneto Institute of Oncology IOV – IRCCS, Padova, Italy; [5]Department of Pathology, University of Padova, Padova, Italy

## ABSTRACT

**Purpose.** The circulating cell-free DNA (cfDNA) in plasma has been reported to be a marker of cancer detection. The aim of this study was to investigate whether the cfDNA has a role as response biomarker in patients receiving preoperative chemoradiotherapy (CRT) for rectal cancer.

**Methods.** Sixty-seven patients (median age 61 years; male/female 42/25) who underwent CRT for rectal cancer were evaluated. After tumor regression grade (TRG) classification was made, the patients were classified as having disease that responded (TRG 1–2) and that did not respond (TRG 3–5) to therapy. Plasma samples were obtained from patients before and after CRT. The cfDNA levels were analyzed by quantitative real-time polymerase chain reaction of $\beta$-globin. On the basis of the Alu repeats, the cfDNA was considered as either total (fragments of 115 bp, Alu 115) or tumoral (fragments of 247 bp, Alu 247). The association between the pre- or post-CRT levels and between variations during CRT of the Alu 247, Alu 115 repeat, and Alu 247/115 ratio (cfDNA integrity index) and the pathologic tumor response was analyzed.

**Results.** The baseline levels of cfDNA were not associated with tumor response. The post-CRT levels of the cfDNA integrity index were significantly lower in responsive compared to nonresponsive disease ($P = 0.0009$). Both the median value of the Alu 247 repeat and the cfDNA integrity index decreased after CRT in disease that responded to therapy ($P < 0.005$ and $P < 0.005$, respectively) compared to disease that did not respond to therapy ($P = 0.83$ and $P = 0.726$, respectively). The results of the multivariable logistic regression analysis showed that only the cfDNA integrity index was significantly and independently associated with tumor response to treatment.

**Conclusions.** The plasma levels of the longer fragments (Alu 247) of cfDNA and the cfDNA integrity index are promising markers to predict tumor response after preoperative CRT for rectal cancer.

Preoperative chemoradiotherapy (CRT) is currently considered the standard treatment for locally advanced rectal cancer. The oncological outcomes using this approach are encouraging, with rates of local and distant recurrence at the 5-year mark ranging 6–9 and 33–36%, respectively.[1–3] However, the rates of chemotherapy-, radiotherapy-, and surgery-related toxicity, as well as bowel and sexual dysfunction, are disappointing. The peculiar aspects of this approach are related to clinical overstaging, which may result in an unnecessary neoadjuvant treatment in almost one-fifth—18% in a German trial—of patients.[2] In addition, the disease of approximately 40% of patients shows poor or no response to preoperative CRT, while a

Presented as a podium presentation at the American Association Cancer Research annual meeting, April 2009, Denver, CO.



First Received: 1 October 2010;
Published Online: 17 March 2011

S. Pucciarelli, MD
e-mail: puc@unipd.it

complete pathologic response has been reported in up to 44% of cases.[4,5] For patients with disease that shows complete clinical response after preoperative CRT, less aggressive approaches have been advocated, such as a wait-and-see policy or transanal local excision.[6] These considerations have prompted researchers to attempt to find biomarkers that may be used to predict pathologic tumor response before and after neoadjuvant treatment.

The cell-free DNA (cfDNA) circulating in plasma or serum shows characteristics of a potential candidate biomarker of tumor response. Although circulating cfDNA is present in healthy individuals, it has been implicated as a strong diagnostic and prognostic marker of malignancy.[7–9] Its limitations are related to the contradictory findings reported concerning the proportion of tumor- and non-tumor-derived cfDNA. Interestingly, it has been hypothesized that cfDNA released into circulation from tumor necrosis varies in size, whereas cfDNA released from non-tumoral apoptotic death is uniformly truncated into fragments shorter than 200 bp.[10–12] In healthy individuals, the main source of cfDNA is thought to be apoptotic cells. In contrast, necrotic cell death is a frequent event in solid tumors, and DNA fragments released from the tumor cells are variable in length.[10] As a consequence, the amount of longer DNA fragments and the ratio between the longer and shorter fragments, known as the integrity index, may reflect the presence of cancer.[13] Recently, Umetani et al.[14] developed a highly sensitive method to measure the integrity of the cfDNA in serum by using quantitative real-time polymerase chain reaction (qPCR) for Alu repeats in colorectal cancer patients. They found that DNA integrity was a reliable biomarker for the detection of colorectal cancer. Accordingly, recent studies have reported an increased ratio between the long and short fragments in patients with cancer.[13–16]

The principal aim of the current pilot study was thus to investigate whether the variations in the plasma levels of cfDNA during neoadjuvant therapy and the size distribution of its short and long fragments are associated with tumor response after preoperative CRT for rectal cancer. In addition, we aimed to investigate whether the pre- or postoperative plasma levels of cfDNA and the cfDNA integrity can predict the pathologic tumor response. Finally, we sought to preliminarily confirm that the levels of cfDNA may be used to differentiate patients with rectal cancer from healthy control subjects.

## MATERIALS AND METHODS

### *Patient, Tumor, and Treatment Characteristics*

Between June 1998 and April 2008, a total of 216 patients with primary adenocarcinoma of the rectum underwent CRT followed by surgery. The pretreatment evaluation of the patients included a complete clinical history and physical examination, colonoscopy, complete blood cell count, transrectal ultrasound, pelvic computed tomography scan or magnetic resonance imaging, abdominal/chest computed tomography, and carcinoembryonic antigen test. The inclusion criteria for CRT were as follows: biopsy-proven adenocarcinoma of the mid–low rectum (<11 cm from the anal verge); clinical stage T3–4 and/or node-positive disease; and Eastern Cooperative Oncology Group performance status score of 0–2.

After excluding 149 patients because both pre- and post-CRT plasma samples were not available for molecular analysis, 67 cases were included in the study group. These patients were provided preoperative external-beam radiotherapy with high-energy photons (>6 MV) with conventional fractionation (≥50 Gy in 28 fractions, 1.8 Gy per day, 5 sessions per week), and 5-fluorouracil (5-FU)-based chemotherapy administered by bolus or continuous venous infusion. A standard total mesorectal excision was performed 4 to 8 weeks after the completion of preoperative CRT.

To confirm the ability of the cfDNA to discriminate the patients with rectal cancer from healthy subjects, blood samples of healthy individuals without a history of cancer were obtained after colonoscopy revealed neither cancer nor polyps. A ratio of patients to healthy subjects of 2/1 was considered adequate.

The study protocol was reviewed and approved by the local ethics committee (protocol 740 P), and each patient provided written informed consent.

### *Evaluation of Tumor Response*

The surgical specimens, assessed in a standardized manner, were reviewed by one pathologist (C.M.), who was unaware of patient outcome. The histopathology findings and definition of radical surgery were reported following the 2002 American Joint Committee on Cancer tumor, node, metastasis system. The tumor response to CRT was defined as the tumor regression grade (TRG) and was scored following the criteria proposed by Mandard et al.: TRG 1, pathological complete response, i.e., absence of viable cancer cells in the resected specimen; TRG 2, presence of residual cancer cells; TRG 3, fibrosis outgrowing residual cancer cells; TRG 4, residual cancer cells outgrowing fibrosis; and TRG 5, absence of response.[17] By means of this system, disease was classified as being responsive (TRG 1–2) and nonresponsive (TRG 3–5) to therapy.[18,19]

### *Extraction of DNA from Plasma Samples*

The pre- and post-CRT plasma samples were obtained 15 days before the CRT and 15 days before surgery,

respectively. Seven milliliters of peripheral blood was drawn into a purple-top blood collection tube (containing EDTA additive) before physical examination or biopsy. Plasma samples were left at room temperature for molecular assays; samples were transferred to the study laboratory within 4 h of collection for processing. The plasma samples were obtained by centrifugation of 7 ml of peripheral blood at 3000 *g* for 10 min. The plasma samples were carefully collected from the upper portion of the supernatant and stored in aliquots at −80°C. The DNA was purified from 500 µl of plasma with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.[20] The washes with the buffer AW2 were increased to two to remove inhibitors of the PCR.[20] The DNA preparations were eluted in 80 µl of elution buffer. The eluted DNA was stored at −20°C until further use.

### *qPCR of Plasma DNA Fragments*

The quantification of the DNA fragments was performed by qPCR, which amplified and quantified the shorter and longer fragments. To maximize the sensitivity of the DNA quantification, the Alu repeats, which are the most abundant repeat sequence in the human genome, were used as a target of the qPCR.

Two primer pairs were used, as previously reported.[15] One set of primers (Alu 115) amplified both the shorter (115 bp) and longer fragments, whereas the second primer set (Alu 247) amplified only the longer (247 bp) DNA fragments. The results obtained using the Alu 115 primers represent the total free plasma DNA, while the results of the Alu 247 primers reflect the amount of DNA released from nonapoptotic cells. The quantitative values from the 115-bp primers represent the total level of cfDNA (ng/ml), while the ratio of longer to shorter fragments show the integrity of cfDNA in each sample.

The sequences of the Alu 115 primers were as follows: forward, 5′-CCTGAGGTCAGGAGTTCGAG-3′ and reverse, 5′-CCCGAGTAGCTGGGATTACA-3′; the Alu 247 primers were as follows: forward, 5′-GTGGCTCA CGCCTGTTAATC-3′ and reverse, 5′-CAGGCTGGAGTG CAGTGG-3′ (Primm, Milan, Italy).

The reaction was performed using 7300 Real-Time PCR System (Applied Biosystem, Milan, Italy) as previously reported.[15] The absolute amount of cfDNA in each sample was determined by a standard curve using 3.3-fold dilutions (from 10 ng to 1 pg) of genomic DNA from the peripheral lymphocytes of clean-colon healthy subjects.

A negative control (without the template) was performed in each plate. All qPCR assays were performed in a blinded fashion without knowledge of the specimen identity. After amplification, melting curves were obtained to confirm the accurate amplification signal. In this setting, each amplicon displays a specific melting behavior. The 115-bp amplicon produced a peak at approximately 79°C, whereas the 247-bp amplicon had a higher melting temperature of approximately 83°C. The quantitative ratio of Alu 247 to Alu 115 reflected the integrity of the cfDNA (cfDNA integrity index = Alu 247/115 ratio).

The total plasma cfDNA was also measured by a qPCR assay of the $\beta$-globin gene: $\beta$-globin forward 5′-TGAG TCCAAGCTAGGCCCTTT-3′; $\beta$-globin reverse 5′-CCAG GAGCTGTGGGAGGAA-3′ (Applied Biosystem, Milan); and a labeled fluorescent TaqMan $\beta$-globin probe (FAM), 5′-CTAATCATGTTCATACCTCTTAT-3′ (MGB) (Applied Biosystem, Milan).

### *Statistical Analysis*

The levels of each biomarker at baseline and after CRT were compared between groups (rectal cancer patients and healthy clean-colon subjects at baseline; characterized as having disease that responded and that did not respond to therapy at baseline and after CRT) by the Mann–Whitney test.

To study the variation in the levels of cfDNA, the baseline levels of patients with and without disease that responded to therapy were compared with the post-CRT levels. The Wilcoxon signed rank test was performed to assess the significance of changes within each group.

All $P$ values were two-sided, and a $P$ value of $< 0.05$ was considered statistically significant.

To evaluate the diagnostic or predictive performance of the cfDNA quantification, the area under the receiver-operating characteristic curve (AUC-ROC) was calculated. An AUC-ROC equal to 1 denotes perfect discrimination between distinct groups of patients, while a value equal to 0.5 denotes the lack of discrimination.

Moreover, to investigate the independent role of the cfDNA in predicting tumor response, a multivariable logistic regression analysis was performed using the following covariates: age (years), gender (male vs. female), distance from anal verge (cm), radiotherapy dose (Gy), administration of 5-FU (continuous infusion vs. bolus vs. oral), drugs other than 5-FU (yes vs. no), pre- and posttreatment cfDNA Alu 247, pre- and posttreatment cfDNA Alu 115, and pre- and posttreatment cfDNA integrity index.

As a result of the relatively low number of events (response, $n = 25$) compared to the number of investigated covariates, univariate logistic regression analysis was performed to select the three variables with the smallest $P$ value to be input in the multivariable analysis. Multivariable logistic regression was fit to the data using the forward stepwise mode for variable selection.

Statistical analyses were performed by SAS software, release 9.1.3 (SAS Institute, Cary, NC).

## RESULTS

### *Patient, Tumor, and Treatment Characteristics*

Of the 67 patients included in the study group, the tumor was located in the lower rectum for most patients (63%). Before CRT, 69% and 88% of disease were clinically staged as T3–4 and lymph node positive, respectively; 58 patients (87%) received a total dose of radiotherapy higher than 50 Gy, and in 30 cases (45%), drugs other than 5-FU were administered ($n = 27$, oxaliplatin; $n = 2$, carboplatin; $n = 1$, leucovorin). In 45 patients (67%), 5-FU was administered by continuous venous infusion. The median (range) interval time between the completion of preoperative CRT and surgery was 48 (34–120) days. Complete details of the patient, tumor, and treatment characteristics are summarized in Table 1.

### *Tumor Response at Histopathology*

The following TRG distribution was found: TRG 1, $n = 10$; TRG 2, $n = 15$; TRG 3, $n = 21$; TRG 4, $n = 16$; and TRG 5, $n = 5$. On the basis of the TRG distribution, 25 patients (37%) were considered to have disease that responded to therapy (TRG 1–2), and 42 (63%) had disease that did not respond to therapy (TRG 3–5).

### *cfDNA Distribution in Healthy Individuals and Rectal Cancer Patients*

The ability of cfDNA to discriminate the patients with rectal cancer and the healthy clean-colon subjects was confirmed by using a control group of plasma samples from healthy subjects; these plasma samples were obtained immediately after a colonoscopy yielded negative findings of cancer and polyps.

In the patients, the baseline levels of Alu 115, Alu 247, and $\beta$-globin were significantly higher ($P < 0.0001$) than in the control group (Table 2), and the cfDNA integrity index was 10 times higher in the patients than in the healthy control subjects.

By means of ROC curve analysis, each method of cfDNA quantification showed a high ability to discriminate healthy individuals from patients ($AUC_{Alu\ 115} = 0.92$, 95% confidence interval [CI] 0.8–1.0; $AUC_{\beta\text{-globin}} = 0.93$, 95% CI 0.9–1.0; and $AUC_{Alu\ 247} = 0.97$, 95% CI 0.9–1.0). It should be noted that, with a cutoff of 2.00 ng/ml, the diagnostic power of Alu 247 showed 100% specificity (95% CI 89.9–100) and 94% sensitivity (95% CI 85.4–98.3) (Fig. 1).

**TABLE 1** Patient, tumor, and treatment characteristics of the 67 patients included in the study

| Characteristic | Value[a] |
|---|---|
| Age, years, median (range) | 61 (20–79) |
| Sex | |
| Male | 42 (63%) |
| Female | 25 (37%) |
| Tumor distance from the anal verge | |
| ≤7 cm | 42 (63%) |
| >7 cm | 25 (37%) |
| Total radiotherapy dose delivered | |
| ≥50 Gy | 63 (94%) |
| <50 Gy | 4 (6%) |
| 5-Fluorouracil administration | |
| Continuous infusion | 45 (67%) |
| Bolus | 11 (17%) |
| Oral (capecitabine) | 9 (13%) |
| Not available | 2 (3%) |
| Other drugs | |
| 5-FU alone | 37 (55%) |
| Oxaliplatin | 27 (40%) |
| Carboplatin | 2 (3%) |
| Leucovorin | 1 (2%) |
| Surgical procedure | |
| APR | 7 (10%) |
| Local excision | 4 (6%) |
| Hartmann's | 3 (5%) |
| LAR | 53 (79%) |
| ypTNM | |
| 0 | 9 (13%) |
| I | 24 (36%) |
| II | 14 (21%) |
| III | 7 (11%) |
| IV | 9 (13%) |
| Not evaluable | 4 (6%) |
| Radical surgery | |
| Yes | 51 (76%) |
| No | 11 (17%) |
| Not evaluable | 5 (7%) |

[a] Data are expressed as *n* (%) unless otherwise indicated

*APR* abdominoperineal resection, *LAR* low anterior resection

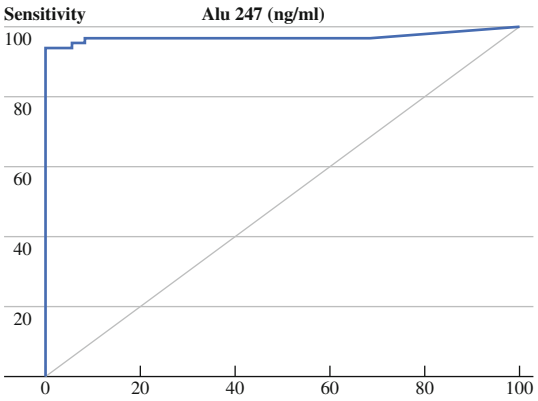
### *Baseline or Post-CRT cfDNA Levels and Tumor Response*

The median baseline levels of the Alu 115 and Alu 247 fragments and the cfDNA integrity index were lower in responsive disease compared to nonresponsive disease (3- to 4-fold smaller); however, the difference was not statistically significant (data not shown).

**TABLE 2** Baseline plasma levels of cfDNA in patients with rectal cancer and healthy control subjects

| Marker | Healthy control subjects ($n = 35$), median (Q1; Q3) | Patients ($n = 67$), median (Q1; Q3) | $P$ value |
|---|---|---|---|
| Alu 115 (ng/ml) | 1.0 (0.0; 2.1) | 47.0 (8.6; 218.6) | <0.0001 |
| Alu 247 (ng/ml) | 0.3 (0.0; 0.7) | 47.5 (10.0; 342.7) | <0.0001 |
| $\beta$-Globin (ng/ml) | 7.7 (3.0; 12.0) | 61.3 (18.3; 267.9) | <0.0001 |
| Alu 247/115 ratio | 0.1 (0.0; 0.4) | 1.1 (0.7;1.9) | <0.0001 |

*Q1* 25% quartile, *Q3* 75% quartile




| | AUC (95%CI) | p-value | Cut-off (ng/ml) | Sensitivity (95%CI) | Specificity (95%CI) |
|---|---|---|---|---|---|
| Alu247 | 0.97 (0.9–1.0) | 0.0001 | > 2.0065 | 94.0 (85.4–98.3) | 100.0 (89.9–100.0) |

**FIG. 1** ROC for discriminating patients with rectal cancer from healthy clean-colon subjects using the Alu 247 plasma levels

Comparing the levels of cfDNA, after preoperative CRT, between the response and nonresponse groups, the Alu 115 fragments and $\beta$-globin did not show any differences ($P = 0.928$ and 0.0992, respectively). On the other hand, the levels of the Alu 247 fragments approached statistical significance ($P = 0.0731$), and the cfDNA integrity index showed a highly statistically significant difference ($P = 0.0009$). By means of ROC curves, the post-CRT levels of Alu 115, $\beta$-globin, and Alu 247 were poorly accurate in distinguishing between response versus nonresponse to therapy ($AUC_{Alu\ 115} = 0.51$, 95% CI 0.4–0.6; $AUC_{\beta\text{-globin}} = 0.62$, 95% CI 0.5–0.7; $AUC_{Alu\ 247} = 0.63$, 95% CI 0.5–0.7). Meanwhile, the ROC curve analysis related to the cfDNA integrity index revealed a significant discriminative capacity (AUC = 0.76, 95% CI 0.6–0.8, $P < 0.05$); moreover, with a cutoff at 0.44, the sensitivity and specificity were 83 and 60%, respectively.

### *Variations of Pre- and Post-CRT Levels of cfDNA and Tumor Response*

In the nonresponsive group, the median pre- and post-CRT values of cfDNA did not show a statistically significant difference (Table 3; Fig. 2). Conversely, in the responsive group, the median levels of Alu 247 and the cfDNA integrity index were significantly lower after CRT compared to baseline ($P = 0.0048$ and 0.0005, respectively) (Table 3; Fig. 2).

### *Multivariable Analysis*

At univariate logistic regression analysis, the covariates with the smallest $P$ values were the following: posttreatment cfDNA integrity index ($P = 0.021$), posttreatment cfDNA Alu 247 ($P = 0.158$), and gender ($P = 0.166$).

The results of the multivariable logistic regression analysis showed that only the cfDNA ratio was significantly and independently associated with tumor response to

**TABLE 3** Variations of the cfDNA levels before and after chemoradiotherapy in disease with response and nonresponse

| Response to therapy | Marker | Before CRT, median (Q1; Q3) (ng/ml) | After CRT, median (Q1; Q3) (ng/ml) | $P$ value |
|---|---|---|---|---|
| Yes ($n = 25$) | Alu 115 | 55.2 (11.4; 218.6) | 50.7 (10.3; 186.1) | 0.3871 |
| | Alu 247 | 58.1 (17.4; 342.4) | 14.3 (7.1; 84.5) | 0.0048 |
| | $\beta$-globin | 77.9 (18.2; 173.0) | 35.3 (21.2; 93.1) | 0.0574 |
| | Ratio Alu 247/115 | 0.8 (0.6; 2.1) | 0.4 (0.2; 0.8) | 0.0005 |
| No ($n = 42$) | Alu 115 | 31.2 (6.8; 179.4) | 48.9 (10.7; 137.3) | 0.9071 |
| | Alu 247 | 32.3 (10.0; 336.7) | 44.7 (12.6; 317.9) | 0.8394 |
| | $\beta$-Globin | 52.9 (22.7; 325.3) | 73.9 (28.3; 158.0) | 0.7822 |
| | Ratio Alu 247/115 | 1.2 (0.7; 1.8) | 1.1 (0.7; 2.1) | 0.7262 |

*Q1* 25% quartile, *Q3* 75% quartile

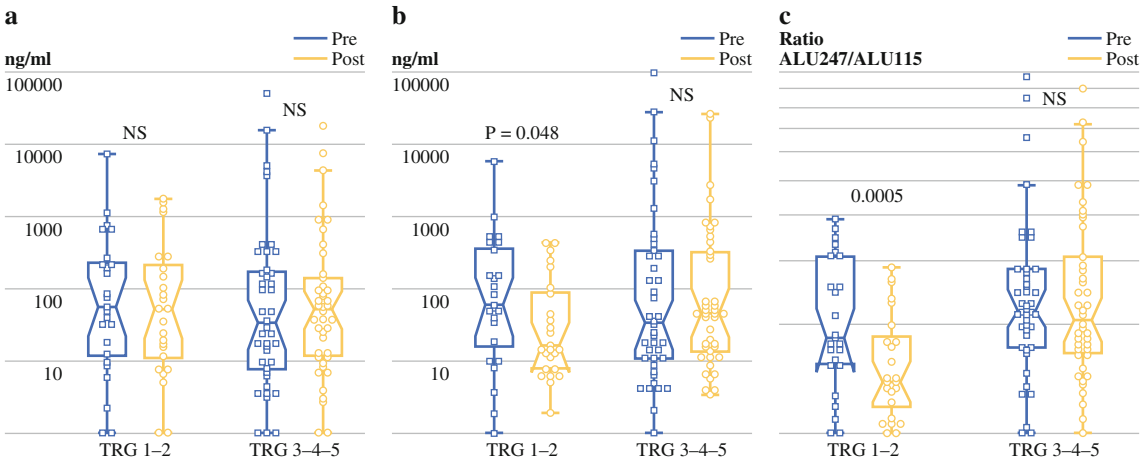



**FIG. 2** Pre- and post-CRT levels of Alu 115 (**a**) and Alu 247 (**b**), and the DNA integrity index (**c**) in plasma samples of patients with disease that responded (TRG 1–2) and did not respond (TRG 3–5) to therapy

**TABLE 4** Predictive factors of tumor response by multivariable logistic regression analysis (forward stepwise model)

| Factor | *B* | SE | Wald statistic | df | *P* value | OR | 95% CI for OR | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower | Upper |
| Ratio 247/115 post-CRT | 0.801 | 0.348 | 5.290 | 1 | 0.021 | 0.449 | 0.227 | 0.888 |

Hosmer–Lemeshow goodness-of-fit test: $\chi^2 = 4.19$, df = 8, $P = 0.84$

*OR* odds ratio, *SE* standard error, *df* degree of freedom

**TABLE 5** Confusion matrix according to the logistic regression model

| Observed | Predicted[a] | | Percentage correct |
|---|---|---|---|
| | Nonresponse | Response | |
| Nonresponse | 35 | 7 | 83.3 |
| Response | 13 | 12 | 48.0 |
| Overall percentage | | | 70.1 |

[a] Cutoff value: 0.50

treatment. In particular, the increasing posttreatment cfDNA Alu 247/115 ratio was negatively correlated with the dependent variable (Table 4).

The predictive efficiency of the logistic model is summarized in the confusion matrix reported in Table 5.

## DISCUSSION

In this translational pilot study, we sought to preliminarily confirm the potential diagnostic ability of the cfDNA circulating in plasma to discriminate cancer patients from healthy control subjects. Moreover, we focused on its ability to be a marker of tumor response in patients with rectal cancer who receive preoperative CRT.

The main findings of the study were that the plasma level of cfDNA permitted us to differentiate between patients with cancer and healthy clean-colon subjects, and that the variations in the plasma levels of cfDNA long fragments and the cfDNA integrity index are associated with pathologic tumor response.

Currently, the major concerns of performing preoperative CRT for locally advanced mid–low rectal cancer are related to the prediction of response and to the diagnosis of a complete pathological response after neoadjuvant therapy. Both of these issues are clinically relevant because the prediction of response might spare patients whose disease does not respond to therapy from unnecessary toxic treatments, while the prediction of a complete pathological response has the potential to modify the conventional surgical treatment with less aggressive (e.g., the wait-and-see policy or transanal local excision) approaches.[21] Currently, the complete pathological response after preoperative CRT is poorly predicted by conventional imaging modalities.[22]

Unfortunately, although many clinical, pathological, and molecular markers have been investigated as markers of response after preoperative CRT, none of them has yet gained clinical acceptance.[23,24] The proposed markers are often found to be contradictory and nonreproducible as a result of the complexity of the biological phenomena

related to the cancer treatment. Nevertheless, the molecular characteristics of the tumor are thought to play an important role.

Recently, circulating cfDNA and the cfDNA integrity index have been shown to be promising diagnostic biomarkers of colorectal and breast cancers.[14,15,25,26] Many different hypotheses concerning the origin of the circulating cfDNA have been considered, including the following: active liberation by the tumor itself, connection to the events of necrosis, apoptosis, mitotic catastrophe, autophagia, rupture of tumor cells, and circulation of micrometastases.[10] Among the various concepts of DNA liberation, the most studied are the events of apoptosis and necrosis.[10,27] These two phenomena may be distinguished by the dimension of the DNA fragments: the apoptotic death of cells releases DNA fragments shorter than 200 bp into circulation, whereas tumor necrosis is characterized by the presence of fragments that vary in size and are generally greater than 200 bp. In some patients, fragments are present that indicate the event of apoptosis, whereas in other patients, fragments of both longer and shorter dimensions are present.[11] The findings of our preliminary study seem to confirm this hypothesis. Compared with healthy control subjects, far higher levels of cfDNA were present in patients with cancer, and the level of long DNA fragments (Alu 247) was the most accurate circulating cfDNA marker in discriminating the cancer from noncancer subjects.[28] It should be noted that with a cutoff of 2.0065 ng/ml, the levels of Alu 247 showed a sensitivity and specificity of 94 and 100%, respectively, with an overall diagnostic accuracy of 97%. The limitations of this finding are related to the relatively small sample size and the lack of this marker to be cancer-site specific.

However, the major end point of our study was to evaluate the role of cancer-related circulating cfDNA in indicating tumor response to preoperative CRT in rectal cancer. Our hypothesis was that if the cfDNA is associated with cancer diagnosis, then the patients who experience relevant tumor regression after neoadjuvant therapy should have lower levels of circulating cfDNA compared to patients whose disease does not respond. In fact, while the baseline levels of circulating cfDNA did not predict tumor regression, the post-CRT cfDNA integrity was associated with response ($P = 0.0009$). In addition, after CRT, we found significantly reduced levels of Alu 247, and consequently higher ratios of the DNA integrity, in patients with responsive compared to nonresponsive disease ($P = 0.0048$ and 0.0005, respectively). The multivariable logistic regression analysis confirmed that posttreatment cfDNA integrity index was negatively correlated with tumor response to treatment ($P = 0.021$). These findings seem to confirm that notion of plasma cfDNA long fragments represent the cfDNA related to the tumor, while the short fragments represent a heterogeneous source of DNA. Moreover, the cfDNA may have clinical significance only when considered as a dynamic process. The variations in the cfDNA long fragments and the cfDNA integrity index during the treatment seem to have better clinical value than a single pre- or post-CRT assessment.[29–31]

Indeed, considering each individual patient, the measured cfDNA quantity showed an increased variability, indicating that the clinical use of this marker will require further studies and refinements. The reason for the wide range of concentrations could be the result of different factors related to the release of cfDNA into the plasma. In this context, the roles of cell turnover, immunological response, aggressiveness of disease, and apoptosis induced by the CRT require further studies.[10,32] Future research will show whether this marker could also be used during preoperative CRT.

Although basal levels of cfDNA cannot be used as markers of response, statistically significant differences between response and nonresponse could be found during treatment (e.g., after the first 2–3 weeks of preoperative CRT) with the association with instrumental investigations (transrectal ultrasound, pelvic computed tomography scan or magnetic resonance imaging, abdominal/chest computed tomography, and carcinoembryonic antigen test). This would permit a modification of the therapy in real time. For patients with nonresponsive disease, the treatment could be stopped and the patients could undergo surgery, thereby avoiding unnecessary toxic adverse effects and sparing resources.

The limitations of the present study are related to the relatively small number of patients evaluated, the variability of the drugs, and the total dose of radiotherapy provided. For these reasons, this study should be considered a pilot study. Our findings should be validated in a prospective study and on a larger series of patients receiving more homogeneous treatments before it can be incorporated into clinical decision-making algorithms.

In conclusion, this study confirmed that circulating cfDNA and the ratio between the long and short cfDNA fragments (cfDNA integrity index) have potential to be used as diagnostic markers of rectal cancer. In addition, the long cfDNA fragments and the cfDNA integrity index were found to be associated with tumor response, and the best way to use these markers is to evaluate their variation in plasma levels during treatment.

**ACKNOWLEDGMENT** This study was supported in part by grants from the Banca AntonVeneta, CARIPARO, and the AIRC Foundation. Biological samples were provided by 2nd Surgical Clinic, Tumor Tissue Biobank. The article was reviewed and edited for English-language usage by American Journal Experts.

## REFERENCES


1. Bosset JF, Collette L, Calais G, et al. Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med.* 2006;355: 1114–23.
2. Sauer R, Becker H, Hohenberger W, et al. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med.* 2004;351:1731–40.
3. Gerard JP, Conroy T, Bonnetain F, et al. Preoperative radiotherapy with or without concurrent fluorouracil and leucovorin in T3–4 rectal cancers: results of FFCD 9203. *J Clin Oncol.* 2006; 24:4620–5.
4. Minsky BD, Cohen AM, Kemeny N, et al. Enhancement of radiation-induced downstaging of rectal cancer by fluorouracil and high-dose leucovorin chemotherapy. *J Clin Oncol.* 1992;10: 79–84.
5. Mohiuddin M, Hayne M, Regine WF, et al. M. Prognostic significance of postchemoradiation stage following preoperative chemotherapy and radiation for advanced/recurrent rectal cancers. *Int J Radiat Oncol Biol Phys.* 2000;48:1075–80.
6. Habr-Gama A, Perez RO, Nadalin W, et al. Operative versus nonoperative treatment for stage 0 distal rectal cancer following chemoradiation therapy: long-term results. *Ann Surg.* 2004;240: 711–7.
7. Taback B, Hoon DS. Circulating nucleic acids and proteomics of plasma/serum: clinical utility. *Ann N Y Acad Sci.* 2004;1022:1–8.
8. Taback B, Hoon DS. Circulating nucleic acids in plasma and serum: past, present and future. *Curr Opin Mol Ther.* 2004;6: 273–8.
9. Fujimoto A, O'Day SJ, Taback B, et al. Allelic imbalance on 12q22–23 in serum circulating DNA of melanoma patients predicts disease outcome. *Cancer Res.* 2004;64:4085–8.
10. Gormally E, Caboux E, Vineis P, et al. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: practical aspects and biological significance. *Mutat Res.Mutat Res.* 2007;635: 105–17.
11. Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res.* 2001;61: 1659–65.
12. Giacona MB, Ruben GC, Iczkowski KA, et al. Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. *Pancreas.* 1998;17:89–97.
13. Wang BG, Huang HY, Chen Y, et al. Increased plasma DNA integrity in cancer patients. *Cancer Res.* 2003;63:3966–8.
14. Umetani N, Kim J, Hiramatsu S, et al. Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: direct quantitative PCR for repeats. *Clin Chem.* 2006;52:1062–9.
15. Umetani N, Giuliano AE, Hiramatsu, et al. Prediction of breast tumor progression by integrity of free circulating DNA in serum. *J Clin Oncol.* 2006;24:4270–6.
16. Jiang WW, Zahurak M, Goldenberg D, et al. Increased plasma DNA integrity index in head and neck cancer patients. *Int J Cancer.* 2006;119:2673–6.
17. Mandard AM, Dalibard F, Mandard JC, et al. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. *Cancer.* 1994;73:2680–6.
18. Beddy D, Hyland JM, Winter DC, et al. A simplified tumor regression grade correlates with survival in locally advanced rectal carcinoma treated with neoadjuvant chemoradiotherapy. *Ann Surg Oncol.* 2008;15:3471–7.
19. Ryan R, Gibbons D, Hyland JMT, et al. Pathological response following long-course neoadjuvant chemoradiotherapy for locally advanced rectal cancer. *Histopathology.* 2005;47:141–6.
20. Huang ZH, Li LH, Hua D. Quantitative analysis of plasma circulating DNA at diagnosis and during follow-up of breast cancer patients. *Cancer Lett.* 2006;243:64–70.
21. Habr-Gama A. Colon and rectal surgery without mechanical bowel preparation: a randomized prospective trial. *Ann Surg.* 2003; 237:363–7. *Tech Coloproctol.* 2004;8:128.
22. Maretto I, Pomerri F, Pucciarelli S, et al. The potential of restaging in the prediction of pathologic response after preoperative chemoradiotherapy for rectal cancer. *Ann Surg Oncol.* 2007;14: 455–61.
23. Goebel G, Zitt M, Zitt M, et al. Circulating nucleic acids in plasma or serum (CNAPS) as prognostic and predictive markers in patients with solid neoplasias. *Dis Markers.* 2005;21:105–20.
24. Locker GY, Hamilton S, Harris JJ, et al. ASCO. 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol.* 2006;24:5313–27.
25. Tsang JC, Lo YM. Circulating nucleic acids in plasma/serum. *Pathology.* 2007;39:197–207.
26. Kamat AA, Sood AK, Dang D, et al. Quantification of total plasma cell-free DNA in ovarian cancer using real-time PCR. *Ann N Y Acad Sci.* 2006;1075:230–4.
27. Stroun M, Lyautey J, Lederrey C, et al. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. *Clin Chim Acta.* 2001;313:139–42.
28. Stroun M, Lyautey J, Lederrey C, et al. Alu repeat sequences are present in increased proportions compared to a unique gene in plasma/serum DNA: evidence for a preferential release from viable cells? *Ann N Y Acad Sci.* 2001;945:258–64.
29. Deligezer U, Eralp Y, Akisik EE, et al. Size distribution of circulating cell-free DNA in sera of breast cancer patients in the course of adjuvant chemotherapy. *Clin Chem Lab Med.* 2008;46: 311–7.
30. Deligezer U, Eralp Y, Akisik EZ, et al. Effect of adjuvant chemotherapy on integrity of free serum DNA in patients with breast cancer. *Ann N Y Acad Sci.* 2008;1137:175–9.
31. Zitt M, Muller HM, Rochel M, et al. Circulating cell-free DNA in plasma of locally advanced rectal cancer patients undergoing preoperative chemoradiation: a potential diagnostic tool for therapy monitoring. *Dis Markers.* 2008;25:159–65.
32. Lindsay KJ, Coates PJ, Lorimore, et al. The genetic basis of tissue responses to ionizing radiation. *Br J Radiol.* 2007;80(Spec No 1):S2–6.