

Detection and Monitoring of Cell-Free DNA in Blood of Patients with Colorectal Cancer

Heidi Schwarzenbach,^a Jan Stoecklacher,^b Klaus Pantel,^a
and Eray Goekkurt^b

^a*Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf,
Hamburg, Germany*

^b*Department of Internal Medicine I, University Hospital Carl Gustav Carus,
University Dresden, Dresden, Germany*

Cell-free tumor-specific and normal DNA are released into the blood circulation by cellular necrosis and apoptosis. We examined whether circulating DNA in blood of patients with colorectal cancer could be used as an additional marker for diagnosis and response to therapy. The concentration of circulating cell-free DNA in the blood of 55 patients with advanced colorectal cancer and 20 healthy donors was measured. One to four follow-up serum samples from 14 patients were also available. Patients with colorectal cancer had a wide range of DNA concentrations in their blood. The calculated values were between 22 and 3922 ng/mL DNA, with a mean and median value of 1157 ng/mL and 868 ng/mL, respectively. In contrast, the average concentration of cell-free DNA in the serum of healthy donors was significantly lower (5–16 ng/mL). During therapy the levels of serum DNA were not constant, but fluctuated, regardless of the chemotherapy used. High DNA levels of >1000 ng/mL of blood significantly correlated with a shorter survival ($P = 0.02$). Quantitative analysis demonstrates that elevated DNA levels can be detected in blood of patients with colorectal cancer and may be a useful tool in combination with other tumor markers for detection of colorectal cancer.

Key words: colon cancer; circulating DNA; serum DNA concentrations

Introduction

Colorectal tumors are still a leading cause of cancer deaths worldwide. Despite significant improvements for some patients by the introduction of active drugs, such as oxaliplatin, bevacicumb, or cetuximab, a relevant number of patients get mostly toxic side effects from the treatment, while lacking response of the tumor.^{1,2}

Whereas surgery is the treatment of choice only in earlier stages of the disease, chemotherapy and targeted therapy are able to provide clinical benefit for the patient in both early and

late stages of the disease. But, the necessity for adjuvant treatment and the choice of an effective palliative regime with minimal toxicity still need to be better defined.² Currently, the type and quantity of therapy are based on results from Phase II–III studies and on the patient's body surface. Great efforts have been made to identify patients with increased chances of responding to treatment, and many factors have been associated with efficacy, such as interactions with other drugs, histologic differentiation of the tumor, age, gender, and severity of the disease. Available treatment regimens for patients with gastrointestinal tumors have a narrow therapeutic index and administration of maximum doses to achieve the best response may put some patients at increased risk for severe side effects. There is a need to tailor the therapy.^{1–3}

Address for correspondence: Dr. Heidi Schwarzenbach, Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany. Voice: +49-40-42-803-7494; fax: +49-40-42-803-6546. hschwarz@uke.uni-hamburg.de

The multistep progression of carcinogenesis is characterized by the successive accumulation of genetic alterations in genes controlling the cell cycle and apoptosis. Allelic imbalances, such as DNA losses and gains, are common features of tumors. Colon carcinoma develops from a preceding noninvasive adenoma. Mutations in at least three distinct signaling pathways cause gastrointestinal polyposis syndromes. First, mutations of the *adenomatous polyposis coli* (*APC*) gene are capable of initiating clonal cell growth in the colon and generate the familial adenomatous polyposis syndrome. Second, mutations which disrupt gene expression of *SMAD4* and *endoglin* have been found in the juvenile polyposis syndrome; and, third, mutations that affect receptor tyrosine kinase signaling pathways have been detected in inherited polyposis syndromes. In addition, mutations affecting the stability of genes occur in DNA mismatch repair genes and have been found in hereditary nonpolyposis colorectal cancer.^{4,5}

Moreover, increased concentrations of circulating DNA in blood of tumor patients were detected.⁶ According to the noninvasive and rapid blood sample collection by simple venous puncture, plasma or serum may therefore be a convenient source for detection of tumor-specific DNA. Cell-free DNA is supposed to be released into the blood circulation by apoptotic and necrotic cells of the primary tumor early during tumorigenesis. Moreover, it has been suggested that living cells may actively release their DNA into the blood.⁷ However, elevated DNA levels are not only measured in the blood of tumor patients, but also in the blood of patients with benign lesions, inflammation, trauma, and acute or chronic diseases, albeit the DNA yields in blood of these patients are lower.^{8,9} The observation that patients have elevated DNA levels in the blood stream indicates that a rise of cell-free DNA levels as a result of cell death may be a reflection of physiological and pathologic processes. For these reasons, detection of circulating DNA in peripheral blood of tumor patients could provide a useful, additional tool for diagnosis of col-

orectal cancer. Measurements of cell-free DNA have previously been shown to have diagnostic potential in a number of malignant diseases and also to be a noninvasive approach for early detection of carcinoma.⁶ A further important feature is the possibility of taking repeated blood samples during treatment, consequently allowing the changes in DNA levels in blood to be traced.

A recent study described the quantification of cell-free plasma DNA as an appropriate implement for monitoring patients with colorectal cancer and, prospectively, for identifying high-risk individuals.¹⁰ Furthermore, the presence of free-circulating tumor-associated DNA in plasma seems to be a relevant prognostic marker for these patients and may be used to identify patients at high risk of recurrence.¹¹ The DNA released from dead cancer cells into the blood circulation varies in size. Umetani *et al.* developed a novel method to measure the DNA integrity in serum—the ratio of longer to shorter DNA fragments—and showed that the DNA integrity may be a potential serum biomarker for detection and evaluation of colorectal carcinoma.¹² Investigations have revealed that patients with colorectal cancer possess even submicrogram per milliliter amounts of DNA in urines and showed that the smaller DNA fragments are derived from the blood circulation. The possibility that the transrenal DNA originates from apoptotic cells, and the potential use of this finding for detection of colon cancer, have been discussed.¹³ In addition to the increased DNA concentrations, elevated RNA levels are detectable in blood of patients with colon cancer. The presence of plasma RNA, which is supposed to be derived from the primary tumor, is associated with advanced stages of the colorectal tumors and tumor cells circulating in blood.¹⁴

On the basis of these promising findings, we scrutinized the clinical relevance of elevated DNA levels in blood of patients with advanced colorectal cancer and compared the serum DNA yields with the established parameters. Additionally, we followed the changes

in the DNA levels during treatment and chemotherapy.

Material and Methods

Between March 2004 and October 2005 blood samples were collected from 55 patients with sporadic advanced colorectal carcinoma (UICC tumor stage IV) who underwent primary surgical resection at the Department of Internal Medicine I, University Hospital Carl Gustav Carus, University Dresden. All patients analyzed had histologically proven epithelial colorectal cancer and, together with 20 healthy donors, gave their informed consent before the blood was taken. The examination of blood samples was approved by the local ethics review board.

Follow-up blood samples of 14 patients with advanced colorectal cancer were obtained during treatment, from 2 weeks until 9 months after the beginning of first-line chemotherapy. Patients received either FOLFIRI (irinotecan, 5-fluorouracil, and leucovorin) or FOLFOX (oxaliplatin, 5-fluorouracil, and leucovorin), or Xeloda plus irinotecan or irinotecan plus cetuximab. One patient received Xeloda plus local radiofrequency ablation of liver metastasis.

After blood sample collection in citrate-containing tubes, the samples were at once stored at 4°C and processed within 4 hours to prevent any sampling/post-processing bias. Blood fractionation was performed by initial centrifugation of 20 mL of blood at 2,500 *g* for 10 minutes. The upper phase contained the serum, from which 3 to 4 mL were removed for extraction of circulating DNA. Genomic DNA was extracted from serum of each patient using the QIAamp DNA Mini Kit and a vacuum chamber (Qiagen QIAvac24) according to the manufacturer instructions (Qiagen, Hilden, Germany). Quantification and quality of the isolated DNA were spectrophotometrically determined at 260 and 280 nm, respectively, on a NanoDrop photometer (Peqlab Biotechnologie, Erlangen, Germany).

Statistical analysis was performed using the SPSS software package, version 13.0 (SPSS Inc., Chicago, IL, USA). Chi-square and Fisher's exact test were employed to identify possible associations of the DNA concentrations with the established parameters (e.g. gender, age, tumor localization, tumor stage, chemotherapy, and date of death). Kaplan–Meier plots were used to estimate overall survival and the log rank test was used for statistical analyses. A *P*-value ≤ 0.05 was considered as statistically significant.

Results

Elevated DNA Levels in Blood Serum of Patients with Colorectal Cancer

Blood was withdrawn from 55 patients with colorectal cancer just before beginning palliative chemotherapy and from 20 healthy donors. The spectrophotometric measurements of the DNA concentrations revealed a wide range, between 22 and 3922 ng/mL of blood, with a mean value of 1157 ng/mL and a median value of 868 ng/mL. As compared to the significantly higher DNA concentrations in blood serum of patients with colorectal cancer, healthy donors had minor DNA levels in their serum. The DNA concentrations of serum from 20 healthy controls were between 5 and 16 ng/mL, with a mean value of 8 ng/mL and a median value of 7 ng/mL (data not shown).

Statistical Evaluations of Serum DNA Concentrations with the Established Risk Parameters in Patients with Colorectal Cancer

To determine whether a correlation exists between clinical parameters of the patients with colorectal cancer and the elevated DNA levels in their blood, we compared tumor localization (colon and rectal carcinoma), chemotherapy, age, gender, and overall survival. The majority of the patients (77%) were male. The patients were between 32 and 83 years old, and

their mean age was 63. Seventy and 30% of the patients had colon or rectal carcinoma, respectively. All patients had UICC tumor stage IV, and 40% of the patients were still alive at time of analyses. Fifty-three and 47% of the patients had DNA concentrations of <1000 ng/mL and >1000 ng/mL of blood, respectively. Of the patients who had DNA concentrations >1000 ng/mL, 27% were still alive and 73% were dead. No statistically significant associations between the serum DNA values and the clinical features of the patients were detected. However, Kaplan–Meier analyses showed that high DNA concentrations of >1000 ng/mL of blood significantly correlated with a shorter survival ($P = 0.02$; Fig. 1).

DNA Concentrations in the Follow-Up Serum Samples and Their Relationship to Treatment

To examine the impact of the palliative chemotherapy regimen on the DNA levels in blood of 14 patients with colorectal cancer, the DNA concentrations of 1–4 follow-up serum samples were measured. These patients were randomly chosen from the entire patient cohort (Table 1).

Of these, eight patients received a FOLFIRI regimen (irinotecan, 5-fluorouracil and leucovorin), two patients a FOLFOX regimen (oxaliplatin, 5-fluorouracil, and leucovorin), one patient Xeloda and radiofrequency ablation of liver metastasis, one patient Xeloda and irinotecan, and two patients were treated with irinotecan and cetuximab.

As shown in Table 1, in general, the serum DNA levels of the patients decreased after the beginning of the treatment and increased some weeks later upon disease progression. These fluctuations of the DNA yields were observed in the majority of cases, regardless of the treatment. In addition, whereas three patients (2–4) showed a rise in DNA after the beginning of the treatment, the serum DNA level of one patient (5) continuously decreased.

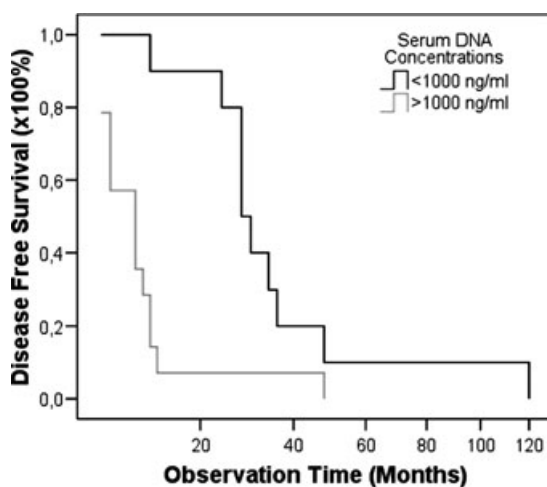


Figure 1. Kaplan–Meier disease-free survival analysis among patients with serum DNA concentrations of >1000 ng/mL and <1000 ng/mL blood at the time of primary diagnosis.

Discussion

Although an increased DNA concentration in blood is not specific for a defined disease, quantitative analysis of cell-free DNA as a non-invasive approach may have diagnostic value in combination with other clinical parameters or molecular markers.^{15,16} Also, the elevated DNA levels in blood of patients with colorectal cancer may be a useful tool for detection of this tumor entity and the evaluation of therapeutic response. A recent study compared the sensitivity and specificity of free circulating DNA with that of the more conventional serum marker carcinoembryonic antigen (CEA) and showed that cell-free circulating DNA, especially when used in combination with CEA, represents a potentially useful tool for the diagnosis of early-stage colorectal cancer.¹⁷

In the current study, we measured the DNA yields in serum from 55 patients with advanced colorectal cancer, which revealed a wide range of DNA concentrations. In general, the patients had a high DNA level in their blood and only single cases displayed low DNA levels, as documented by the high mean and median values of the patients. In contrast, healthy donors had scarce DNA concentrations in serum. A

TABLE 1. Quantification of the DNA concentrations in the follow-up serum samples

Patient No.	Cancer	Sex	Serum DNA (ng/ml)	Follow-up Sampling*	Treatment
1	Rectum	male	3014	1st	FOLFIRI
			1733	2nd: 9 weeks	
			3444	3rd: 13 weeks	
			1647	4th: 22 weeks	
2	Rectum	male	2529	1st	FOLFIRI
			2890	2nd: 2 weeks	
3	Rectum	female	3922	1st	FOLFIRI
			5944	2nd: 9 weeks	
			1769	3rd: 14 weeks	
			1891	4th: 20 weeks	
4	Colon	male	1183	1st	FOLFOX
			1324	2nd: 10 weeks	
5	Colon	male	3100	1st	FOLFIRI
			811	2nd: 7 weeks	
			484	3rd: 10 months	
6	Colon	male	2261	1st	FOLFIRI
			1941	2nd: 16 weeks	
			3700	3rd: 21 weeks	
			2394	4th: 27 weeks	
			539	5th 34 weeks	
7	Colon	female	1753	1st	Xeloda + Radiofrequen zablation
			1242	2nd: 9 weeks	
			2320	3rd: 13 weeks	
			925	4th: 19 weeks	
8	Colon	male	3217	1st	FOLFIRI
			1760	2nd: 7 weeks	
			1946	3rd: 18 weeks	
			656	4th: 23 weeks	
9	Colon	male	2974	5th: 35 weeks	Irinotecan + Xeloda
			1221	1st	
10	Colon	male	614	2nd: 3 weeks	FOLFIRI
			2458	1st	
			158	2nd: 11 weeks	
11	Colon	male	453	3rd: 16 weeks	Irinotecan + Cetuximab
			3060	1st	
			1505	2nd: 4 months	
12	Colon	male	1806	3rd: 9 months	FOLFIRI
			1953	1st	
			705	2nd: 16 weeks	
			547	3rd: 20 weeks	
13	Colon	male	1731	4th: 31 weeks	FOLFOX
			2944	1st	
			1144	2nd: 20 weeks	
14	Colon	male	1921	3rd: 32 weeks	Irinotecan + Cetuximab
			1850	1st	
			1415	2nd: 13 weeks	
			311	3rd: 15 weeks	
			612	4th: 25 weeks	

*The number of the weeks of the follow-up sampling is related to the 1st blood withdrawal. **NeoGenomics**
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comparison of the mean and median values showed that patients with colorectal cancer had DNA yields that were significantly (more than 100 times) higher than those of the healthy donors. We found higher DNA concentrations in blood of our palliative patient cohort than did a recent study that reported that at the time of surgery, cell-free DNA levels in plasma of all tumor patients were about only 25 times higher in comparison with healthy controls.¹⁰ Furthermore, Diehl *et al.* found increased DNA concentrations in blood of their patient cohort and revealed that the proportion of mutant *APC* DNA fragments in plasma averaged 11% of the total DNA fragments, even in large, metastatic tumors. Their scrutiny was consistent with a model wherein hypoxia induces necrosis of tumors, leading to phagocytosis of tumor cells and the subsequent release of digested DNA into the blood circulation.¹⁸

Our measurements of the follow-up samples from 14 patients showed that the DNA yields varied and fluctuated after treatment with chemotherapy. Our results are in line with the findings of a recent study which demonstrated that during a follow-up period of 4 to 10 months, cell-free DNA levels decreased progressively in tumor-free patients, whereas they increased in those developing recurrences or metastases.¹⁰ Our study population consisted of patients with advanced colorectal cancer with a high initial tumor burden. The very high tumor DNA concentrations measured in our study cohort represent this high initial tumor mass; the decrease in the DNA concentrations in the follow-up patients represents the effect of the cytostatic treatment; and the increase in the DNA levels in some patients was due to disease progression. Furthermore, a report showed for another tumor entity that tumor-specific cell-free DNA levels declined in response to therapy for ovarian carcinoma and additionally, correlated with increasing burden of this tumor. The investigators deduced that tumor-specific DNA may be a useful surrogate biomarker of therapeutic response.¹⁹

Moreover, a study investigated 58 patients who underwent primary colorectal cancer resection and demonstrated that the 2-year overall survival rate was 48% in the group where free-circulating tumor-associated DNA was detected in plasma and 100% in the one where free-circulating tumor-associated DNA was not detected in plasma.¹¹ In addition, our study showed that high DNA concentrations of >1000 ng/mL of blood significantly correlated with a shorter survival.

In conclusion, our data show that serum DNA levels are significantly increased in patients with colorectal cancer of stage IV and fluctuate during chemotherapy. These observations suggest that measurements of cell-free DNA may be an additional implement for detection of colorectal cancer. Long-term follow-up studies of a larger cohort of patients are planned to explicitly record the changes in DNA concentrations in blood.

Acknowledgments

We are grateful to the Erich and Gertrud Roggenbuck-Stiftung, Hamburg, for supporting this work. We thank Ms. Jessica Gädicke and Imke Müller for their excellent technical assistance.

Conflicts of Interest

The authors declare no conflicts of interest.

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