Utility of Cell-free Tumour DNA for Post-surgical Follow-up of Colorectal Cancer Patients

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Abstract. *Background: While efficient surgical treatment is the key to prolonged survival of patients with colorectal cancer, post-surgical follow-up is important for the early detection of relapsing disease or of disease progression. Current dispensarization, typically based on imaging CT, PET, MR, is frequently supported by the observation of tumour markers (CEA, CA19-9). Due to their limited sensitivity and selectivity, better tools for monitoring of the disease are desirable. Tumour cell-free DNA (cfDNA) has been recently demonstrated as a new promising molecular marker for observation and early detection of disease progression. Patients and Methods: We present results of post-surgical monitoring tumour cfDNA in the cases of seven patients suffering from advanced forms of CRC. We applied a mutation-based approach in which the total cfDNA was screened for a specific somatic mutation present in the primary tumour. We screened a panel of the most frequent somatic mutations covering the genes APC, KRAS, TP53, PIK3CA and BRAF. All patients were tested positive for tumour cfDNA prior to surgery. cfDNA was then evaluated within a week after surgery and subsequently in monthly intervals. Results: We present typical cases of colorectal cancer patients who underwent surgical treatment at different levels of radicality with or without adjuvant chemo/biotherapy. The tumour cfDNA status was found to be always closely correlated with the actual clinical status of the patient. Conclusion: The cfDNA appears to be a viable tool for the monitoring of the clinical progression of CRC in patients with cfDNA positivity prior to surgery.*

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The mortality and the survival rates of colorectal cancer are highly influenced by the stage of the disease at the time of diagnosis as well as at the onset of the treatment. Despite adequate surgical intervention with or without adjuvant chemotherapy, 30% to 50% of patients with colorectal cancer will experience disease relapse, will develop metastases and will propably die within 5 years of the diagnosis (1). Long-term follow-up approaches based on imaging techniques, including computed tomography (CT), nuclear magnetic resonance (MR), positive emission tomography (PET) and ultrasonography (US), are complemented by the monitoring of the levels of tumour markers. The traditionally used markers in colorectal cancer are CEA (Carcinoembryonic antigen) and CA19-9 (carbohydrate antigen 19-9). Their relative low sensitivity and specificity prompted a search for biological indicators that may better assist the evaluation of the risk for recurrence (2-4).

New improvements in molecular-genetic testing, as well as better insight into the basic mechanisms of carcinogenesis, are enabling for better approaches in early diagnosis, as well as for better monitoring of progression, resulting thus, in more efficient surgical and oncological treatments. Among various approaches, cell-free DNA (cfDNA), present in blood and the lymphatic systems, is of great interest (5). Originally described in 1948 (6), cfDNA is a product of apoptotic and necrotic cellular processes (7). As the cfDNA levels are found to be significantly increased when comparing cancer patients to healthy controls (8, 9), cfDNA has been studied in various human cancers (10-15).

In cancer patients, necrosis of the tumour mass produces the main component of cfDNA, referred to as the tumour cfDNA (16, 17). These are key elements for diagnostic testing and subsequent monitoring of the disease. The fundamental molecular similarity of the tumour cfDNA to its parent tissue can be utilized to eliminate any interference of

the remaining non-tumour cfDNA, naturally present in the plasma (18). The most common approach to the examination of tumour cfDNA is based on tumour-specific mutations. A panel of somatic mutations is first evaluated in the primary tumour. The presence of tumour cfDNA is then detected upon occurrence of the tumour-specific mutations in the plasma (19, 20).

It was a primary objective of the current study to investigate the presence of tumour cfDNA and its correlation to the clinical course of the disease. We applied a routine mutation detection in order to monitor tumour cfDNA levels during post-surgical follow-up. We present several clinical cases, in which tumour cfDNA monitoring may become an important part of the clinical management of the disease.

Patients and Methods

The group of patients consisted of four females and three males 54 to 76 years of age, with clinically confirmed colorectal cancer and detectable tumour cfDNA prior to surgery. There were three rectal, two rectosigmoidal, one sigmoidal and one splenic flexure tumour in stages III or IV. All patients were directed to either curative or palliative surgical treatment. For each patient, multiple samples were acquired including an initial plasma sample obtained one day prior to surgery, tumour tissue sample collected during the surgery and up to three more plasma samples, acquired during the subsequent follow-up.

The plasma was separated from the blood cells by centrifugation, immediately after blood collection, and was then frozen at –20˚C before transport to the laboratory. cfDNA was isolated from the plasma by NucleoSpin Plasma XS (Macherey-Nagel, Dueren, Germany). The detection of tumour cfDNA was based on a mutation-specific approach in which the plasma is screened for the presence of specific somatic mutations previously found in the tumour tissue. Thus firstly the presence of any, of the most frequent, somatic mutations was examined in the tissue collected during the surgery. The mutation panel included *APC* (Adenomatous polyposis coli, mutation cluster region), *KRAS* (Kirsten rat sarcoma viral oncogene homolog, exon 1), *TP53* (tumor protein 53, exons 5-8), *PIK3CA* (phosphoinositide-3-kinase, catalytic, alpha polypeptide, exon 9 and exon 20) and *BRAF* (v-Raf murine sarcoma viral oncogene homolog B1, exon 15). For each patient, only mutations detected in the tumour tissue were evaluated in the plasma samples.

All mutation detections were performed by a denaturing capillary electrophoresis technique (DCE) using Genoscan mutation detection kit (Genomac International, Prague, Czech Republic) in which a fluorescently labelled PCR, amplified regions of up to 140 bp fragments and separated them in forms of homo- and heteroduplexes at a precise given temperature on a standard DNA sequencer (ABI PRISM 3100, Applied Biosystems, Foster City, California) (21, 22). The technique provides the relative fraction of mutated alleles and has previously been applied for the detection of mutants in various tumour tissues (23-25).

Overall, seven samples from primary tumour tissue and 35 plasma samples were examined, resulting in a total of 119 mutation analysis tests performed. In addition to tumour cfDNA, the levels of standard tumour markers CEA and CA19-9 were also assessed.

Results

Data regarging the monitored patients are summarized in Table I. The levels of tumour cfDNA were characterized according to the estimated fraction of mutated alleles present in the plasma cfDNA sample as follows: (–) no mutated alleles found; (+) ca. 5% of mutated alleles; (++) ca. 5% –30% of mutated alleles and (+++) more than 30% of mutated alleles. There were three cases (two in Stage III, one in Stage IV) of curable R0 resections (including one total pelvic exenteration) with subsequent long-lasting periods without detectable tumour cfDNA and no signs of disease progression. In addition there was one case of stage IV disease with a curable R0 resection without detectable tumour cfDNA after surgery, but a re-appearance of tumour cfDNA and detection of disease progression seven months after the surgery. Finally, levels of tumour cfDNA fluctuated in one patient with stage IV disease, in palliative chemotherapy following an R2 pelvic exenteration and during palliative biological therapy of inoperable stage IV tumour. The detailed descriptions of the clinical courses of the disease for individually monitored patients follow.

Patients after R0 resection with no signs of relapse.

A) Female, 62 years old, operated on for sigmoidal colon cancer invading the small bowel (T4N0M0), in whom an *en bloc* R0 resection was performed. No neoadjuvant chemothery was indicated, and preoperative tumour marker levels were 1.3 μg/l CEA and 14.4 kU/l CA19-9. Tumour cfDNA was identified in the plasma prior to surgery. Adjuvant chemotherapy was administered. Neither in the clinical postoperative course, nor during cfDNA detection were there any signs of relapse.

B) Female, 76 years old, operated on for synchronous splenic flexure adenocarcinoma and segment VI liver metastasis. No neoadjuvant chemothery was indicated and a simultaneous left colectomy with liver resection was performed (R0). Preoperative staging was cT3NXM1hep with tumour markers of 3.4 μg/l CEA and 14.1 kU/l CA19-9. The postoperative course was uneventfull and adjuvant chemotherapy was administered. Tumour cfDNA, detected prior to surgery, disappeared after surgery and remained undetected in consecutive postsurgical blood samples. Twenty seven months after surgery, the patient remains without clinical signs of relapse, as shown by PET/CT, colonoscopy and tumour markers.

C) Male, 68 years old, operated on urgently for obstructive ileus, rectosigmoidal cancer invading the small bowels and the bladder, with rectovesical fistula. R2 enteroanastomosis and sigmoido stomy was performed. Radiotherapy and chemotherapy were contraindicated due to the malnutrition and the poor general condition. With 35.3 μg/l CEA and 12.4 kU/l CA19-9, radical surgery was indicated and a supralevator total pelvic exenteration was performed. The operation included

Table I. *Comprehensive overview of all patients showing cfDNA positivity prior to surgery and their subsequent monitoring after surgery.*

Patient	Age, gender tumor site stage(+)	Treatment	Marker	Before surgery (<1 week)	After surgery <1 week	<6 months	6-12 months	12-18 months	18-24 months	Clinical status
A	62y female	Primary	cfDNA	+	–	–	–	–		No signs of progression
	sigmoid colon	*en bloc* R0 resection	CEA	–		–		–		18 months after surgery
	T4N0M0	adjuvant chemo	CA19-9	–		–		–		
B	76y female	Primary + liver mt	cfDNA	++	–	–	–	–	–	No signs of progression
	splenic flexure	R0 resection	CEA	–	–		–		–	27 months after surgery
	T3N1M1hep	adjuvant chemo	CA19-9	–	–		–		–	
C	68y male		cfDNA	++	–	–	–	–	–	Full recovery with no signs of
	rectosigmoid	R0 pelvic exenteration	CEA	++		–	–			relapse 29 months after surgery
	T4N0M0		CA19-9	–		–	–			
D	59y male	Primary + liver mt	cfDNA	+	–	++	++			Lung metastases detected 7 months
	rectum	R0 resection	CEA	–		+	++			after surgery, alive on chemotherapy
	T3N0M1hep	adjuvant chemo	CA19-9	–		+	+			12 months from surgery
E	54y female		cfDNA	+	–	–	++	–		Adjuvant chemotherapy and biological
	rectosigmoid	R2h resection	CEA	++		+	++	+++		therapy with slow progression,
	T4N1M1hep		CA19-9	–		–	–	–		death 24 months after surgery
F	62y male	Inoperable	cfDNA	+++	++	–	+		++	Palliative biological therapy
	rectum	stoma	CEA	++	++	–	–			with slow progression,
	T4NxM1hep/pulm	palliative chemo	CA19-9	++	++	+	+			death 25 months after surgery
G	71y female	Neoadjuvant biological	cfDNA	+	++	++	+++			Palliative chemotherapy
	rectum	R2 pelvic exenteration	CEA	++			+++			with steady progression, death
	T4N2M1hep		CA19-9	–			+			9 months after surgery

cfDNA	CEA	CA19-9	+) according to TNM classification
–) undetectable	–) <5 μg/L	–) <40 U/mL	
+) ca. 5% mutated alleles	+) 5-10 μg/L	+) 40-1000 U/mL	
++) ca. 30% mutated alleles	++) >10 μg/L	++) >1000 U/mL	
+++) >30% mutated alleles	+++) >100 μg/L.		

triple small bowel resection, including resection of internal illiac vessels. No adjuvant oncological therapy was indicated due to sacral bed sore. Histology confirmed massive tumour infiltration from the rectosigmoideum to the bladder, pT4N0M0, and poorly differentiated mucinous tubular carcinoma. Tumour cfDNA was only detected prior to pelvic exenteration, but remained undetected in sampling following surgery. Twenty nine months after the surgery, the patient has fully recovered with no clinical, CT, colonoscopy or marker signs of relapse.

Patients with subsequent disease progression after R0 resection. D) Male, 59 years old, indicated for laparoscopic sigmoidostomy and insertion of port catheter due to stenosing rectal cancer with multiple liver metastases before neoadjuvant chemotherapy (T3N0M1hep). Initial marker levels were 8.4 μg/l CEA and 79.1 kU/l CA19-9. After downsizing of the tumour (2.2 μg/l CEA, 31.7 kU/l CA 19-9), radical R0 surgery was performed with simultaneous low anterior resection, with 11 non-anatomical resections for the liver metastases. The postoperative course was without complications and the patient continued chemotherapy. Tumour cfDNA was detected preoperatively as shown in Figure 1A, and was undetected at the days following the surgery (Figure 1B). Six months after the surgery tumour cfDNA was again detected and the CT scan revealed multiple lung metastases, as shown in Figure 1C, also confirmed by histology exams. The patient is continuing chemotherapy with good performance 17 months after the original surgical treatment.

E) Female, 54 years old, after a previous sigmoidorectal bypass operation and a year of chemotherapy for rectosigmoidal cancer, was found to have positive para aortic lymphnodes and multiple synchronous liver metastases (pT3N1M1hep). Extended low anterior rectosigmoidal resection was performed, and the liver metastases were unresectable. A pre-operatively elevated level of CEA at 14.1 μg/l (0.6 kU/l CA19-9) was accompanied by positive tumour cfDNA. After the operation adjuvant chemotherapy and biological therapy was administered, there was no observable remission of the liver metastases; however, the levels of tumour cfDNA were suppressed and the patient had a good performancestatus. Over the next 12 months the disease slowly progressed, both clinically and on CT; the tumour cfDNA re-emerged, with CEA of 125.3 μg/l and CA19-9 of 0.6 kU/l. The patient died 25 months after the start of tumour cfDNA monitoring.

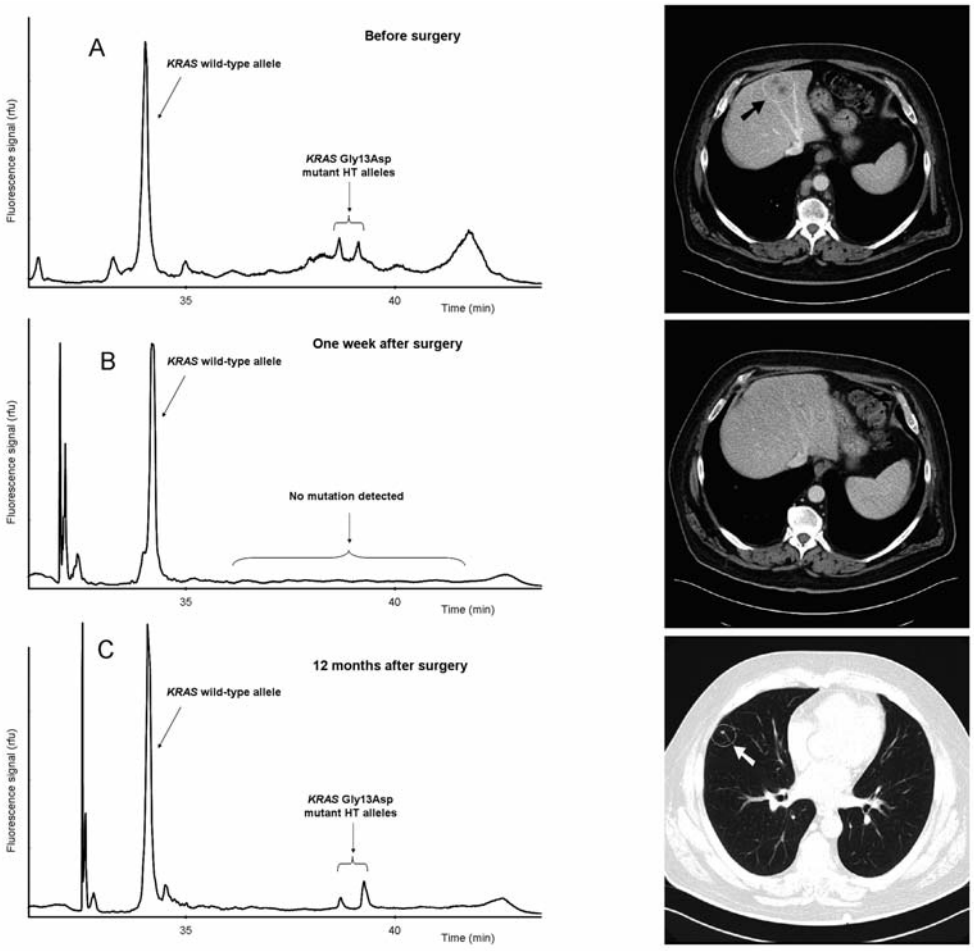

Figure 1. *Monitoring of the clinical course of metastatic disease in patient D after the resection of the primary tumour and of liver metastases. Tumour cfDNA was found prior to the surgery by detecting a KRAS mutation (Gly13Asp) from the primary tumour presence in plasma, Figure 1A. The tumour of cfDNA was subsequently absent 1 week after R0 resection of the primary tumour and the liver metastases, as shown in Figure 1B. 6 months from operation the disease has progressed with the presence of lung metastases and, at the same time, tumour cfDNA reemerged as shown in (Figure 1C).*

Patients with subsequent disease progression after R2 resection.

F) Male, 62 years old at the time of surgery, was admitted to the surgical department for rectal bleeding; rectal cancer was confirmed by biopsy. CEA was 26.9 μg/l and CA19-9 was 1668 kU/l. PET/CT described multiple liver and pulmonary metastases. The primary tumour had invaded the prostate (cT4NXM1hep, pulm). During surgery, multiple diffuse inoperable liver metastases were confirmed and palliative sigmoidostomy was performed (R2). Tumour cfDNA was positive before as well as after surgery. The postoperative course was uneventful (CEA=16.1 μg/l, CA 19-9=2989 kU/l). Palliative chemotherapy was administered and the tumour markers decreased (CEA to 4.4 μg/l, CA19-9 to 78.4 kU/l), accompanied by tumour cfDNA temporary disappearance. Despite this, CT demonstrated a gradual slow progression of both liver and lung secondary tumours. Tumour cfDNA reappeared one year after the surgery and the mutated allele fraction steadily increased over the subsequent year. The patient died 25 months after the operation date.

G) Female, 71 years old, was diagnosed with synchronous rectal cancer invading the rectovaginal septum with three liver metastases and a histologically confirmed duplicity of bilateral ductal breast cancer (cT4NXM1hep). A rectal adenocarcinoma with poor differentiation was diagnosed; biopsy from the liver secondaries proved their rectal origin, CEA was 26.9 μg/l and CA19-9 2.8 kU/l. Neoadjuvant therapy was indicated with concomitant chemoradiotherapy and bevacizumab biological therapy. When the tumour progressed and became symptomatic, surgery was indicated. Subsequently, abdominoperineal resection with *en bloc* hysterectomy and resection of the part of the infiltrated vagina was performed.

Due to the extent of the disease, simultaneous liver resection was not indicated. Histology confirmed pT4, pN2 (13 positive nodes), pM1. The postoperative course was complicated with deep ileofemoral thrombosis. Even after the change of the chemotherapy regiment, permanent progression of the disease was confirmed clinically and by CT (CEA=1740 μg/l, CA 19-9=313 kU/l). The liver metastases doubled in diameter and number within two months and pulmonary metastases also appeared. The patient died 18 months after diagnosis. Tumour cfDNA was detected preoperatively and during the postoperative chemotherapy.

Discussion

A high level of free circulating DNA is generally considered to be a negative prognostic factor. In patients who underwent curative resection of liver metastases from colorectal cancer, a high level of free circulating DNA was reported to be a predictive factor of recurrence (26). In our study, during the follow-up, tumour cfDNA typically disappeared after curative R0 surgery, while in patients with non curative treatment (R2 surgery or palliative surgery) tumour cfDNA remained detectable immediately after operation. In the case of concurrent resection of the primary tumour and of hepatic metastases (Patient D), the tumour cfDNA was undetectable one week after surgery, but re-appeared in a sample acquired seven month after the surgery. The patient was subsequently examined by CT and a progression of the disease, through new liver metastases, was confirmed.

Although the actual levels of mutated allele fractions in plasma cfDNA do not correlate with the tumour stage among different patients, they seem to closely follow the actual development of the disease in each individual. As seen in Table I, a progression of the disease was typically translated into an increasing fraction of mutated cfDNA alleles in plasma (patients D and E), (Figure 1). At the same time, the outcome of anticancer therapy can be observed as a decrease in the tumour cfDNA levels, for positive response to biological palliative therapy, up to six months after the surgery. In contrast, no response to palliative chemotherapy is evident from the steadily increasing levels of mutated cfDNA (Patient G).

When comparing to the levels of CEA and CA19-9, the tumour cfDNA appears to be a more universal marker for monitoring of the clinical course of the disease, correlating to either of the two tumour markers. Indeed, such a notion needs to be confirmed by the study of additional patients.

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References

1 Gan S, Wilson K and Hollington P: Surveilance of patients following surgery with curative intent for colorectal cancer. World J Gastronterol *13*: 3816-3823, 2007.
2 Kaneko I, Tanaka S, Oka S, Yoshida S, Hiyama T, Arihiro K, Shimamoto F and Chayama K: Immunohistochemical molecular markers as predictors of curability of endoscopically resected submucosal colorectal cancer. World J Gastroenterol *13*: 3829-3835, 2007.
3 Ogino S, Kawasaki T, Kirkner GJ, Kraft P, Loda M and Fuchs CS: Evaluation of Markers for CpG Island Methylator Phenotype (CIMP) in Colorectal Cancer by a Large Population-Based Sample. J Mol Diagn *9*: 305-314, 2007.
4 Famulski W, Sulkowska M, Wincewicz A, Kedra B, Pawlak K, Zalewski B, Sulkowski S, Koda M and Baltaziak M.: P53 correlates positively with VEGF in preoperative sera of colorectal cancer patients. Neoplasma *53*: 43-48, 2006.
5 Vlassov VV, Laktionov PP and Rykova EY: Circulating nucleic acids as a potential source for cancer biomarkers. Curr Mol Med *10*: 142-165, 2010.
6 Mandel P, Metais P: Les acidos nucleiques du plasma sanguin chez l´homme. C R Seances Soc Biol Fil *142*: 241-243, 1948.
7 Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD and Knippers R: DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res *61*: 1659-1665, 2001.
8 Bendich A, Wilczok T and Borenfreund E: Circulating DNA as possible factor in oncogenesis. Science *148*: 374-376, 1965.
9 Leon SA, Shapiro B, Sklaroff DM and Yaros MJ: Free DNA in the serum of cancer patiens and the effect of therapy. Cancer Res *37*: 646-650, 1977.
10 Shapiro B, Chakrabarty M, Cohn EM and Leon SA: Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer *51*: 2116-2120, 1983.
11 Behera D, Gumbhir K, Malik SK and Dani HM: Lung cancer and circulating DNA. Indian J Chest Dis Allied Sci *28*: 54-55, 1986.
12 Anker P, Lefort F, Vasioukhin V, Lyautey J, Lederrey C, Chen XQ, Stroun M, Mulcahy HE and Farthing MJ: K-ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. Gastroenterology *112*: 1114-1120, 1997.
13 Silva JM, Dominguez G, Garcia JM, Gonzalez R, Villanueva MJ, Navarro F, Provencio M, San Martin S, Espana P and Bonilla F: Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations. Cancer Res *59*: 3251-3256, 1999.
14 Kamat AA, Baldwin M, Urbauer D, Dang D, Han LY, Godwin A, Karlan BY, Simpson JL, Gershenson DM, Coleman RL, Bischoff FZ and Sood AK: Plasma cell free DNA in ovarian cancer: an independent prognostic biomarker. Cancer *116*: 1918-1925, 2010.
15 Jung K, Stephan C, Lewandowski M, Klotzek S, Jung M, Kristiansen G, Lein M, Loening SA and Schnorr D: Increased cell-free DNA in plasma of patients with metastatic spread in prostate cancer. Cancer Lett *18*: 173-180, 2004.
16 Choi JJ, Reich CF and Pisetsky DS: The role of macrophages in the in vitro generation of extracellular DNA from apoptotic and necrotic cells. Immunology *115*: 55-62, 2005.

17 Stroun M, Lyautey J, Lederrey C, Olson-Sand A and Anker P: About the possible origin and mechanism of circulating DNA: apoptosis and active DNA repase. Clin Chim Acta *313*: 139-142, 2001.
18 Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C and Beljanski M: Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology *46*: 318-322, 1989.
19 Diehl F, Schmidt K, Durkee KH, Moore KJ, Goodman SN, Shuber AP, Kinzler KW and Vogelstein B: Analysis of mutations in DNA isolated from plasma and stool of colorectal cancer patients. Gastroenterology *135*: 489-498, 2008.
20 Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D: BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat Methods *3*: 551-559, 2006.
21 Bjørheim J, Minarik M, Gaudernack G and Ekstrøm PO. Evaluation of denaturing conditions in analysis of DNA variants applied to multi-capillary electrophoresis instruments. J Sep Sci *26*: 1163-1168, 2003.
22 Ekstrom PO, Khrapko K, Li-Sucholeiki XC, Hunter IW and Thilly WG: Analysis of mutational spectra by denaturing capillary electrophoresis. Nat Protoc *3*: 1153-1166, 2008.
23 Minarik M, Minarikova L, Hrabikova M, Minarikova P, Hrabal P and Zavoral M: Application of Cycling Gradient Capillary Electrophoresis (CGCE) to Clinical Screening of *APC*, *K-ras* and *DCC* Point Mutations in Patients with Sporadic Colorectal Tumors. Electrophoresis *25*: 1016-1021, 2004.
24 Pesek M, Benesova L, Belsanova B, Mukensnabl P, Bruha F and Minarik M: Dominance of EGFR and insignificant KRAS mutations in prediction of tyrosine-kinase therapy for NSCLC patients stratified by tumor subtype and smoking status. Anticancer Res *29*: 2767-2773, 2009.
25 Salek C, Minarikova P, Benesova L, Nosek V, Strnad R, Zavoral M and Minarik M: Mutation status of K-ras, p53 and allelic losses at 9p and 18q are not prognostic markers in patients with pancreatic cancer. Anticancer Res *29*: 1803-1810, 2009.
26 Thinssen MA, Swinkels DW, Ruers TJ and de Kok JB: Difference between free circulating plasma and serum DNA in patiens with colorectal liver metastases. Anticancer Res *22*: 421-425, 2002.

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