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Persistence of K-ras Mutations in Plasma after Colorectal Tumor Resection

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Abstract. *Free DNA in the circulation is increased five-to ten-fold in patients with solid tumours compared to healthy controls. A range of tumor-specific mutated DNA has been shown to be readily extractable and possible to analyse from plasma and serum in these patients. K-ras oncogene mutations are an early event in a subset of colorectal tumors and have been found in 30-60% of patients with colorectal carcinoma (CRC). The presence of tumor-derived k-ras gene mutations in the circulation has previously been described before surgery. The aim of this study was to characterize the presence of mutant k-ras in plasma in the short-term postoperative period after radical surgery of CRC patients, and further to characterize this in relation to relapse of the disease. Tumors and corresponding plasma pre- and postoperatively on day three after surgery were collected from 25 patients with CRC (Dukes' stage A-D). Biopsies for DNA extraction from the tumors were collected from the most invasive parts microscopically. After PCR amplification of the k-ras gene (codon 12 and 13), the presence of mutations was analysed by TGGE (temperature gradient gel electrophoresis). Twenty four/25 patients underwent putatively curative resections. Sixteen of the 25 patients (64%) expressed k-ras mutations in their tumor. Of these, 9 patients (56%) also had detectable k-ras mutations in preoperative plasma samples. On day three postoperatively, 8 of these patients persistently were found to have mutant k-ras in the plasma. This was not correlated with tumor stage. None of the 9 tumor mutation-negative cases expressed mutated k-ras in their plasma pre- or postoperatively. The results indicate that plasma mutant k-ras can be detected pre- and early postoperatively in all stages of colorectal neoplasia. No correlation between short-term postoperative persistence of mutant plasma-DNA and disease recurrence at follow-up was found. However, the use of k-ras as a marker during postoperative follow-up and as a possible tool for early detection of recurrent disease must be further characterized.*

Abbreviations: CRC, colorectal carcinoma; PCR, polymerase chain reaction; TGGE, temperature gradient gel electrophoresis.

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Key Words: K-ras mutations, TGGE (temperature gradient electrophoresis), colorectal carcinoma, circulating nucleic acids.

During recent years, increasing attention has been drawn to the analysis of tumor DNA in blood. Free plasma-DNA in the circulation is increased five-to ten-fold compared to normal in patients with solid cancer tumors (1, 2). A range of tumor-specific mutated DNA has been shown to be readily extractable and possible to analyse from plasma and serum in these patients (3-7). The mechanism of DNA release into the serum or plasma is not yet fully explained, but it is postulated that this DNA is excreted directly into the circulation from tumor cells undergoing apoptosis or being destroyed by the immune system. The presence and quantity of corresponding circulating tumor DNA are likely to be a reflection of tumor burden or metastasis and may have a prognostic value.

Mutation of the k-ras oncogene is associated with a number of gastrointestinal malignancies and this mutation has been described as an early event in the development of colorectal carcinoma (CRC). K-ras mutations reportedly occur in 30% to 60% of all CRC (3, 4, 8-17). Mutant k-ras in plasma has been studied in different malignancies, but its disappearance and characteristics after radical surgery of CRC has not been fully characterized.

The aim with this study was to characterize the occurrence of mutant k-ras in the tumor in relation to its presence in free, circulating DNA preoperatively and in the short-term, postoperative period after radical surgery and further to correlate these data to relapse of the disease.

Materials and Methods

Patients. Twenty-five patients with CRC, age 49-90 years (median age 72 years), undergoing surgery at our department, were studied. Informed consent was obtained from each patient prior to surgery.

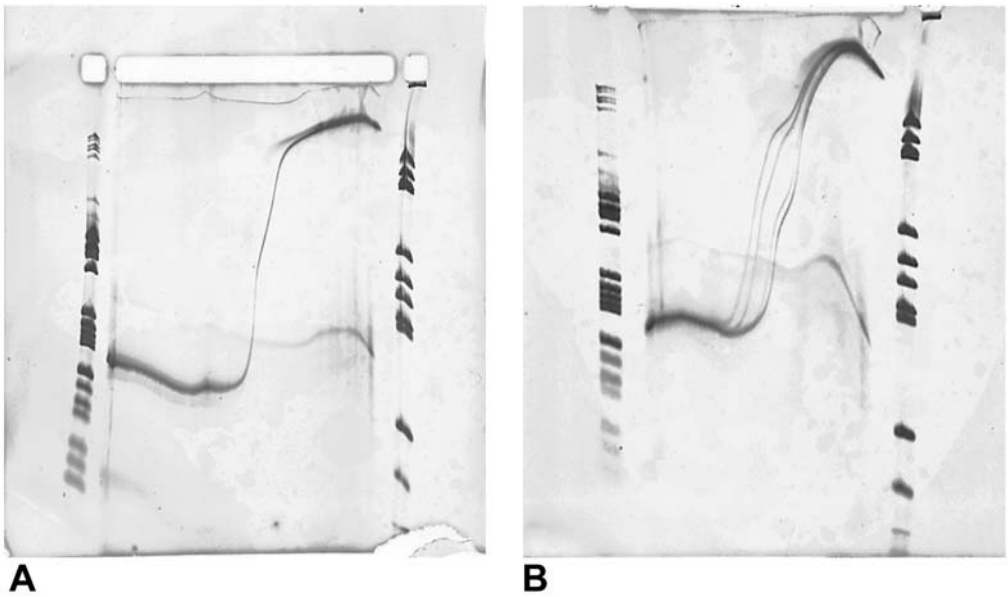

Figure 1. *A. Perpendicular TGGE showing wild-type DNA confirmation. B. Perpendicular TGGE with k-ras mutation resulting in four melting bands.*

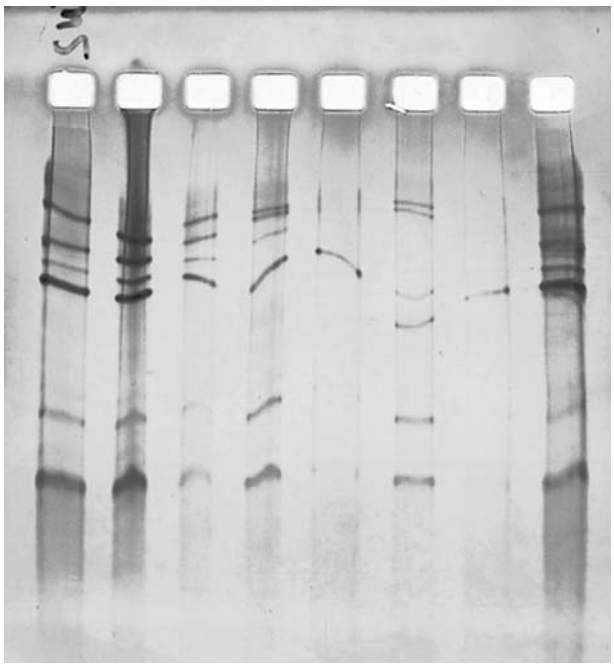

Figure 2. *Parallel TGGE with:*
1. SW 480 cell line (containing k-ras mutation)
2. Tumor with k-ras mutation
3. K-ras mutation in plasma from patient (2)
4. Tumor with k-ras mutation
5. Plasma from patient (4) wild-type configuration
6. Tumor with k-ras mutation
7. Plasma from patient (6) wild-type configuration
8. SW 480 cell line (control)

The material comprised 9 right-sided and 7 left-sided colonic tumors and 9 rectal tumors. According to Dukes' classification, 4 patients had stage A disease, 7 patients had stage B disease and 13 patients had stage C disease. One patient was found to have metastatic disease (liver) during surgery (Dukes' D), but was included in the study. The other 24 cases underwent putative curative resection of the tumor. Blood samples were taken preoperatively and 3 days after surgical resection. The mean follow-up time was 38 months. The study was approved by the Ethical Committee at Huddinge University Hospital, Stockholm.

DNA extraction. Tumor tissue was obtained from the surgical specimens, fixed in formalin and embedded in paraffin, according to standard procedure. Samples for DNA extraction were selected from areas representing only invasive, neoplastic cancer cells (8, 18), outlined with a marker as previously described (8), and then transferred to a 1.5 ml Microfuge tube. DNA was extracted with the Qiamp Tissue kit (Qiagen, Hilden, Germany). Plasma was separated after centrifugation at 3000 rpm immediately after sampling and stored at -70˚C until further use. The samples were then condensated in microtubes, and genomic DNA was extracted with the specific Qiamp DNA Blood kit (Qiagen), according to the manufacturer. The amplification of the PCR product was performed between codons 9 and 30, thus enabling detection of mutations within codons 12 and 13, as described (8, 19).

Temperature gradient gel electrophoresis (TGGE). TGGE of the purified amplification products was performed in a system (20) patented by Qiagen and produced by Biometra (Göttingen, Germany). We used horizontal 8% gels with 4.2 g urea, 2.0 ml acrylamide (stock solution 37.5:1, 40%), 0.1 ml TBE (conc. x 10), 0.5 ml glycerol (50%), 22.5 μl TEMED and 42 ml APS (4%) in

Table I. *Patient data from the material and the results of k-ras analysis of the tumor (k-ras tum), plasma preoperatively (k-ras preop) and three days after surgery (k-ras postop). Figures in brackets represent the individual time of follow-up, except * = time to relapse. n. c. = non conclusive.*

Patient	Age	Sex	Tumor-type	Stage	k-ras tumor	k-ras preop	k-ras postop	Relapse	(months)
1	69	female	rectal	III	+	-	-	liver	(19)*
2	67	female	left-sided	II	-	-	-	no	(52)
3	62	male	rectal	I	-	-	-	no	(50)
4	75	female	right-sided	I	-	-	-	no	(47)
5	49	female	right-sided	III	+	-	-	liver during surgery	
6	81	female	right-sided	II	+	-	n.c.	no	(41)
7	79	male	right-sided	II	+	n.c.	-	no	(49)
8	87	male	rectal	III	-	-	-	carcinosis	(12)*
9	57	male	rectal	III	-	-	-	no	(49)
10	87	male	left-sided	III	+	n.c.	-	no	(12)
11	90	female	right-sided	III	+	+	+	lymphatic	(7)*
12	72	female	left-sided	III	-	-	-	liver	(10)*
13	68	female	left-sided	III	-	-	-	no	(47)
14	79	female	right-sided	II	+	+	+	local	(12)*
15	72	female	rectal	III	+	+	+	liver	(6)*
16	78	female	left-sided	I	+	n.c.	+	no	(41)
17	71	male	rectal	III	+	-	-	pulm	(29)*
18	71	male	right-sided	II	+	+	+	liver	(7)*
19	71	female	rectal	II	+	+	+	no	(48)
20	87	male	rectal	III	+	+	-	no	(6)
21	77	female	right-sided	III	+	+	+	no	(47)
22	79	male	rectal	III	-	-	-	no	(18)
23	51	female	right-sided	II	+	+	+	no	(46)
24	85	female	right-sided	III	-	-	-	no	(12)
25	57	female	left-sided	I	+	+	-	no	(48)

distilled water, filled up to 10 μl. Prior to screening of the amplified k-ras samples, perpendicular TGGE made it possible to identify the different alleles by their individual melting behavior directing at which temperature the different DNA strands would separate. The screening of the multiple samples was done on plates with pre-fixed slots for eight and twelve samples using parallel TGGE. Examples of the perpendicular and parallel gel electrophoresis are shown in Figures 1 and 2. DNA extracted from SW480 cells, expressing k-ras mutations in codon 12 and 13 (21), was used as positive control (Figure 2).

Results

The outcome of the k-ras analysis in the tumor (from invasive cancer cells) and in plasma pre- and postoperatively is shown in Table I. There was no significant correlation between relapse of disease (38 months, mean) during follow-up and the presence of k-ras mutations in the tumor ($p=0.40$) or in plasma ($p=0.99$) postoperatively on day three. The patients did not differ in their survival compared to the expected survival due to tumor stage.

Sixteen/25 (64%) of the patients were found to harbour k-ras mutations within tissue areas of invasive cancer cells in the tumor. Fifty-six% (9/16) of these patients had mutant k-ras in plasma preoperatively. On day three after surgery, 50% (8/16) still expressed mutant k-ras in plasma. One of the patients (patient no. 25) did not exhibit k-ras mutations on day three, despite a positive plasma sample preoperatively. In one patient (no. 16), the analysis was inconclusive in plasma preoperatively and positive for mutant k-ras postoperatively.

In patient no. 5, metastases in the liver were revealed during surgery. Mutant k-ras was not detected either within the tumor or in plasma in this patient.

None of the CRC patients that were k-ras-negative in the tumor exhibited mutant k-ras in plasma pre- or postoperatively.

Discussion

In the present study, we found that the detection rate of the mutant k-ras gene in the circulation preoperatively in plasma is similar to that seen on day three postoperatively. Thus, patients with CRC and a detectable k-ras mutation in the circulation preoperatively undergoing putative radical surgery persist in having a positive plasma analysis in the short-term period postoperatively. We also found that, among patients with k-ras-positive CRC tumors, a

concomitant plasma k-ras mutation does not seem to affect the outcome of the disease. Furthermore, mutant k-ras in plasma in the short-term period does not relate to a higher risk of relapse.

In the early 70s, using quantification by radioimmunoassay of free circulating DNA, cancer patients were shown to have higher levels of plasma and serum DNA compared to patients with benign disease (22) . It was later demonstrated that patients with metastatic disease tended to have even higher levels of serum DNA (1, 2). Further studies established that specific genetic changes seen in primary tumors could also be detected in the serum or plasma of patients with solid tumors. This, in turn, led to the anticipation that any somatic genetic alteration in a primary tumor could serve as a potential target for molecular detection in the serum or plasma (23). The k-ras oncogene, which is mutated in approximately 30-60% of adenocarcinomas of the colon (4, 6, 8-17, 24), is one of the most commonly discussed targets to monitor disease recurrence or to establish disease-free status. Genetically altered circulating DNA could also be a candidate marker for early detection in high-risk patients (*i.e.* those with hereditary disease) or even for screening of larger populations.

Circulating free DNA harbors identical genetic alterations to those seen in the primary tumors (3-6, 8, 9, 11, 24, 25). It has been suggested that persistent k-ras in plasma might indicate a higher risk of developing metastases. We found no correlation between the prevalence of postoperative persistence of mutant k-ras in plasma and disease recurrence within this small group of patients.

The sensitivity of the plasma DNA analysis in relation to positive tumors was low in this study compared to similar reports. On the other hand, specificity with regard to the fact that none of the patients with negative tumors were found to have mutant k-ras in the circulation was high. We used TGGE for the detection of k-ras mutation since it is considered to be a sensitive method to detect point mutations such as k-ras; theoretically, one mutated cell among ten thousand normal cells can be detected and false-positive results are few (20, 26). The choice between using plasma or serum is still a matter of discussion. Serum samples generally generate higher DNA yields, but may contain lower portions of tumor-derived DNA when compared to the plasma (27). The continuous development of methodologies that enable a more reliable detection level can be expected with time. Quantitative PCR, prolonged surveillance of circulating DNA postoperatively, and high-throughput assays are important steps in the evaluation of plasma-DNA as a clinical tool. However, this also requires a characterization of the physiology of circulating nucleic acids, and factors influencing their clearance rate in malignancy. We found that 8/9 patients, all radically treated, still displayed tumor-derived mutated k-ras on day three in plasma. This finding is somewhat surprising since the half-life and elimination of nucleic acids in the circulation has been considered to be much shorter, in fact in some studies it was suggested to be less than 24 hours (28).

In CRC, only 30-60% of all patients have identifiable mutations of the k-ras oncogene intratumorally. In the present study, in total approximately 1/3 of the patients were found to harbor k-ras mutations in plasma. Even though k-ras is readily extractable and possible to analyze with relatively few hot-spots, it is not by itself a sufficient marker for colorectal cancer, neither as a screening method nor for surveillance. Therefore a set-up of genetic, and perhaps epigenetic, markers in combination must be further evaluated.

In conclusion, tumor-specific circulating DNA in plasma persists after radical surgery for CRC during the short-term postoperative period. This does not seem to influence the risk of relapse of the disease. Further studies using quantitative PCR and prolonged surveillance postoperatively are important steps in the evaluation of plasma-DNA as a clinical tool.

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