Detection of K-ras Point Mutations in Mesenteric Venous Blood from Colorectal Cancer Patients by Enriched Polymerase Chain Reaction and Single-strand Conformation Polymorphism Analysis

Shin Fujita¹, Kokichi Sugano², Noriko Fukayama², Yoshihiro Moriya¹, Kenichi Sugihara¹ and Takayuki Akasu¹

¹Department of Surgery and ²Clinical Laboratory Division, National Cancer Center Hospital, Tokyo

In order to confirm the presence of cancer cells in mesenteric venous blood and to examine their relationship with the occurrence of liver metastases, we attempted to detect K-ras codon 12 point mutations in perioperative mesenteric blood using enriched polymerase chain reaction and single-strand conformation polymorphism (PCR-SSCP) analysis in 25 patients with primary colorectal tumors carrying K-ras point mutations. Among these patients, three with synchronous liver metastases were included. The same K-ras point mutation (substitution of GAT for GGT) was detected in both the blood and the primary tumor in a Dukes' C patient. We confirmed this result by colony hybridization and estimated the tumor-to-normal cell ratio to be 1 : 400. This patient has no liver metastases two years after surgery and her carcinoembryonic antigen (CEA) level remains normal. We demonstrated that considerable numbers of cancer cells can be found in mesenteric venous blood during colorectal cancer surgery. However, their potential role in the formation of liver metastases remains unclear.

(Jpn J Clin Oncol 26: 417-421, 1996)

Key words: K-ras—Mesenteric venous blood—Polymerase chain reaction—Single-strand conformation polymorphism—Colorectal cancer

Introduction

The development of liver metastases from colorectal cancer is a multistep process involving the intravasation and circulation of cancer cells preceding their entrapment by, invasion into and progressive growth in the liver. Therefore, the presence of cancer cells in mesenteric venous blood may be used to predict patients at risk of liver metastases. Colorectal cancer cells have been detected in peripheral and mesenteric venous blood using conventional cytology and immunohistochemical methods.¹⁻³ Their incidence, however, varies and the prognostic significance of this phenomenon is unclear.²¹ These studies have highlighted the difficulty in confirming the presence of cancer cells by these methods, suggesting that new identification techniques are necessary. Two recent studies have shown that occult cancer cells can be detected in the regional lymph nodes of, or bone marrow aspirates from colorectal cancer patients using the polymerase chain reaction (PCR).⁴⁻⁵ These methods involve the identification of DNA mutations or cancer-specific mRNA. Reverse transcriptase-PCR (RT-PCR), used to detect overexpression of mRNA in tumors, is a more sensitive method than that used to detect somatic mutations in DNA. However, identification of the same DNA mutation in both mesenteric venous blood and the primary tumor would better confirm the existence of blood-borne cancer cells than detection of overexpressed tumor mRNA by RT-PCR.

In order to confirm the presence of cancer cells in mesenteric venous blood in colorectal cancer, we attempted to detect K-ras codon 12 point mutations in perioperative mesenteric blood using enriched PCR and single-strand conformation polymorphism (SSCP) analysis in 25 patients with primary tumors carrying K-ras point mutations. When a K-ras point mutation was detected in mesenteric blood, it was confirmed to be the same as that of the primary tumor and the ratio of tumor to normal cells in

Received: May 10, 1996
Accepted: June 28, 1996
For reprints and all correspondence: Shin Fujita, Department of Surgery, National Cancer Center Hospital, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104
the blood was estimated by colony hybridization of subcloned PCR products, using oligonucleotide probes specific for either the mutant or wild-type K-ras oncogene. We also examined the relationship between the presence of cancer cells in mesenteric venous blood and the development of liver metastases.

Materials and Methods

Tissue and Blood Samples

Samples of tumor tissue and normal mucosa (10 cm distant from the tumor) were obtained from 60 patients with colorectal cancer immediately after surgery at the National Cancer Center Hospital. Mesenteric venous blood samples were also taken from the mesenteric vein draining the tumor after tumor mobilization. Peripheral blood samples were also taken from an artery during surgery. The blood samples were heparinized and 10 ml of blood from each patient was centrifuged at 10000 x g for 5 min. Cell pellets were washed and centrifuged twice at 700 x g for 5 min in 40 ml 0.2% NaCl solution. The tissues and blood cell pellets were frozen and stored at -80°C until DNA extraction.

Detection of K-ras Point Mutations in Tumor Samples by PCR-SSCP

DNA was extracted from the tissues by a method described previously. One microgram of DNA was used as a template and PCR was performed according to methods described elsewhere. PCR yielded a 108-base-pair DNA fragment spanning exon 1 of the K-ras oncogene. Primer sequences were: 5’GACTGAATATAAACTTG3’ (forward primer) and 5’CTATTGTTGGATCATATTCG3’ (reverse primer). Aliquots of the PCR products of the primary tumors were analyzed by SSCP. Briefly, 2-μl aliquots of the PCR products were mixed with 10-μl of a loading buffer consisting of 90% deionized formamide, 20 mM EDTA and 0.05% bromophenol blue and xylene cyanol. After denaturation at 80°C for 5 min, a 10-μl sample was electrophoresed on 12% polyacrylamide gel at 200 V for 2 h at 18°C. The gels were silver-stained using a silver staining kit (Daiichi Pure Chemical Co., Tokyo).

Detection of a K-ras Point Mutation in Mesenteric Venous Blood by Enriched PCR-SSCP

Mesenteric venous blood DNA was amplified by PCR as before. PCR yielded a 157-base-pair DNA fragment spanning exon 1 of the K-ras oncogene. Primer sequences were: 5’ACTGAATATAAACTTGTTGGGACCT3’ (forward primer) and 5’TCAAAGAATGGTCCTGGACC3’ (reverse primer). Aliquots of the PCR product of mesenteric venous blood DNA were digested with BstNI (New England Biolab, Beverly, MA) at 60°C overnight. After boiling, the mixture was subjected to a second PCR using a different reverse primer, the sequence of which was 5’TAATATGTCGACTAAAA CAAGATTTACCTC3’. The amplified fragments were subjected to SSCP, for which the electrophoresis conditions were 15% polyacrylamide gel at 200 V and 18°C for 4 h.

Colony Hybridization

Mesenteric venous blood DNA was amplified by PCR using the same primers as those used for tumor DNA amplification. The 108-base-pair PCR product containing K-ras codon 12 was cloned into the pUC18 plasmid vector (Pharmacia, Uppsala, Sweden) and JM109 competent cells (Toyobo, Osaka) were transformed by this plasmid according to the manufacturer’s instructions. The transformed cells were plated onto four LB agar (Wako Pure Chemical Co., Osaka) plates, transferred to Hybond-N membranes (Amersham, Buckinghamshire, England).
CANCER CELLS IN MESENTERIC VENOUS BLOOD

Fig. 2. Detection of a K-ras point mutation by colony hybridization. After PCR, amplified DNA fragments were subcloned into the pUC18 vector and used to transform JM109 cells. The transformants were transferred to Hybond-N membranes and hybridized with oligonucleotide probes specific for GGT (A) and GAT (B), because the primary tumor in this patient contained a GGT to GAT point mutation. On this membrane, 433 colonies were hybridized with the normal probe while one colony was hybridized with the mutated probe. Four membranes were hybridized in total, and 1616 and 4 colonies were hybridized with the normal and mutated probes, respectively. In this patient, the tumor-to-normal cell ratio in mesenteric venous blood DNA was 1 : 400.

UK) and hybridized with oligonucleotide probes specific for wild-type or mutant K-ras by a method described previously. After hybridization, the membranes were washed, dried and exposed to x-ray film. The number of hybridized colonies on each membrane was counted manually.

Results

K-ras point mutations at codon 12 were detected in 25 (42%) of 60 primary tumors. In these 25 patients including three patients with synchronous liver metastases, K-ras point mutations in mesenteric venous blood DNA were sought using enriched PCR-SSCP. A K-ras point mutation was detected in one of the 25 patients (4%) (Fig. 1). This patient was a 72-year-old woman with Dukes’ C tumor, and histological examination revealed moderately differentiated adenocarcinoma with vascular invasion. The remaining 24 patients had negative results. One was classified as Dukes’ A, nine as B, 11 as C and three as D.

In order to confirm the K-ras point mutation in mesenteric venous blood DNA and estimate the ratio of tumor to normal cells, colony hybridization was performed. Because the K-ras point mutation in this patient was GGT to GAT, oligonucleotide probes specific for GGT and GAT were used, with CGT as a negative control. In total, 1616 colonies from PCR products amplified from mesenteric venous blood DNA were hybridized with the oligonucleotide probe for GGT while 4 colonies were hybridized with the oligonucleotide for GAT (Fig. 2). No colony was hybridized with the oligonucleotide probe for CGT (data not shown). These results demonstrated the presence of the same point mutation in both mesenteric venous blood and primary tumor DNA. In this patient, the tumor-to-normal cell ratio was 1 : 400. This patient has no liver metastases two years after surgery, and the carcinoembryonic antigen (CEA) level decreased from an abnormal to a normal value after surgery; it currently remains within the normal range.

Discussion

A K-ras point mutation was detected in DNA from perioperative mesenteric venous blood in 1 of 25 colorectal cancer patients using enriched PCR-SSCP analysis, and was confirmed by colony hybridization. These methods are more accurate in confirming the presence of cancer cells than conventional cytological and immunohistochemical methods. The results verified that cancer cells existed in mesenteric venous blood during resection of colorectal cancer.

Enriched PCR-SSCP has been reported to be capable of detecting 0.05% mutant K-ras in the second PCR product. A further study showed that the sensitivity of enriched PCR for detecting K-ras point mutations was 0.01%. Using cytological and immunohistochemical methods, Leather et al.
showed that tumor-to-normal cell ratios in mesenteric venous blood ranged from 1:78746 to 26:520,000. If tumor cells do exist in this ratio, K-ras point mutations would, in some cases, be undetectable by our method. This may be one explanation for the lower detection rate of cancer cells by our method than those reported previously, ranging from 12% to 57%.1-3 However, detection rates of cancer cells in peripheral and mesenteric venous blood by conventional cytology and immunohistochemical methods have varied, partly because these methods are unable to definitely confirm that the atypical cells in the blood are cancer cells. Thus, in some cases, cells detected by these conventional methods have been considered to be false positives. This is thought to explain the lack of a clear correlation between the presence of atypical cells in blood and prognosis.

The existence of cancer cells in mesenteric venous blood during surgery is thought to be a risk factor for liver metastases. In order to reduce perioperative dissemination, a no-touch isolation technique has been advocated. Turnbull et al. demonstrated that the no-touch isolation technique improved the survival rate among colorectal cancer patients. Wiggers et al. also showed a tendency for reduction in the number of liver metastases when the no-touch isolation technique was used. However, this reduction was not significant and the role of this technique in improving survival rates is still controversial. In our study, the tumor-to-normal cell ratio in the mesenteric venous blood DNA of the patient with a K-ras point mutation was 1:400. In spite of this high tumor-to-normal cell ratio, this patient has no liver metastases two years after surgery. Thus, other factors including p53 and DCC alterations may be more important in the development of liver metastases than the existence of cancer cells in mesenteric venous blood.

The viability of cancer cells in mesenteric venous blood is unclear, because DNA point mutation from non-viable cells can also be detected by the enriched PCR-SSCP method. However, in patients with advanced breast cancer, cancer cells have been detected in peripheral blood stem cell collections by immunohistochemistry, and tumor colony formation has been observed in cell cultures. Thus, cancer cells are thought to be viable in blood.

No K-ras point mutations were detected even in three patients with synchronous liver metastases. This probably reflects the fact that single point sampling was carried out during surgery and also that the sensitivity of our method was low. Sampling error is likely to occur with single point sampling, since cancer cells are thought to be released intermittently from the primary site. Therefore, the false negative rate is thought to be high with this method.

RT-PCR is a more sensitive method than the one we employed. Gerhald et al. detected CEA mRNA in bone marrow from colorectal cancer patients by RT-PCR even when only one abnormal cell was present among 10^7 normal cells. This method will therefore be useful for this kind of determination. Circulating malignant cells have also been detected using RT-PCR methods in melanoma, prostate cancer, breast cancer and hepatocellular carcinoma. However, the expression of the genes examined by these methods is not specific to cancer cells and the prognostic usefulness of this method for prediction of cancer metastases is still unclear.

References


oncogene codon 12 mutations by nested PCR using mismatched primers and selective digestion of nonmutated PCR fragments with restriction enzyme. Rinsho Byori 41: 1017-1023, 1993 (in Japanese)


