Detection of K-ras mutations in the plasma DNA of pancreatic cancer patients

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Background. In pancreatic cancers, K-ras mutations have been found frequently (80%–100%), and they could be a good marker to detect tumor DNA in the plasma. Several studies have indicated that polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) analysis of K-ras mutation was a useful method for the detection of hepatic and lymph node metastasis of pancreatic cancer. However, this method sometimes exhibited false-positive results, and the rate of K-ras mutation might thus be overestimated in these tissues. To diagnose pancreatic cancer correctly at an early stage, we attempted to detect tumor DNA in the plasma of pancreatic cancer patients using a more sensitive and specific method. Methods. We examined 28 pancreatic cancer patients using a sensitive mutation-specific mismatch ligation assay for K-ras gene mutations in primary tumors and paired plasma samples. Results. K-ras gene mutations were detected in 26 of the 28 (93%) pancreatic cancers. We also found the same mutations in 9 of these 26 (35%) patients in their plasma DNA. This mutation was found even in the plasma of patients with TNM stage II cancer. Conclusions. Genetic alterations present in the tumors of pancreatic cancer patients can be detected in their plasma, and this approach is potentially applicable for cancer screening and the monitoring of this deadly disease.

Key words: K-ras, pancreatic cancer, mismatch ligation assay

Introduction

Pancreatic cancer is regarded as one of the most aggressive cancers throughout the world.1 Despite the recent advances in diagnosis and treatment, patients with this devastating disease have shown a very poor prognosis.2–4 Fully 50% or more of pancreatic cancers are too far advanced and nonresectable because they are rarely diagnosed at an early stage in affected patients.5 Therefore, methods to detect surgically resectable tumors could significantly reduce deaths from this aggressive disease.

It is now clear that accumulated genetic alterations in dominant oncogenes, such as K-ras, and tumor-suppressor genes, such as p53, p16, and DPC4, are involved in the pathogenesis of pancreatic cancer.6–9 The identification of these genetic changes at sites away from the primary tumor may help to assess the extent of disease and overall tumor burden at the time of initial diagnosis. Previous studies have proposed that tumor DNA is released into the circulation and is enriched in plasma and serum.10,11 Based on these observations, studies have shown that it is possible to detect tumor-specific DNA in the serum of colorectal cancer patients using a mismatch ligation assay for K-ras mutation.12 In pancreatic cancers, K-ras mutations have been found frequently (80%–100%), and they could be a good marker to detect tumor DNA in the plasma.13,14 Several studies have indicated that polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) analysis of K-ras mutation was a useful method for the detection of hepatic and lymph node metastasis of pancreatic cancer.15,16 However, this method sometimes exhibited false-positive results, and the rate of K-ras mutation might thus be overestimated in these tissues.

In the present study, we examined 28 pancreatic cancer patients, using a sensitive mutation-specific mismatch ligation assay for K-ras gene mutations in primary tumors and paired plasma samples. Of the 26 patients with K-ras gene mutations in the tumors, 9 exhibited the same mutation in the matched plasma DNA. This result indicates that genetic alterations present in the tumors of pancreatic cancer patients can
be detected in their plasma, and that this approach is potentially applicable for cancer screening and the monitoring of this deadly disease.

### Patients, materials, and methods

#### Sample collection and DNA preparation

Twenty-eight primary tumors and corresponding normal tissues were prepared from microdissected sections of formalin-fixed, paraffin-embedded tissues obtained from surgical resections in pancreatic cancer patients. Plasma samples were collected from the same patients two times, 1 week before and 3 weeks after surgery, and stored at −80°C. Normal, tumor, and plasma DNAs were prepared as described previously.

#### Mismatch ligation assay

The mismatch ligation assay was performed as described previously. Briefly, the first exon of K-ras was amplified and employed as the template for the mismatch ligation assay in all tumor samples. For each ligation assay, 50 μg of PCR product was mixed with 8 μg each of three mutation-specific oligomers, 100 ng of blocking oligomer, and 8 μg of a common 32P-labeled oligomer in 20 μl of reaction mixture containing 150 mM NaCl, 10 mM MgCl2, 100 mM Tris-HCl (pH 7.5), 1 mM spermidine, 1 mM dithiothreitol (DTT), 1 mM ATP, and 3 μg of T4 gene 32 protein (Boehringer Mannheim, Mannheim, Germany). This mixture was denatured at 95°C for 5 min and allowed to cool at room temperature for 15 min, at which time 1 unit of T4 ligase was added. The ligation was carried out at 37°C for 1 h and was terminated by heat inactivation at 68°C for 10 min. The [32P]phosphate on the unligated oligomer was removed by the addition of 1 unit of alkaline phosphatase and subsequent incubation at 37°C for 30 min. The ligation products were separated on 12% denaturing polyacrylamide gel. The presence and the nature of mutations were determined based on the relative migration of the ligation products formed in control experiments using templates with known K-ras mutations. Oligonucleotide sequences used for the ligation assays were: 12b-Ala, 5'-TGGAGCTGC-3'; 12b-Asp, 5'-TTGGAGCTGA-3'; 12b-Val, 5'-GTGGAGCTGT-3', and 12b-common, 5'-TGCGTAGG-3'. Elevenmers spanning the ligation junction, used as a blocking oligomer, was 5'-TGTTGCGTAG-3' (12N). When a plasma sample was found to be positive, the DNA extraction and PCR reaction were repeated at least once in aliquots of the original plasma samples to confirm the presence of the mutant allele.

#### Results

We first examined K-ras gene status in the tumors, using the mismatch ligation assay. K-ras gene mutations were detected in 26 of the 28 (93%) pancreatic cancers. This result confirmed that most pancreatic cancers had K-ras gene mutations, which may have an important role in the tumorigenic pathway of pancreatic cancer, as previously described. The result also indicated that K-ras mutation might be a good marker to detect pancreatic cancer DNA in plasma because of the high mutation rate in tumors. Subsequently, we tested for the presence of K-ras gene mutation in the paired plasma samples of the above 26 patients (obtained before surgery), and found the same mutations in 9 of the 26 (35%) patients' plasma DNA (Fig. 1). As a control study, we screened for K-ras mutation in the corresponding normal DNA of the 28 patients with pancreatic cancers, using the mismatch ligation assay. No mutation was found in the normal DNA.

We next examined K-ras status in the plasma DNA of the above 26 patients in the plasma samples that had been obtained after surgery. Four of the 9 patients with K-ras gene mutations in their plasma before surgery (44%) had the same mutation in their plasma after surgery; the other 22 of the above 26 patients exhibited no K-ras gene mutations in their plasma after surgery; (Table 1). All 9 patients underwent curative surgical operation, and there were no residual tumors macroscopically. This result, however, indicated the possibility that pancreatic cancers still existed microscopically in the 4 patients who showed K-ras mutations in plasma after surgery.

After completion of the mismatch ligation assay in all specimens, clinicopathological data were correlated...