Telomerase activity, P53 mutation and Ki-ras codon 12 point mutation of the peripheral blood in patients with hepato pancreato biliary diseases

Koji Yamaguchi, Kazuo Chijiiwa, Nobuhiro Torata, Moritoshi Kinoshita, Masao Tanaka

1Department of Surgery and Oncology, Graduate School of Medical Sciences, Fukuoka 812-8582, Japan; 2Gene-Diagnostic Center, Otsuka Assay Laboratories, Otsuka Pharmaceutical Co. Ltd, Tokushima 771-0195, Japan

Background
With progress in molecular biology, the presence of telomerase activity, P53 mutation and Ki-ras codon 12 point mutation has been reported in malignant tumours of the liver, pancreas and biliary tree. The purpose of this paper is to clarify the clinical implications of finding these three biomarkers in the peripheral blood of affected patients.

Methods
Telomerase activity, P53 mutation, and Ki-ras codon 12 point mutation in the peripheral blood were examined among 86 patients with hepato pancreato biliary disease, both benign and malignant, and the results were compared with clinical findings.

Results
Of 20 patients with benign conditions, only one patient with intraductal papillary adenoma showing severe dysplasia exhibited a biomarker (telomerase activity) in the peripheral blood. In total, there were 66 patients with various HPB carcinomas. Of 56 cancer patients studied preoperatively, 16 were positive for more than one biomarker, 13 were positive for telomerase activity, 4 for P53 mutation (three at exon 7 and another at exon 8), and 2 for Ki-ras codon 12 point mutation (both in the second letter). Twelve of the 16 biomarker-positive patients had stage IV disease as opposed to 23 of 40 biomarker-negative patients. The resectability rate of the cancer was 38% in positive patients and 50% in negative patients. The one-year survival rate after resection was zero in positive patients and 15% in negative patients, but the difference was not significant (P = 0.65). Of 32 patients with liver metastasis at the time of the molecular examination, eight were positive and 24 negative. Of 34 patients without liver metastasis, nine were positive and 25 negative. The development of subsequent liver metastases in those without them at the start was not significantly different in those with and without biomarkers (56 vs 36%: P = 0.31).

Conclusions
The three novel biomarkers of the peripheral blood seemed to be of little value for screening of early malignant HPB neoplasms but may help to predict liver metastasis.

Keywords
P53 mutation, Ki-ras codon 12 point mutation, telomerase activity, peripheral blood

Introduction
With the recent advances in molecular biology, new biomarkers of cancer have been reported, including telomerase activity, Ki-ras codon 12 point mutation and P53 mutation. Micrometastasis or circulating cancer cells have been suggested in patients with carcinoma of the colon, breast, pancreas and other organs using these biomarkers. The ras gene family encodes 21-kDa membrane-bound proteins involved in signal transduction. The mutation in Ki-ras oncogene that leads to the activation of GTP/GDP binding protein (RAS p21) involves the 12th codon. These mutations almost exclusively involve a single base pair substitution, which produces an amino acid substitution from glycine to aspartic acid, valine, or arginine. Ki-ras codon 12 point mutation is frequently seen in carcinoma of the gallbladder [1], bile duct [1], intrahepatic duct [2] and pancreas [3]. The P53 gene is located at 17p 13.3 and is a common tumour suppressor gene seen in human carcinomas. This gene is frequently mutated in a variety of human cancers, the incidence being 50% for pancreatic carcinoma [4–6] and 30% for gallbladder carcinoma [7]. The presence of mutations is detected in exon 5 through 8, which occupies
86% of all mutations reported for the P53 gene [5]. Telomerase is an enzyme that contains an RNA template complementary to (GGTTAG) repeats and is believed to be involved in the de novo synthesis of GGTTAG telomeric DNA onto chromosomal ends. Telomerase is important to maintain continuous cell proliferation, by overcoming the end-replication problem, and the activity is seen in more than 80% of various malignant neoplasms, including 95% of pancreatic carcinomas [8] and about 85% of hepatocellular carcinomas [9].

The materials used in the vast majority of such papers are tumour tissues, while minimally invasive or non-invasive sampling of urine, stool or blood is more important in the clinical fields. Very few studies have employed a combination biomarker assay. The present study comprised a combination analysis of telomerase activity, Ki-ras codon 12 point mutation and P53 mutation of the peripheral blood in patients with HPB diseases, both benign and malignant, to determine the clinical implications of these three biomarkers.

**Materials and methods**

**Tissue samples and DNA extraction**

The series included 86 Japanese patients with various HPB diseases treated in the Department of Surgery I, Kyushu University Faculty of Medicine, Fukuoka, Japan, between March 1994 and May 1998; three hepatolithiasis, five hepatocellular carcinoma, five cholangiocarcinoma, three metastatic liver cancer, one cholecholangiocarcinoma, 12 bile duct carcinoma, 13 gallbladder carcinoma, two ampullary carcinoma, six pancreatitis, eight intraductal papillary mucinous adenoma, two islet cell adenoma and 26 pancreatic carcinoma. When more than one molecular marker was positive, the patient was regarded as biomarker-positive, and the others as biomarker-negative.

The diagnosis of malignancy was obtained by cytology and/or histopathology. Fifty-five patients underwent surgical resection for HPB malignancy. The clinical follow-up after the surgical resection was updated as of September 1998.

The blood samples were stored with an RNAase inhibitor at −4°C. Genomic DNA was extracted by proteinase K digestion and phenol/chloroform extraction using the method of Sambrook et al. [11] for the Ki-ras codon 12 point mutation assay and P53 mutation examination. Duplicate assays were performed.

**Primers for Ki-ras codon 12 point mutation and P53 mutation**

In the examination of Ki-ras codon 12 point mutation, we used a mixture of three synthetic oligonucleotides which corresponded to the possible variants of the first nucleotide or three oligonucleotides which corresponded to the three variants of the second nucleotide of codon 12 of the Ki-ras gene for one of the primers for polymerase chain reaction (PCR). Primers used for PCR were synthesized in an ABI DNA synthesizer model 392 (Applied Biosystems, Foster City, CA, USA). The two sets of primers are as follows: set 1: 5’>ACTTGTGGTAGTTGGAGCTC, 5’>ACTTGTGGTAGTTGGAGCTT, 5’>ACTTGTGGTAGTTGGAGCTA and set 2: 5’>CTTGTGGTAGTTGGAGCTG, 5’>CTTGTGGTAGTTGGAGCTG and 5’>CTTGTGGTAGTTGGAGCTG.

In the analysis of P53 mutation, oligonucleotides were synthesized as primers for PCR based on the published P53 gene sequence for each region flanking the intron/exon 5 to 8 [12]. The designations and sequences for each primer were as follows:

- EX-05f, 5’-TCTGTCTCCTTCTCTCTCTC-3’
- EX-05r, 5’-TCTGCAGCCCCACGTGC-3’
- EX-06f, 5’-TGTAGTCTCAGTTGCTC-3’
- EX-06r, 5’-GAGACCCAGGGCTG-3’
- EX-07f, 5’-TCTGTGGCTGTGTATCTC-3’
- EX-07r, 5’-AGGGTGGCAAGTGGCTC-3’
- EX-08f, 5’-GCTTCTCTTCTCTCTCTGA-3’
- EX-08r, 5’-CGCTTCTGGTCTCTGCTGC-3’

The number in each designation indicates the exon of the P53 gene subjected to examination by polymerase chain reaction PCR-fluorescence-based single-strand-conformation-polymorphism (PCR-FSSCP) analysis. ‘f’ and ‘r’ indicate forward and reverse primers for each region. All the primers were labelled at their 5’ ends with fluorescein derivatives by the Fluore prime method (Pharmacia Biotech Co. Ltd, Tokyo, Japan).
Polymerase chain reaction for Ki-ras codon 12 point mutation and P53 mutation

Ki-ras codon 12 point mutation

PCR was performed in 40 cycles of 0.5 min at 95°C, 2 min at 53°C, and 2 min at 70°C, as described by Baker et al. [13]. Mutant-allele-specific amplification (MASA) was performed in 32 cycles of 0.5 min at 95°C, 2 min at 59°C or 60°C, and 2 min at 70°C, by the modified Takeda’s method [14] except for the absence of dimethyl sulfoxide (DMSO). PCR products (5 µl) were electrophoresed in a 3% agarose gel containing 0.5 µg/ml ethidium bromide. Ethidium-bromide staining of PCR products was performed for detection of point mutations in the Ki-ras gene and was visualised under ultra-violet light. The mutant allele specific amplification method employed could detect 5 cancer cells among 10⁸ normal lymphocytes [14].

PCR-FSSCP analysis for P53 mutation

One hundred ng of genomic DNA was amplified in a total volume of 50 µl in the buffer containing 100 µM concentrations of dATP, dCTP, dGTP and dTTP, and 0.125 units of AmpliTaQ (a kind of Taq DNA polymerase) in the buffer recommended for the enzyme, using primers labelled at their 5' ends with fluorescein. The PCR products were diluted 50 times by a stop solution (Pharmacia Biotech Co. Ltd, Tokyo, Japan), heated at 95°C for 5 min and then placed on ice for 5 min. Then 4 µl of this solution was applied to each lane of FSSCP gel fitted to an ALF II automated DNA sequencer (Pharmacia Biotech Co. Ltd, Tokyo, Japan). The FSSCP gel used was 7% polyacrylamide gel containing 5% glycerol. Electrophoresis was performed at 30 W for 3 to 4 h, depending on the length of the amplification product. The temperature of the gel was kept at 30°C during the run. The gel was visualized with UV illumination. The mutant allele specific amplification method employed could detect 5 cancer cells among 10⁸ normal lymphocytes.

Statistical analysis

The distribution of patients was measured by the Chi-square test. Sensitivity, specificity, and positive and negative predictive values were calculated. One-year cumulative survival rate was calculated, and the difference between the survival rates was measured by log-rank test.

Results

Biomarkers in the peripheral blood

Among 10 patients with benign inflammatory diseases, all three biomarkers were negative (Table 1). Among 10 patients with a benign neoplasm, only one with intraductal papillary adenoma and severe dysplasia showed telomerase activity in the peripheral blood.

Of 56 preoperative patients with HPB carcinomas, 16 patients were positive for more than one biomarker in the peripheral blood; 13/51 showed telomerase activity (Figure 1), 4/55 showed a P53 mutation (three at exon 7 and another at exon 8) (Figure 2), and 2/49 showed a Ki-ras codon 12 point mutation (both in the second letter) (Figure 3). Telomerase activity was positive in the blood of 3/10 patients with liver cancer, 4/20 with bile duct cancer and 6/21 with pancreatic cancer. P53 mutation was present in 2/12 patients with liver cancer, 0/20 with bile duct cancer, and 2/23 with pancreatic cancer. Ki-ras codon 12 point mutation was evident in 0/11 with liver cancer, 1/18 with biliary tract cancer and 1/21 with pancreatic cancer. In 47 of the 86 patients, both the tissue samples and peripheral blood were examined for the biomarkers (Table 2). In 15 of these 47, biomarkers were positive in tumour tissue sample, and in 8 of them biomarkers were positive in peripheral blood.
blood. In only two patients were biomarkers positive both in the tumour tissue and the blood; K-ras codon 12 point mutation was positive in the tumour tissue and telomerase activity in the blood.

Of the 56 preoperative patients with HPB cancer, the 16 who were biomarker-positive, included two in stage I, two in stage II and 12 in stage IV (Table 3). The other 40

<table>
<thead>
<tr>
<th>Liver</th>
<th>Telomerase activity</th>
<th>PS3 mutation</th>
<th>Ki-ras codon 12 point mutation</th>
<th>Molecular abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatolithiasis</td>
<td>–</td>
<td>0/3</td>
<td>0/1</td>
<td>0/3</td>
</tr>
<tr>
<td>HCC</td>
<td>1/5</td>
<td>2/5 (Exon 7)</td>
<td>0/5</td>
<td>2/5</td>
</tr>
<tr>
<td>CC</td>
<td>1/3</td>
<td>0/4</td>
<td>0/4</td>
<td>1/5</td>
</tr>
<tr>
<td>Metastatic cancer</td>
<td>1/2</td>
<td>0/3</td>
<td>0/2</td>
<td>1/3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biliary tract</th>
<th>Telomerase activity</th>
<th>PS3 mutation</th>
<th>Ki-ras codon 12 point mutation</th>
<th>Molecular abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choledocholithiasis</td>
<td>4/12 (1/2)</td>
<td>0/12 (0/2)</td>
<td>0/10 (0/1)</td>
<td>4/12 (1/2)</td>
</tr>
<tr>
<td>Bile duct cancer</td>
<td>1/13 (0/5)</td>
<td>0/13 (0/5)</td>
<td>1/11 (2nd letter) (0/5)</td>
<td>2/13 (0/5)</td>
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<tr>
<td>Gallbladder cancer</td>
<td>0/2</td>
<td>0/2</td>
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</table>

<table>
<thead>
<tr>
<th>Pancreas</th>
<th>Telomerase activity</th>
<th>PS3 mutation</th>
<th>Ki-ras codon 12 point mutation</th>
<th>Molecular abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatitis</td>
<td>0/5</td>
<td>0/6</td>
<td>0/3</td>
<td>0/6</td>
</tr>
<tr>
<td>Intraductal papillary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mucinous adenoma</td>
<td>1/7</td>
<td>0/8</td>
<td>0/7</td>
<td>1/8</td>
</tr>
<tr>
<td>Islet cell adenoma</td>
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<td>0/1</td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>6/24 (0/3)</td>
<td>2/26 (Exon 7,8) (0/3)</td>
<td>1/24 (2nd letter) (0/3)</td>
<td>7/26 (0/3)</td>
</tr>
</tbody>
</table>

HCC: hepatocellular carcinoma, CC: cholangio carcinoma. ( / ): patients after resection of HPB cancer

![Figure 1. Telomerase activity examined by the TRAP-eze™ kit M: size marker pUC19/MspI, Lane 1: bile duct carcinoma (negative), Lane 2: pancreatic carcinoma (positive), Lane 3: pancreatic carcinoma (negative), Lane 4: gallbladder carcinoma (negative), Lane 5: positive control, Lane 6: negative control, Lane 7: TSR8 control (0.1 amole), Lane 8: TSR8 control (0.2 amole). Band in lane 2, which is stronger than lane 7 (positive control) is judged as positive for telomerase activity. Bands in lanes 1, 3, 4, which are faintly present but weaker than lane 7, are judged as negative for telomerase activity.](image1.png)

![Figure 2. PS3 mutation (Exon 7). Pancreatic carcinoma (upper curve, positive). Upper curve shows peaks (arrows) which are not seen in the lower curve representing healthy controls (negative control) and indicate PS3 mutation (PCR-FSSCP). Pancreatitis (middle curve, negative).](image2.png)
who were biomarker-negative included one in stage I, four in stage II, 12 in stage III and 23 in stage IV. The positive biomarker in the peripheral blood of the two patients in stage I pancreatic cancer was telomerase activity. Resection was performed in 26 of the 56 cancer patients. The resectability rate in biomarker-positive patients was 38% (6/16) and in biomarker-negative patients 50% (20/40) (Table 4). The sensitivity of the biomarkers to non-resectability was 33%, specificity 77%, positive predictive value 63% and negative predictive value 50%.

The one-year survival rate after surgical resection in 20 biomarker-negative patients was better than that of seven biomarker-positive patients (15% vs 0%), but the difference was not statistically significant ($P = 0.65$).

### Biomarkers in the peripheral blood and liver metastasis

Of 10 patients studied after resection of HPB cancer, one (10%) was positive for telomerase activity but free from liver metastasis and the other nine biomarker-negative patients included eight with liver metastasis (Table 3). The absence of biomarkers indicated the presence of liver metastasis after resection, although the number of patients examined was small ($P = 0.0350$). Of the total 66 patients with HPB cancer, liver metastasis was present at the time of molecular examination in 32 and absent in 34. Among the 32 patients with liver metastasis at the time of the molecular examination, more than one biomarker was positive in eight (25%) (Table 5). Among the other 34 patients without liver metastasis at the time of the molecular examination, more than one biomarker was positive in nine (26%). The sensitivity of the biomarkers to liver metastasis at the time of molecular examination was 25%, specificity 69%, positive predictive value 47% and negative predictive value 51%. Of the 34 patients without liver metastasis at the time of molecular examination, 14 patients developed liver metastasis within six months of the examination and the other 20 did not. Five of the nine biomarker-positive patients without liver metastasis at the time of the examination subsequently developed liver metastasis, as opposed to 9 of 25 patients who were negative for biomarkers (Table 6). The sensitivity

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**Table 2.** Biomarkers in tumour tissue samples and peripheral blood

<table>
<thead>
<tr>
<th>Tumour tissue</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>13</td>
<td>26</td>
</tr>
</tbody>
</table>

Statistical analysis $P = 0.6451$

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**Table 3.** Comparison of AJCC staging before resection, liver metastasis after resection and biomarkers of the peripheral blood in 66 patients with HPB cancer

<table>
<thead>
<tr>
<th>Staging before resection (AJCC Stage)</th>
<th>Liver metastasis after resection</th>
<th>Liver metastasis after resection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

Statistic analysis $P = 0.1275$ $P = 0.0350$

Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; AJCC: American Joint Committee on Cancer.
of the biomarkers to the occurrence of liver metastasis within six months was 35%, specificity 80%, positive predictive value 56% and negative predictive value 64%.

**Discussion**

Telomerase activity, P53 mutation and Ki-ras codon 12 point mutation in the peripheral blood were examined to clarify the clinical implications of the three biomarkers in 86 patients with HPB diseases. Of 20 patients with benign conditions, only one (5%) with intraductal papillary adenoma and severe dysplasia showed a biomarker (telomerase activity). Of 56 preoperative patients with HPB carcinomas, 16 patients were positive for more than one biomarker. The biomarkers seemed to correlate with advanced stages of HPB carcinoma but not with the resectability rate or the one-year survival rate after surgical resection. Biomarkers were positive in 25% of patients with liver metastasis at the time of the molecular examination, while 56% of marker-positive patients without initial metastasis developed clinical liver metastasis within six months.

Some normal adult somatic tissues exhibit telomerase activity, including male germ cells, the basal layer of the epidermis, the proliferative zone of the intestine, activated lymphocytes and blood stem cells. In addition, telomerase activity is detectable in normal human peripheral blood...
leucocytes including T cells and B cells [15–17]. This evidence might suggest that telomerase activity in the peripheral blood produces a false positive result. However, the telomerase activity in these cells is extremely low, being about 1–2% of the activity found in cancer cells [18]. In the present study, positive telomerase activity from the activated peripheral lymphocytes was eliminated to the best of our ability. Nine patients with acute inflammatory diseases were negative for telomerase activity in peripheral blood (unpublished data). The TRAP-eze™ kit used in the present series contains both negative and positive controls, a control cell pellet of 10⁶ telomerase positive cells and a TRS8 control template, which give a telomerase ladder in the presence of Taq DNA polymerase and the TS and RP primers. We carefully evaluated the results comparing the bands obtained with the negative and positive control bands. Therefore, the false positivity of telomerase activity by the peripheral lymphocytes was eliminated as far as possible.

Although all the steps leading to cancer are not yet known, progression to a cancerous state requires accumulation of a series of genetic alterations. An emerging hypothesis suggests that the upregulation or re-expression of telomerase is critical to cancer cell growth. In contrast to normal cells, tumour cells show no net loss of the telomere length with cell division, suggesting that telomere stability may be required for cells to escape replicative senescence and proliferate indefinitely. Immortalisation may occur through mutation of a gene in the telomerase repression pathway, permitting the expression of telomerase and the maintenance of telomere stability in cancer cells. Thus it is becoming generally accepted that the upregulation or reactivation of telomerase activity may be a rate-limiting, if not critical, step required for the continuing proliferation of cancers. In the present study of the peripheral blood, telomerase activity was the most sensitive of the three biomarkers. The activity was evident in 16 of the 56 preoperative patients with HPB cancer but in only one of the 20 patients with benign conditions (29 vs 5%).

Interestingly, one patient with intraductal papillary adenoma showing severe dysplasia was positive for telomerase activity. This case might therefore be one of intraductal papillary adenocarcinoma. Telomerase activity in this patient might indicate cancer cells in the peripheral blood. At present, the patient is being followed up very carefully.

Several studies demonstrate a relationship between biomarkers and clinical outcome in certain cancers. The presence of telomerase activity has been shown to correlate with poor clinical outcome in gastric cancer [19]. High levels of enzyme correlate with poor clinical outcome in neuroblastoma, whereas patients with metastatic neuroblastoma without telomerase activity experience spontaneous regression of the tumours [20]. Clinical outcome and several prognostic indicators of breast cancer have been shown to have a statistically significant correlation with the level of telomerase activity [21]. High telomerase activity is detected frequently in primary non-small-cell lung cancers that exhibit high tumour cell proliferation rates and an advanced pathological stage [22]. The presence of P53 mutation correlated with poor prognosis in pancreatic carcinoma [23]. In the present series, the biomarkers were seen in more advanced HPB carcinomas, and there was a trend towards better survival after surgical resection in biomarker-negative patients.

The presence of biomarkers may indicate minimal residual disease in patients undergoing surgical resection, and their detection may be a useful surrogate end-marker for following chemotherapy. They are examined by highly sensitive molecular biology. It was reported that with the application of a mutant allele specific amplification method to examine cytokeratin, a single cancer cell could be detected among 10⁴ normal cells in lymph nodes. Telomerase activity is seen in 80–90% of human cancers and telomerase activity in the peripheral blood was studied to determine the development of liver metastasis after surgical resection. In the present series, five of nine biomarker-positive patients developed liver metastasis, as opposed to nine of 25 biomarker-negative patients. Thus biomarker positivity faintly predicted liver metastasis, albeit with low sensitivity and accuracy.

Clinical demands for biomarkers are to:

1) detect early malignant disease;
2) screen malignant disease by non-invasive or minimally invasive sampling;
3) predict biological behaviour; and
4) identify an important subgroup of patients who might benefit from post-operative adjuvant therapy.

Combination assays can be expected to compensate for low individual sensitivity of the biomarkers, but the combination of P53 mutation or Ki-ras point mutation with telomerase has been reported by few other authors. In the present series, the three biomarkers were positive in the peripheral blood in 26% of HPB carcinoma patients. The vast majority of biomarker-positive patients were positive for telomerase activity, and the patients with all three biomarkers...
were invariably in stage III or IV. Therefore, these three biomarkers were not useful for screening of early HPB cancer, but could have some value for prediction of liver metastasis.

In conclusion, telomerase activity was the most sensitive of the three biomarkers in the peripheral blood in the patients with HPB cancer. Their presence seemed to correlate with advanced-stage disease but not with resectability or clinical outcome after resection.

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References