short insertions, the hybridization probe bridges the whole mutation site (12, 13). For the detection of a long insertion, a general solution could involve designing the probe to overlap 1–5 bases on the insertion. The scheme of our solution is shown in Fig. 1A.

Genomic DNA was extracted from 200 μL of peripheral blood anticoagulated with EDTA with the QIAamp blood reagent set (Qiagen) according to the manufacturer’s instruction. All blood samples were kept at −20°C until DNA isolation.

PCR was performed in disposable capillaries (Roche Diagnostics). The reaction volume was 10 μL, containing 1 μL of DNA (40–80 ng), 0.2 μM each of the primers reported by Rigat et al. (1), 1 μL of reaction buffer (LightCycler DNA master hybridization probes 10× buffer; Roche Diagnostics), 0.4 μL of 25 mM MgCl₂ stock solution, 0.5 μL of dimethyl sulfoxide, and 0.1 μM each of the probes. The detection probe specific for the 3’ end of the insertion (underlined four bases in the 5’ end of the probe) was labeled at the 3’ end with fluorescein (5’-CGT CAG AT ACA GTC ACT TTT ATG-3’). The anchor probe (5’-GGT TTC GCC AAT TTT ATT CCA GCT CTG-3’) was labeled with LightCycler Red 640 at the 5’ end and was modified at the 3’ end by phosphorylation to block extension.

The PCR conditions were as follows: initial denaturation at 95°C for 60 s, followed by 40 cycles of denaturation (95°C for 0 s, 20°C/s), annealing (61°C for 10 s, 20°C/s), and extension (72°C for 15 s, 20°C/s). The melting curve analysis consisted of 1 cycle at 95°C for 60 s, followed by 40 cycles of denaturation (95°C for 0 s, 20°C/s), annealing (61°C for 10 s, 20°C/s), and extension (72°C for 15 s, 20°C/s). The fluorescence signal (F) was monitored continuously during the temperature ramp and then plotted against the temperature (T). These curves were transformed to derivative melting curves (dF/dT) vs T. The derivative melting curves for the three genotypes (I/I, I/D and D/D) are depicted in Fig. 1B.

The PCR conditions were the same when the confirmatory primer pair were used, except that the annealing temperature was 67°C. In this case, the derivative melting curve exhibited only the peak characteristic of the insertion allele.

Of 103 patient samples tested, 23.3% were I/I, 44.7% were I/D, and 33.0% were D/D.

The proposed technique and the electrophoresis yielded identical results. No ACE D/D misclassification was found even when the confirmatory, independent primer pair was used.

References
logic diagnosis. After centrifugation, sera were divided into aliquots and stored at −80 °C. The ELISA for anti-survivin antibodies was established. Recombinant His-tagged T7-TAT-myc-survivin protein was prepared by the following procedure. We received pcDNA3-myc-survivin as a generous gift from Dr. J. C. Reed (Burnham Institute, La Jolla, CA) (2). SacI and XhoI were used to digest pcDNA3-myc-survivin for ligation into pET21a. In turn, pET21a-T7-TAT-myc-survivin was digested with NdeI and XhoI and ligated into pET15b. The resulting pET15b-T7-TAT-myc-survivin was transfected into BL21 cells, which then overexpressed recombinant survivin protein. Recombinant protein was purified with a nickel-nitrilotriacetate column (QIAGEN) according to the manufacturer’s instructions. Purity of the recombinant protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie blue staining. As a control, His6-T7-TAT-green fluorescent protein was used (provided by the Department of Pathology at Sapporo Medical University, Sapporo, Japan).

As an antigen for coating wells in the ELISA, purified recombinant His6-T7-TAT-myc-survivin was diluted in 50 mmol/L bicarbonate buffer (pH 9.5) to a final protein concentration of 5 mg/L as determined by the method of Bradford, using a commercially available reagent set (Bio-Rad). Recombinant His6-T7-TAT-green fluorescent protein was used as a control antigen at the same concentration in the same buffer.

Survivin or control antigen solution was placed in wells of 96-well plates (Corning) and incubated overnight at 4 °C. After removing antigen solutions and washing five times with phosphate-buffered saline (PBS) with 0.5 mL/L Tween 20 (T-PBS), plates were blocked with 10 g/L bovine serum albumin in PBS for 2 h at room temperature. After emptying the wells and washing five times with T-PBS, 100 μL of serum sample diluted in PBS (1:100 by volume) was added to each well and incubated for 1 h at room temperature. Samples were then removed and wells were washed five times with T-PBS, after which each well was incubated for 30 min with a 1:2000 dilution of rabbit anti-human IgG F(ab′)2 conjugated with horseradish peroxidase (DAKO). After removal of this antibody solution and washing five times with T-PBS, each well was developed by o-phenyldiamine. After a 10-min incubation in darkness, the reaction was stopped with 0.25 mol/L H2SO4, and absorbance was measured at 492 nm.

Anti-p53 antibodies were detected with a commercially available ELISA method (Pharma Cell), using recombinant wild-type p53 according to the manufacturer’s instructions.

The cutoff value for positivity in the anti-survivin ELISA, determined from healthy donor samples as the mean absorbance +2 SD, was 0.936. The cutoff value for anti-p53 positivity, determined from healthy donor samples as the mean absorbance +2 SD, was 0.236. Sera from 25 of 63 gastrointestinal cancer patients (39.7%) were reactive with recombinant survivin protein by ELISA, whereas none of the control sera from healthy donors was reactive. Subgroup results for gastrointestinal cancers were as follows: 13 of 30 positive sera for biliary-tract cancer; 4 of 10 positive for gastric cancer; 3 of 10 positive for colorectal cancer; 1 of 3 positive for hepatoma; 3 of 7 positive for pancreatic cancer; and 1 of 3 positive for esophageal cancer. Sera from 15 of 63 gastrointestinal cancer patients (23.8%) and 2 of 33 healthy donors (6.1%) reacted with recombinant wild-type p53 protein in the anti-p53 ELISA. Overall, sera from 30 of 63 gastrointestinal cancer patients (47.6%) reacted by ELISA with recombinant survivin protein, recombinant wild-type p53 protein, or both (Fig. 1A).

To determine the analytical specificity of the survivin ELISA, all serum samples (100 μL of a 1:100 dilution) were incubated with 30 mg/L recombinant survivin protein or green fluorescent protein control antigen for 1 h at 37 °C and then subjected to the survivin ELISA described above. Reactivity of sera decreased substantially after adsorption to recombinant survivin protein, but not after adsorption to control antigen.

In addition, anti-survivin monoclonal antibodies (mAbs) and sera reacting or not reacting with recombinant survivin protein in the survivin ELISA were tested against recombinant survivin protein by Western analysis. Recombinant survivin protein was separated by 100 mL/L sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoro...
ride membrane (Milipore). The membrane was blocked with 10 g/L bovine serum albumin solution in PBS and cut into strips. After washing with T-PBS, strips were incubated overnight at 4 °C with serum samples diluted 1:100 by volume or anti-survivin mAbs (1.0 mg/L; Clone 91618.11; Genzyme/Technet). After washing with T-PBS, strips were incubated for 30 min at room temperature with a 1:1000 dilution of rabbit anti-mouse immunoglobulin or a 1:2000 dilution of rabbit anti-human IgG (H+L) antibodies conjugated with horseradish peroxidase (DAKO). After washing with T-PBS, strips were developed by use of a diaminobenzidine solution (Sigma). Staining patterns of anti-survivin mAbs (lane A) and sera from two patients with gastric cancer (lanes B and C) and two healthy donors (lanes D and E) are shown in Fig. 1B. Anti-survivin mAb and sera from gastric cancer patients recognized the 32-kDa recombinant survivin protein (His6-T7-TAT-myc-survivin). No reactivity was found in sera from the two healthy donors.

We demonstrated anti-survivin antibodies in patients with various gastrointestinal cancers. When anti-p53 antibodies were additionally considered, the overall prevalence of antibody reactivity was not increased. However, determination of survivin antibodies may be useful for detecting gastrointestinal cancers.

Survivin expression has been detected in 53.2% of colorectal cancers (6), and mRNA transcripts encoding survivin were detected in 63.5% of recurrent colorectal cancers by a reverse transcriptase-PCR (8). However, Rohayem et al. (11) reported that 4 of 49 colorectal cancer patients (8.2%) and 11 of 51 sera from lung cancer patients (21.6%) reacted with His-tagged recombinant survivin protein in an anti-survivin ELISA. In the present study, antibody responses against survivin were not always apparent in all patients whose cancers expressed survivin. In addition, survivin expression has been detected in 70.7% of breast cancer patients (10), but antibody responses against survivin were 6.3% (1 in 16) of breast cancer patients (unpublished data). Our results and the reported data suggest that the site of tumor origin influences anti-survivin reactivity.

We found anti-survivin reactivity 1 year after operation in a biliary-tract cancer patient who had no anti-survivin antibodies before or shortly after surgery (data not shown). At the time reactivity was detected, a local recurrence of cancer was found. In addition, anti-survivin reactivity in three biliary-tract cancer patients who had high anti-survivin reactivity before their operations did not show a decrease at 1 week after operation (data not shown). In one of three patients, a local recurrence of cancer was found at the time of writing.

References

EDTA Samples Are Stable for Prothrombin Time Measurement by Combined Thromboplastin Reagent, Juha Horsti (Valkeakoski District Hospital, Tampere University Hospital Laboratory, 37600 Valkeakoski, Finland; fax 358-3-586-7435, e-mail juha.horsti@tays.fi)

The prothrombin time (PT) test is used to detect preoperative bleeding tendencies and to monitor anticoagulant therapy (prevention of venous thromboembolism, treatment of deep vein thrombosis, primary prevention of myocardial ischemia, acute myocardial infarction, prothrombotic conditions such as atrial fibrillation, and other indications) (1). Anticoagulant therapy is widely used, and requests for PT tests are increasing continually (2). Two major methods are used worldwide to measure PT, namely the “Quick method” and the Owren method (combined thromboplastin reagent). Properties of these methods were compared in a recent study (3). Most studies in the scientific literature have used the Quick method, and the results do not always correspond to the combined thromboplastin method.

The materials of which evacuated tubes are constructed affect the stability of samples for PT assay. The surface of borosilicate glass activates factor VII at 4 °C, which shortens the coagulation time(s) for PT (4, 5). In polypropylene or siliconized borosilicate glass tubes, surface activation is lower.

According to current recommendations, PT measurement should be made within 2 h for samples stored at room temperature or within 4 h of sample collection for samples stored at 4 °C (6–8), or within 8 h for samples stored at room temperature (9). The use of polypropylene tubes and storing evacuated tubes unopened extend PT stability at room temperature or 4 °C to 24 h (10–12). Baglin and Luddington (2) noted an overall mean difference in the International Normalized Ratio (INR) after 3