mL, and mean arterial pressure was significantly correlated with PHF concentrations [Fig. 1C; slope, 0.008014 ± 0.003734; y-intercept, -0.6246 ± 0.3908 units/mL; R = 0.394, P = 0.0417 (significant); n = 27].

Our goal was to develop MAbs with high affinities to both the rat and human PHF antigens. The antibodies produced were selective for PHF, as demonstrated in the bioassay and cross-reactivity studies. Results generated with the PHF ELISA to detect PHF in biological samples correlated well with results obtained for PHF with the bioassay. The ELISA offers a distinct advantage over the bioassay in that it is faster, less expensive, and quantitative, making it a more reliable system. Although the antigen used in this ELISA was not highly purified and the structure has not been completely elucidated, the strong correlation between this ELISA and the bioassay indicate that this assay may be useful in studies that will further our understanding of this substance. Highly pure antigen, as well as improved understanding of the etiology of PHF-related disease, will be required for this assay to be used as a routine clinical tool.

In the normotensive group, PHF was not correlated with blood pressure, but in the hypertensive group PHF was correlated with blood pressure. The number of hypertensive samples was much lower than the number of normotensive samples because of the restriction placed on sample selection. In most cases, once people are diagnosed with high blood pressure, therapy is initiated. Blood pressures in the hypertensive group were therefore significantly correlated with blood pressure. The number of hypertensive patients with high blood pressure, therapy is initiated.

References


Detection of Anti-Livin Antibody in Gastrointestinal Cancer Patients, Aitsuhi Tsyuihushi, Koichi Asanuma, Naoki Tsuiji, Yoshiki Torigoe, Noriyuki Sato, Koichi Hirata, and Naoki Watanabe (Departments of 1 Clinical Laboratory Medicine, 2 Pathology, and 3 Surgery, Sapporo Medical University School of Medicine, South, 1 West, 16, Chuo-ku, Sapporo 060-8543, Japan; * author for correspondence: fax 81-11-622-7502, e-mail watanabn@sapmed.ac.jp)

Livin, a recently described member of the inhibitor of apoptosis protein (IAP) family, contains a single baculovirus IAP repeat and a carboxyl-terminal RING finger (1–3). Like other proteins in the IAP family, livin binds specifically to a terminal effector cell-death protease, in this instance, caspase-9 (1–3). The consequences are substantially reduced caspase activity and reduced cell death in response to diverse apoptotic stimuli (1–3). Semi-quantitative reverse transcription-PCR methods have detected human livin mRNA in fetal kidney, heart, and spleen and in adult tissues such as heart, lung, spleen, ovary, and placenta (4). In addition, livin mRNA is overexpressed by some cancer cells, including melanoma, breast cancer, cervical cancer, colon cancer, prostate cancer, leukemia, and lymphoma cells (4).

As with survivin overexpression (5–7), livin overexpression by cancer cells may lead to anti-livin antibody responses and cytotoxic T-lymphocyte responses against the cancer. In the present study, we examined livin mRNA expression in gastrointestinal cancer cell lines and

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the prevalence of antibody responses against livin in patients with various gastrointestinal cancers.

We cultured human pancreatic cancer cell lines (PANC-1, Capan-1, AsPC-1, MIAPaCa-2, and BxPC-3), gastric cancer cell lines (MKN-1, MKN-45, and TMK-1), colon cancer cell lines (HT-29, SW480, SW620, and LS180), and a hepatoma cell line (HepG2) in RPMI-1640 with 100 mL/L calf serum at 37°C, and a hepatoma cell line (HepG2) in RPMI-1640 with 100 mL/L calf serum at 37°C. Livin mRNA expression was quantified by a previously reported method (8). The sequence of the forward primer was 5’-TCAGTTCTGCTCCGGTGCA-3’, that the reverse primer was 5’-CGTCTTCCGTTCTCCCA-3’, and the sequence of the TaqMan probe was 5’-CCACAGTGTG-CAGGAGACTCACTCCC-3’. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was used and amplified with TaqMan control reagents (Perkin-Elmer Applied Biosystems). The conditions of the one-step reverse transcription-PCR have been described previously (8). The calibration curves were constructed with known dilutions of RNA obtained from PANC-1 cells (8). The normalized concentration of livin mRNA, an arbitrary number that can be used to compare the relative amount of livin mRNA in different samples, was determined by dividing the concentration of livin mRNA by the concentration of GAPDH mRNA (8). Livin mRNA was highest in a pancreatic cancer line, AsPC-1 (livin mRNA:GAPDH mRNA = 0.78) and in a colon cancer line, HT-29 (livin mRNA:GAPDH mRNA = 0.42). No livin mRNA [no amplification plot was detected at ΔRn ≥0.05 (8)] was detected in two other pancreatic cancer lines (MIAPaCa-2 and BxPC-3), a gastric cancer line (MKN-1), or two other colon cancer lines (LS180 and SW620). Other cell lines contained intermediate concentrations of livin mRNA (livin mRNA:GAPDH mRNA = 0.22–0.01).

Blood samples were collected from 10 healthy blood donors (age range, 40–65 years) and 35 gastrointestinal cancer patients (age range, 43–67 years) after histologic diagnosis. In addition, blood samples were collected from 15 patients with gastric cancer admitted to Sapporo Medical University. These gastric cancer patients were diagnosed as being in stage I to IV based on TNM classification (UICC). Informed consent was obtained from all blood donors. After centrifugation, sera were divided into aliquots and stored at −80°C.

To measure anti-livin antibodies, we prepared recombinant His-tagged T7-livin protein by the following procedure. Total RNA was extracted from a human melanoma cell line (888mel) as a template. The full-length livin gene was amplified by reverse transcription-PCR with livin-specific primers (forward primer, 5’-CCGGATCCATGGGACCTAAAGACAGTG-3’; reverse primer, 5’-CCGAATTCTAGCACGGAAGTGGTCAGCA-3’). The amplified product was digested with BamHI and EcoRI and inserted at the same site in a pET15b-T7 vector (5). The resulting pET15b-T7-livin was transfected into BL21 cells, which then overexpressed recombinant livin protein (His-T7-livin). The recombinant protein was purified with use of a nickel-nitrioltriacetate column (QIAGEN) according to the manufacturer’s instructions. The purity of the recombinant protein (50 kDa) was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Coomassie blue staining. As a control, His-T7-green fluorescent protein (GFP) was used (5).

As an antigen for coating wells in the anti-livin assay, purified recombinant livin was diluted in 50 mmol/L bicarbonate buffer (pH 9.5) to a final protein concentration of 5 mg/L as determined by the Bradford method (Bio-Rad). Recombinant His-T7-GFP was used as a control antigen at the same concentration in the same buffer.

Livin or control antigen solution was placed in the wells of 96-well plates (Corning) and incubated overnight at 4°C. After the antigen solutions were removed and the wells were washed five times with phosphate-buffered saline (PBS) containing 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 the wells were emptied and washed five times with T-PBS, 100 μL of serum sample diluted 1:100 in PBS was added to each well and incubated for 1 h at room temperature. The samples were then removed, and the wells were washed five times with T-PBS, after which each was incubated for 30 min with a 1:2000 dilution of rabbit anti-human IgG F(ab’)2 conjugated with horseradish peroxidase (Dako). After this antibody solution was removed and the wells were washed five times with T-PBS, each well was developed by addition of o-phenylenediamine. After a 10-min incubation in the dark, the reaction was stopped with 0.25 mol/L H2SO4, and the absorbance was measured at 492 nm. Data were obtained in triplicate for each sample.

The cutoff value for positivity in the anti-livin assay, determined from healthy donor samples as the mean absorbance + 2 SD, was 0.272. Sera from 17 of 35 gastrointestinal cancer patients (47%) were reactive with recombinant livin protein by the ELISA, whereas only 1 control serum from healthy donors was reactive (Fig. 1A). Subgroup results for gastrointestinal cancers were as follows: 5 positive sera of 9 for biliary tract cancer; 6 of 9 for gastric cancer; 6 of 11 for colorectal cancer; 1 of 3 for hepatoma; 2 of 7 for pancreatic cancer; and 2 of 3 for esophageal cancer. All 17 sera reacting with recombinant livin protein in the anti-livin assay were subjected to the anti-survivin assay described previously (5). Anti-survivin antibodies were detected in 13 of 17 sera tested. The intensities of the anti-livin antibody responses did not correlate with those of the anti-survivin antibody responses (y = 0.804x − 0.096; r = 0.411). In addition, sera from 6 of 15 gastric cancer patients (4 positive sera of 7 for stage III, 1 of 2 for stage IV, 1 of 1 for recurrent gastric cancer) were reactive with recombinant livin protein in the assay.

To examine the analytical specificity of the anti-livin assay, all serum samples (100 μL of a 1:100 dilution) were incubated with 30 mg/L recombinant livin protein or GFP control antigen for 1 h at 37°C and then were subjected to the assay. Serum reactivity decreased dramatically after incubation with recombinant livin protein, but not after incubation with recombinant control GFP.
In addition, anti-livin polyclonal antibody and sera reacting or not reacting with recombinant livin protein in the anti-livin plate-coating assay were tested against recombinant livin protein by Western blot analysis. Recombinant livin protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with bovine serum albumin solution (10 g/L) in PBS and cut into strips. After being washed with T-PBS, membrane strips were incubated overnight at 4°C with serum samples diluted 1:100 or anti-livin polyclonal antibody (1.0 mg/mL). After being washed with T-PBS, the strips were incubated for 30 min at room temperature with a 1:2000 dilution of rabbit anti-human IgG F(ab')2 conjugated with horseradish peroxidase (Dako). After being washed with T-PBS, the strips were developed with a diaminobenzidine solution (Sigma). The anti-livin polyclonal antibody and sera from three gastrointestinal cancer patients all recognized the 50-kDa recombinant livin protein (Fig. 1B), whereas serum from the healthy donor was not reactive.

Kasof and Gomes (1) reported that the overall protein identity of livin to survivin based on GAP pairwise sequence alignment (GCG) was 26.3%. Survivin, a member of another IAP family, is overexpressed by most cancers (9, 10). Rohayem et al. (6) reported that 11 of 51 sera from lung cancer patients (22%) and 4 of 49 sera from colorectal cancer patients (8%) reacted with recombinant survivin protein in an anti-survivin assay. We previously reported detection of anti-survivin antibodies in 25 of 63 gastrointestinal cancer patients (40%) when the cutoff value for positivity was set at the mean absorbance for healthy donor samples +2 SD (5). Survivin is recognized not only by antibodies but also by cytotoxic T-lymphocytes (7, 11). Thus, survivin is considered a major cancer antigen.

Livin is expressed by most cancers, including carcinomas of the breast, cervix, colon, and prostate, as well as by melanomas and by leukemia and lymphoma cells (1–4). In the present study, gastric cancer, colon cancer, pancreatic cancer, and hepatoma cells showed overexpression of livin mRNA. The relatively high prevalence of anti-livin antibodies in gastrointestinal cancer patients (47%) and the lack of correlation with anti-survivin antibodies suggest that testing for anti-livin antibodies may be useful for detecting these cancers. Together with previously reported findings, our results suggest that livin might serve as a novel cancer antigen.

References