ORIGINAL ARTICLE

Anti-livin antibodies: novel markers of malignant gastrointestinal cancers

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KEY WORDS

anti-livin antibodies, gastrointestinal cancers, radioimmunoassay

ABSTRACT

INTRODUCTION Livin represents apoptosis inhibitors and may be important in cancer.
OBJECTIVES The aim of the study was to develop an anti-livin autoantibody assay and investigate its usefulness in the clinical practice in relation to gastrointestinal cancers (GIC).
PATIENTS AND METHODS We studied sera obtained from 36 patients with GIC and 59 healthy controls. A solid-phase radioimmunoassay to detect anti-livin antibodies in serum was developed. Polipropylene tubes were coated with recombinant human livin, and 100-fold dilutions of sera were incubated in these tubes. On the next day, the tubes were decanted, washed and labeled 125-I protein A was added. After 2-hour incubation, the tubes were washed and radioactivity was measured using the γ counter.
RESULTS We observed a statistically significant difference between the presence and levels of anti-livin antibodies in sera of patients with GIC and in control subjects. Anti-livin autoantibodies were detected in 9 patients with GIC. Of note, the level of anti-livin antibodies was significantly elevated in 25% of GIC patients. The presence of anti-livin antibodies was confirmed with sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting.
CONCLUSIONS A high prevalence of anti-livin antibodies in patients with GIC indicates that they may be useful in the diagnosis of these malignancies.

INTRODUCTION Livin belongs to the family of protein inhibitors of genetically programmed cell death (apoptosis). To date, 8 apoptosis inhibitors have been described, namely cellular inhibitors of apoptosis protein (cIAP)-1, cIAP-2, neuronal IAP, survivin, X-linked IAP, baculovirus repeat containing ubiquitin conjugating enzyme, ILP-2, and livin[1-10] (FIGURE 1).

IAPs are present in most normal human adult tissues, except for survivin and livin, which are found in fetal tissues but not in adults. An increased expression of livin and survivin was observed in gastrointestinal malignancies as well as breast, prostate, and lung cancers.[2-6]

The IAP family is characterized by the presence of the BIR domain, which suppresses apoptosis through binding and inhibition of caspase-3, -7, and -9. The caspases are cysteine proteases that lead to apoptosis. The BIR domain is evolutionarily conserved in baculoviruses, flies (*Drosophila melanogaster*), several mammalian species, and humans.

Numerous IAPs contain the RING domain, which plays a role in ubiquitination and degradation of apoptosis regulators, including caspase-3, -7, -9 and mitochondrial proapoptotic protein, SMAC.

Livin, a novel member of the IAP family, was identified, isolated from malignant melanoma, and described as the melanoma IAP by Vućić et al.[7] and Franklin et al.[8] Further, more detailed studies on this novel inhibitor were performed by Kasof and Gomez,[9] who introduced the term "livin", which has been generally accepted by researchers.

Kasof and Gomez evaluated the amino acid sequence of livin, which consists of 298 amino acids and shows homology to the amino acid sequence

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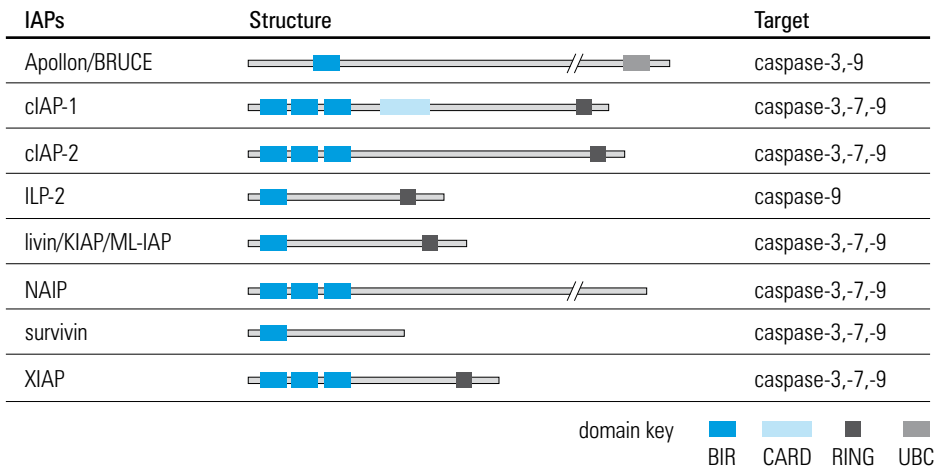

FIGURE 1 Inhibitors of apoptosis proteins (IAPs) present in humans (illustration courtesy of R&D Systems) Abbreviations: BIR – baculovirus IAP repeat, BRUCE – baculovirus repeat containing ubiquitin conjugating enzyme, CARD – caspase recruitment domains, cIAP – cellular IAP, XIAP – X-linked IAP

of baculoviruses (baculoviruses IAP repeat), highly conserved in other IAPs.[9]

In normal adult tissues, only traces of livin can be detected, but its overexpression in malignant tumors may cause immunological response, secretion of autoantibodies, and production of cytotoxic lymphocytes.

Livin has been extensively studied by oncologists and radiotherapists because of its potential role in the destruction of malignant cells by restoring their sensitivity to chemo- and radiotherapy.[1,10-13] Therefore it is crucial to investigate whether anti-livin antibodies can be novel markers of cancer.

The aim of the present study was to develop a novel radioimmunoassay that could enable us to detect and evaluate the prevalence of anti-livin antibodies in the serum of patients with gastrointestinal cancer (GIC).

PATIENTS AND METHODS

Serum samples were obtained from 36 patients with malignant tumors of the gastrointestinal tract (stomach, small intestine, colon, and pancreas). The material was obtained from the Department of Clinical Pathomorphology, Poznan University of Medical Sciences, Poznań, Poland. The diagnosis was confirmed by histological examination; however, in 11 cases it was based on identification of the tumor in clinical examination and observation of a high level of carcinoembryonic antigen (CEA). The control group consisted of serum samples obtained from 59 healthy individuals.

Livin was purchased from R&D Systems, protein A from SIGMA and GE Healthcare, 125-I isotope and polyethylene tubes from OBI Świerk. Electrophoresis reagents including sodium dodecyl sulfate (SDS), acrylamide, methylenebisacrylamide, and molecular weight markers were purchased from SIGMA. Chemiluminiscence reagent kit (ECL system), X-ray films, and antibodies against human γ globulin, labeled with horseradish peroxidase, were obtained from GE Healthcare/Amersham Biotech.

We used 3 methods for the determination of anti-livin antibodies: solid-phase radioimmunoassay, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and preadsorption of serum samples, which was used to test antibody specificity.

Development of the solid-phase radioimmunoassay

Tube coating Livin was dissolved in 0.05 M phosphate buffer saline (PBS) with sodium azide (NaN_3) and the tubes were coated by adding 1 µl of livin in 200 µl of the buffer. After 20 h of incubation at +4°C, the tubes were decanted and washed twice with PBS/0.1% Tween 20. Subsequently, all the remaining active sites were blocked for 20 h at 4°C in 1 ml 0.5% bovine serum albumin (BSA) dissolved in PBS with the addition of NaN_3. The tubes were ready for use after triple washing with PBS containing 0.1% Tween 20.

Determination of anti-livin antibodies The serum samples were diluted for testing 1:100 and a volume of 200 µl was added to the coated tubes. The samples were mixed on vortex and incubated for 20 h at 4°C. Subsequently, the tubes were decanted and washed twice with PBS with the addition of 0.1% Tween 20. A volume of 200 µl of freshly labeled protein A (125I- protein A, approximately 100,000 impulses [cpm] per tube) diluted in PBS/BSA was added to each tube. After 2 h of incubation at 37°C, the tubes were decanted, washed 3 times with PBS buffer containing 0.1% Tween 20, and finally decanted. Their activity was measured on the γ counter.

Polyacrylamide gel electrophoresis

To evaluate autoantibodies SDS-PAGE and Western blotting (immunoblotting) were used. Separation procedure was performed with 12% stacking and 4% separating gel (30% acrylamide, 0.8% methylenebisacrylamide, 1M Tris-HCl buffer, 20% SDS and ammonium persulphate [TEMED, Sigma]). Livin was denatured in appropriate proportions (to obtain livin concentration of 1 mg/ml) in a solution consisting of 0.3 mol/l Tris-HCl buffer (pH 6.8), 6% SDS, 30% glycerol, 6% mercaptoethanol, and 0.1% bromophenol blue. The samples were placed for 5 min on a thermal block set to 95°C, and then 5 µl of livin in a volume of 40 µl was transferred

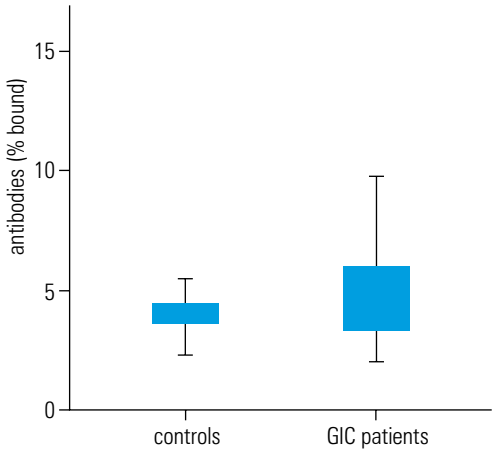

FIGURE 2 Anti-livin antibodies detected by radioimmunoassay in sera of patients with gastrointestinal cancers (GIC) (n = 36) compared with control subjects (n = 59)

on the lanes in polyacrylamide gel. The same procedure was applied to marker proteins (MP Biomedicals, Germany). When SDS-PAGE had been finished, livin was electrophoretically transferred to a nitrocellulose membrane (Millipore) in the presence of a buffer composed of 25 Mm TRIS, 190 Mm glycine, 20% methanol (pH 8.3). The serum samples (1:200 dilution) were incubated for 16 h at 4°C. Incubation with secondary antibody (antihuman IgG labeled with horseradish peroxidase) was conducted at room temperature for 1 h with a subsequent chemiluminescence reaction and autoradiography (ECL kit, Amersham). The final step was autoradiography on X-ray films which were exposed for 3 to 15 min.

Statistical analysis The data were analyzed using Statistica V.8. The results were expressed as means and standard deviations, unless otherwise stated. Statistical significance was assessed using the nonparametric Mann-Whitney test, applied to determine differences between non-normally distributed groups.

RESULTS **Radioimmunoassay** Our study has shown a difference in sample radioactivity between the control group and the group of patients with gastrointestinal malignancies. We have detected anti-livin antibodies in 9 patients. Our results have exceeded 2 standard deviations of the reference value calculated for the control group. Thus, in 25% of cases, the level of anti-livin

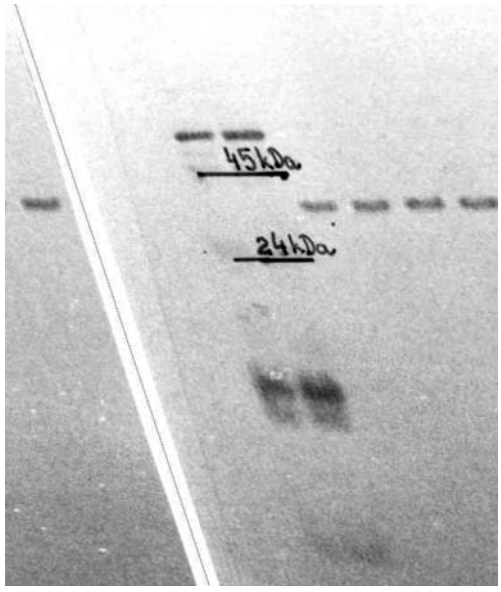

FIGURE 3 SDS-PAGE and Western blotting results of sera with elevated anti-livin antibody levels

antibodies was remarkably elevated. The mean and standard deviation of anti-livin antibodies in healthy subjects was 3.9 ±0.84% bound, while in patients with GIC it was 5.2 ±2.61% bound. The difference between the groups was statistically significant, $P < 0.05$ (FIGURE 2).

Polyacrylamide gel electrophoresis and Western blotting We used SDS-PAGE and Western blotting to test 7 patients with anti-livin antibody positivity (determined by the RIA method) and 3 controls. In 5 patients, after incubation of their serum samples with nitrocellulose straps containing separated livin, the results were definitely positive – there were visible bands in the spots of livin presence – molecular weight ca. 38 kDa corresponding to the known livin size from 37 or 39 kDa. The results were negative in 2 patients, (FIGURE 3). Anti-livin antibodies were not detected in any of the control samples using the present method.

Testing for specificity of anti-livin antibodies To test antibody specificity, i.e., the reaction of antibodies only with livin, we conducted serum preadsorption tests with positive antibodies. On

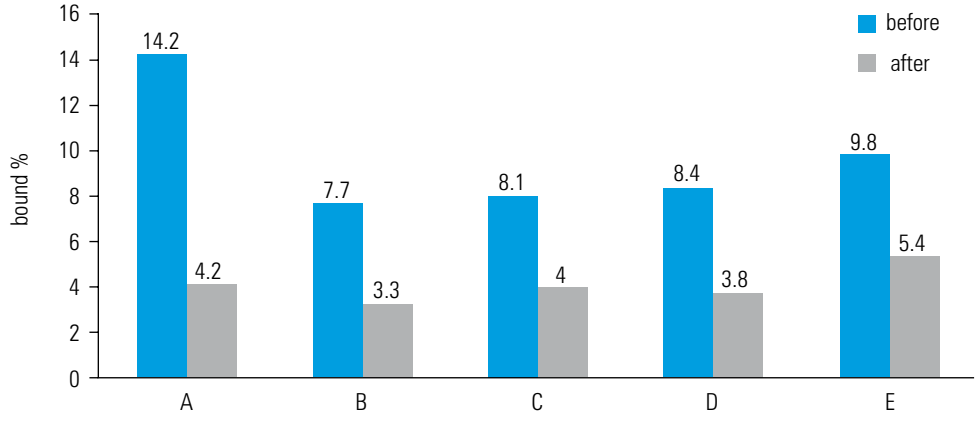

FIGURE 4 Anti-livin autoantibodies before and after preabsorption of the sera with livin. Letters A–E indicate sera from the individual study subjects.

the preceding day, we added 10 μg of livin per tube to the serum samples (1:200 dilution) in order to obtain saturation of antibodies. The next day, we determined the presence of anti-livin antibodies using the solid-phase radioimmunoassay. The activity of all samples preincubated with livin serum decreased, which meant that these antibodies were selectively directed against livin, as shown in **FIGURE 4**.

DISCUSSION The presence of anti-livin antibodies in patients with GIC was first described by Yagihashi et al.,[14] who used an enzyme-linked immunoassay (ELISA) and considered results surpassing 2 standard deviations of the normal as positive. The authors observed the presence of anti-livin antibodies in 47% of 35 serum samples obtained from these patients. However, their control group consisted only of 7 patients.

There have been no other reports on anti-livin antibodies so far. Livin has only recently become commercially available, thus Yagihashi et al. are considered as pioneers in this field.[14]

In our study, conducted using several methods (solid-phase radioimmunoassay and SDS-PAGE/Western blotting), we confirmed the findings of Yagihashi et al.[14] by detecting anti-livin antibodies in 25% of patients with GIC. The discrepancy between our study and that of Yagihashi et al. might have been caused by the size of the control groups, comprising 59 and 7 patients, respectively. A larger control group enabled us to determine the reference range more precisely.

So far, the determination of CEA and α-fetoprotein (AFP) antigen levels has been the most common approach in clinical practice. Overexpression of CEA occurs in various types of cancers, especially in adenocarcinomas, including GIC. However, elevated CEA levels are observed in benign diseases, such as enteritis, hepatitis, pneumonia, and pancreatitis, as well as in the serum of current smokers. Therefore, CEA antigen has only minor diagnostic significance and is usually useful in the evaluation of surgical treatment efficacy, because in successfully treated patients a decrease in CEA levels is observed. CEA levels rise in the case of recurrence or metastasis. AFP is secreted by liver cells and, physiologically, it is present only in the fetus and in pregnant women. In pathological conditions, high AFP levels are found in patients with liver, pancreatic, and gastrointestinal neoplasms and in patients with liver metastases. However, an elevated AFP concentration is also detected in nonneoplastic diseases, such as hepatitis, cirrhosis, Crohn's disease, and intestinal polyposis.

In their review, Saif et al.[15] discussed antibodies used in biochemical diagnosis of malignant diseases. They included antibodies against p-53, mucin, livin, survivin, Fas proteins, and antinuclear antibodies. However, there is no single universal marker of GIC. That is why new methods, namely SEREX and SAX, have been suggested for identification of a set of markers directed against various antigens specific for particular neoplasms. Koziol et al. have demonstrated that it is possible to distinguish between serum samples of patients with breast, colon, gastric, liver, lung, or prostate cancers using a set of 7 cancer antigens.[16]

So far, there have been no studies on anti-livin antibodies in other gastrointestinal and autoimmune diseases reported in the literature, so it is too early to consider anti-livin antibodies as markers of malignant tumors.

In conclusion, the solid-phase radioimmunoassay enables to detect anti-livin antibodies in patients with GIC. The prevalence of anti-livin antibodies in patients with GIC is 25%. SDS-PAGE confirmed the presence of anti-livin antibodies and preabsorption serum testing proved specificity of the method.

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ARTYKUŁ ORYGINALNY

Przeciwciała antyliwinowe – nowy marker nowotworów złośliwych przewodu pokarmowego

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SŁOWA KLUCZOWE

badanie radioimmunologiczne, nowotwór przewodu pokarmowego, przeciwciała antyliwinowe

STRESZCZENIE

WSTĘP Liwina należy do białek inhibitorów apoptozy i może odgrywać rolę w patogenzie nowotworów.

CELE Celem badania było opracowanie metody oznaczania przeciwciał antyliwinowych i ocena ich przydatności w praktyce klinicznej w odniesieniu do chorych na złośliwe nowotwory przewodu pokarmowego.

PACJENCI I METODY Do badań użyto surowicy 36 osób chorych na nowotwory przewodu pokarmowego. Przebadano też surowice 59 osób zdrowych stanowiących grupę kontrolną. W celu oceny obecności przeciwciał antyliwinowych opracowano technikę radioimmunologiczną. Probówki polipropylenowe opłaszczono ludzką rekombinowaną liwiną. Inkubowano je z surowicą rozcieńczoną stukrotnie. Następnego dnia probówki płukano i dodawano 125-I Protein A. Po 2 godzinach inkubacji probówki płukano, a następnie mierzono radioaktywność za pomocą licznika promieniowania γ.

WYNIKI Wykonane badania wykazały statystycznie znamienną różnicę w radioaktywności próbek między grupą osób zdrowych i chorych na raka przewodu pokarmowego, odzwierciedlającą różnice w obecności i poziomach przeciwciał antyliwinowych w surowicy pacjentów. U 9 chorych z nowotworami przewodu pokarmowego wykryto obecność przeciwciał antyliwinowych. Należy podkreślić, że u tych 25% pacjentów stężenie przeciwciał antyliwinowych było znacznie zwiększone. Obecność tych przeciwciał została potwierdzona metodą elektroforezy w żelu poliakrylamidowym w obecności dodecylosiarczanu sodu oraz immunoblottingu.

WNIOSKI Duża częstość występowania przeciwciał antyliwinowych u chorych na nowotwory złośliwe przewodu pokarmowego wskazuje, że przeciwciała te mogą być przydatne w diagnostyce tych nowotworów.

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