

Expression of Livin in Renal Cell Carcinoma and Detection of Anti-livin Autoantibody in Patients

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OBJECTIVES	To assess the expression of livin, a member of the inhibitor of apoptosis protein family, in renal cell carcinoma (RCC) and to determine its prognostic relevance.
METHODS	Immunohistochemical staining for livin was performed using paraffin-embedded tissues from 45 cases of RCC. Then we assessed anti-livin antibodies (Abs) in patient sera by enzyme-linked immunosorbent assay and Western blotting. Disease-specific survival of patients was assessed, and differences between the immunohistologically livin-positive and livin-negative groups and between the anti-livin Ab-positive and anti-livin Ab-negative groups were compared with recurrence-free survival using the Kaplan-Meier method and log-rank test.
RESULTS	Of the 45 RCC specimens, 26 (57.8%) showed positive staining of livin immunohistochemically. In the RCC patients, anti-livin antibodies were detected and their levels were significantly higher than those in healthy volunteers. However, there was no difference in disease-specific survival between the livin-positive and livin-negative patients or between the anti-livin-positive and anti-livin-negative patients.
CONCLUSIONS	Although livin expression may not provide predictive information, it may be recognized as a tumor antigen by the immune system in RCC patients. UROLOGY 70: 38–42, 2007. © 2007 Elsevier Inc.

Livin is a member of the inhibitor of apoptosis protein (IAP) family.¹ It is a protein with a single baculoviral IAP repeat domain and a COOH-terminal RING domain, and it can inhibit apoptosis by inhibiting proteolytic activation of caspases 3, 7, and 9.² Livin is not present in most normal adult differentiated tissues, although its messenger ribonucleic acid and the protein are present in the fetal brain, kidney, and placenta.^{1,3} It is expressed in malignant melanoma cells, and it could be one of the immunodominant tumor antigens in melanoma patients.⁴ It is also expressed in lung cancer,⁵ colon cancer, prostate cancer, B cell lymphoma, erythroleukemia, and promyelocytic leukemia cell lines.⁶ The antiapoptotic activity of livin is more robust than that of survivin,¹ another member of the IAP family that has been demonstrated in colorectal cancer, non-small-cell lung cancer, gastric cancer, bladder cancer, breast cancer, melanoma, neuroblastoma, hepatocellular carcinoma, diffuse large B cell lymphoma,⁷ and renal cell

carcinoma (RCC).⁸ Though there have been a number of reports^{1,5,6} demonstrating its expression in various malignancies, livin expression in RCC and its antigenic potential have not yet been elucidated.⁸

Herein we assess the expression of livin in RCC cells and tissues and study the correlation between its expression and prognosis. Serologic responses against livin were also examined in RCC patients and healthy volunteers. Our study highlights livin as a novel tumor-specific antigen in RCC.

MATERIAL AND METHODS

Immunohistochemical Staining for Livin

We reviewed the clinical pathology archives of 138 consecutive patients who underwent radical or partial nephrectomy and were diagnosed as having clear cell RCC at the Sapporo Medical University Hospital, Sapporo, Japan from May 1991 to August 1998. Patients whose medical records were incomplete or who had a history of another cancer were excluded. We selected 45 patients on the basis of the availability of sufficient material for immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). The median age at operation for the 27 male and 18 female patients was 61 years (range, 24 to 80). All hematoxylin and eosin-stained slides were reviewed, and clinical stage was assigned according to the 2002 TNM classification of malignant tumors. All of these specimens were histopathologically diagnosed as clear cell carcinoma. There

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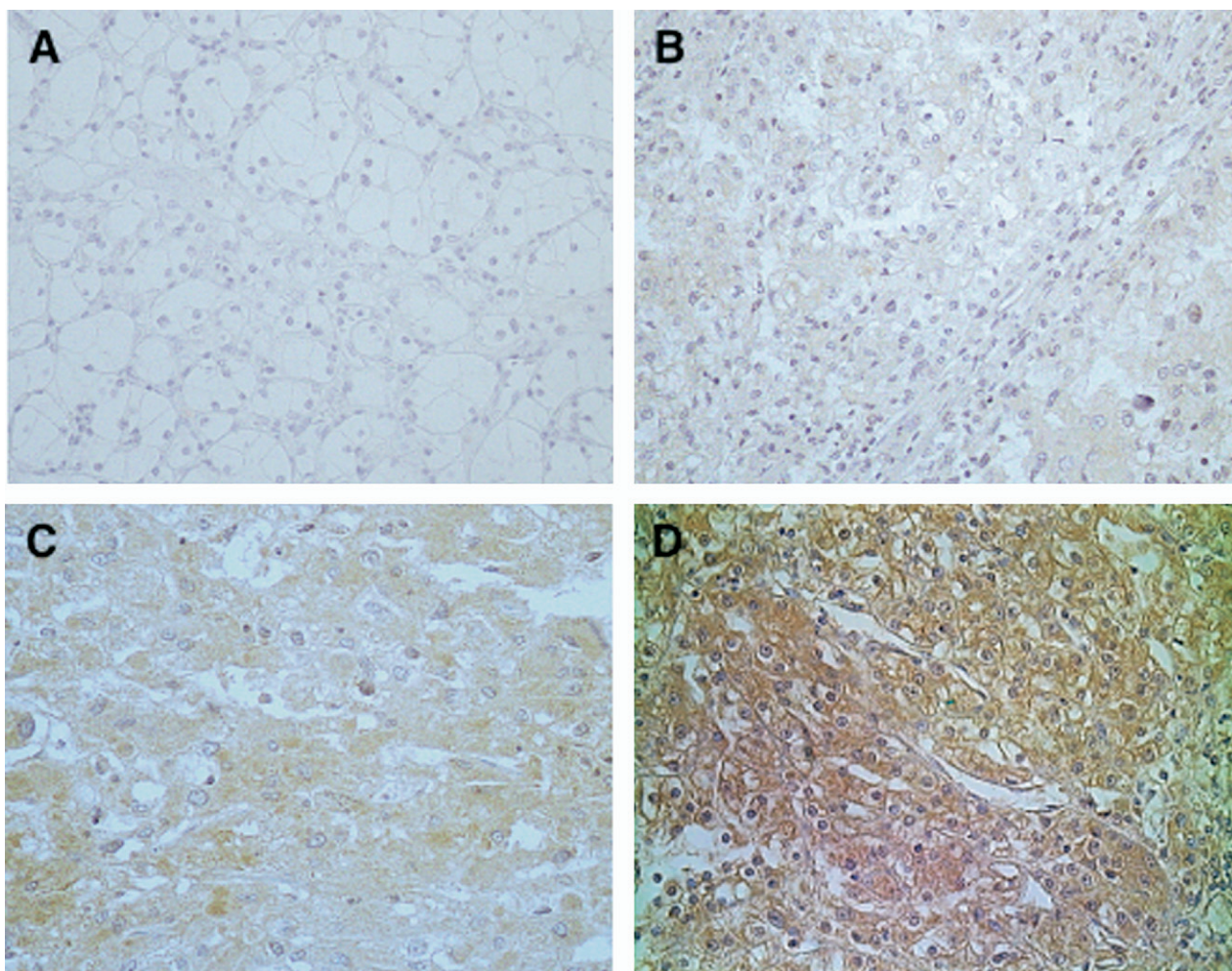


Figure 1. Representative pictures of immunohistochemical staining with a monoclonal antibody reacting to livin in RCC. (A) Score 0; (B) score 1; (C) score 2; (D) score 3.

were 22 cases of clinical Stage 1, 9 cases of Stage 2, 7 cases of Stage 3, and 7 cases of Stage 4. The Fuhrman grade distribution was 15 cases with grade 1 to 2, 22 cases with grade 3, and 8 cases with grade 4. Median tumor diameter was 5.5 cm (range, 1.2 to 18 cm). All patients were followed up every 3 months during the first 3 years after surgery, every 6 months from 3 to 10 years, and annually after 10 years. The median follow-up period was 63 months (range, 3 to 117 months). All patients with Stage 3 and 4 disease underwent cytokine-based immunotherapies after surgery. Six (19.4%) of the 31 Stage 1 and 2 patients underwent such therapies when a recurrent tumor was detected during follow-up.

Immunohistochemical staining with a monoclonal anti-livin antibody established in our laboratory was performed on 5- μ m-thick, formalin-fixed, paraffin-embedded tissue sections by the method previously described.⁵ All specimens were reviewed independently using light microscopy by investigators who were blinded to clinicopathologic data (H.K., I.H., and T.T.). For the staining of livin, the mean percentage of positive tumor cells was determined in at least five areas at $\times 400$ magnification. The cells that expressed livin were classified qualitatively into four categories (0, 1+, 2+, and 3+) according to the intensity of staining and the percentage of cells: 0, less than 10% of cancer cells with positive staining; 1+, low to moderate staining in 11% to 30% of cells; 2+, moderate to strong staining in 31% to 50% of cells; and 3+, diffuse strong staining

in more than 50% of cells (Fig. 1).⁹ If a specimen showed a staining pattern that did not fit into one of the categories, the pathologists scored it according to their judgment. The specimens were classified as normal (category 0) or as abnormal (categories 1 to 3). Any immunoreactivity was considered positive because livin is undetectable in normal adult tissue.⁵

ELISA for Anti-livin Antibody in Patient Sera

Blood samples were collected from the 45 RCC patients who underwent the immunohistochemical analysis described above before surgery and from 21 healthy volunteers (age range, 22 to 65 years) after receipt of informed consent. After centrifugation, sera were divided into aliquots and stored at -80°C . ELISA was performed as previously described.¹⁰ Data were obtained in triplicate for each sample.

Western Blot Analysis of Serum Anti-livin Antibody

The livin complementary deoxyribonucleic acid (cDNA) fused with the myc tag was cloned into BamHI and EcoRI sites of pcDNA3.1 mammalian expression vector (Invitrogen, Carlsbad, Calif). 293T human embryonal kidney cells were transfected with the plasmid by using Lipofectamine 2000 (Invitrogen). Forty-eight hours after the transfection, cell lysates were prepared from livin-transfected cells and wild-type 293T cells,

Table 1. Relationship between livin expression and clinicopathologic factors of patients with renal cell carcinoma

Factors	Total (n = 45)	Livin Expression		P Value
		Negative (n = 19)	Positive (n = 26)	
Clinical stage				0.344
1	25	13 (52.0)	12 (48.0)	
2	6	1 (16.7)	5 (83.3)	
3	7	2 (28.6)	5 (71.4)	
4	7	3 (42.9)	4 (57.1)	
Fuhrman grade				0.093
1/2	8	6 (75.0)	2 (25.0)	
3	26	10 (38.5)	16 (61.5)	
4	11	3 (27.3)	8 (72.7)	
Tumor diameter (cm)				0.460
≤7	28	21 (75.0)	7 (25.0)	
>7	17	11 (64.7)	6 (35.3)	
Tumor necrosis				0.060
No	33	26 (78.8)	7 (21.2)	
Yes	12	6 (50.0)	6 (50.0)	
Perirenal fat invasion				0.744
No	42	18 (42.9)	24 (57.1)	
Yes	3	1 (33.3)	2 (66.7)	
Tumor thrombus				0.418
No	38	17 (44.7)	21 (55.3)	
Yes	7	2 (28.6)	5 (71.4)	
Distant metastasis				0.971
No	38	16 (42.1)	22 (57.9)	
Yes	7	3 (42.9)	4 (57.1)	
Anti-livin antibody				0.005
Negative	20	13 (65.0)	7 (35.0)	
Positive	25	6 (24.0)	19 (76.0)	

Values in parentheses are percentages.

respectively, in 1 mL of radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor mixture tablet (Roche Molecular Biochemicals, Indianapolis, Ind). Then the lysates were used for immunoprecipitation with an anti-myc monoclonal antibody conjugated to agarose (Santa Cruz Biotechnology, Santa Cruz, Calif). The samples were washed three times with RIPA buffer, boiled in Laemmli buffer, and resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Milipore, Bedford, Mass), which were then incubated with serum samples diluted 1:10 or an anti-livin polyclonal antibody (1.0 mg/L, original) followed by a 1:2000 dilution of rabbit antihuman immunoglobulin G (IgG) F(ab')₂ or goat antirabbit IgG F(ab')₂ conjugated with horseradish peroxidase (DakoCytomation, Glostrup, Denmark). The immunocomplex was visualized by enhanced chemiluminescence according to the manufacturer's specifications (Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

The chi-square test was used for comparison of data among groups. We compared the values for the anti-livin antibody between the two groups by using the Wilcoxon test. Disease-specific survival was assessed by the Kaplan-Meier method, and differences between the two groups were compared using the log-rank test. Univariate and multivariate regression analyses according to the Cox proportional hazards regression model, with disease-specific survival as the dependent variable, were used to evaluate livin expression and anti-livin antibody as potential independent prognostic factors. $P < 0.05$ was consid-

ered to indicate statistical significance. The calculations were performed with JMP software (SAS Institute, Cary, NC).

RESULTS

Expression of Livin in RCC Tissues

By immunohistochemical staining, livin was detected in 26 (57.8%) of the 45 RCC specimens. There were 19 cases of staining score 0, 15 cases of score 1, 3 cases of score 2, and 8 cases of score 3. The association between livin expression and clinicopathologic characteristics is shown in Table 1.

Serum Anti-livin Antibody in RCC Patients

The absorbance value of ELISA detecting the anti-livin antibody in sera from RCC patients and healthy volunteers was 0.17 ± 0.09 and 0.08 ± 0.03 , respectively (Fig. 2A). The cutoff value for the positivity in the anti-livin ELISA was determined from the absorbance values of healthy volunteers' samples as the mean absorbance value + 2 standard deviations (SD) (0.14). On the basis of this criterion, serum samples from 25 (55.6%) of the 45 patients were positive for the anti-livin autoantibody. However, there was no significant relationship between anti-livin autoantibody and any clinicopathologic factor.

Western blot analysis for the serum anti-livin antibody in an RCC patient demonstrated immunoreactivity to

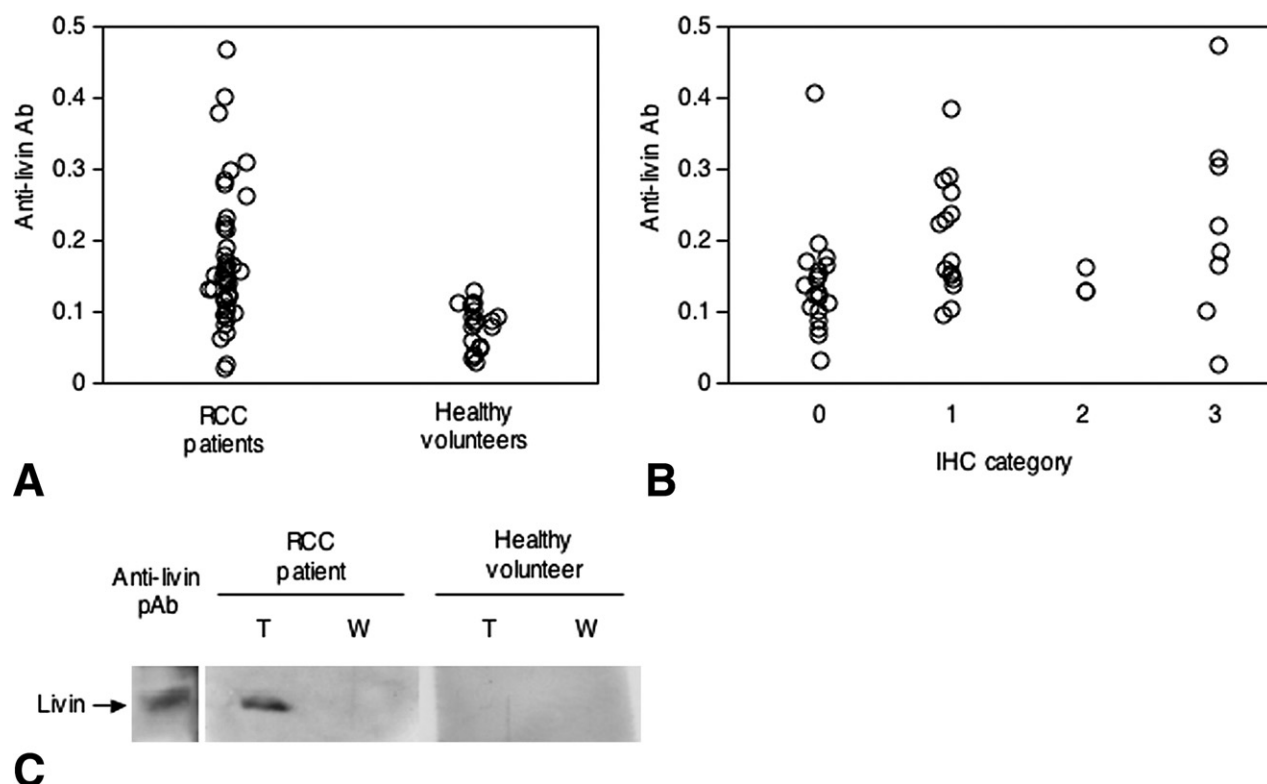


Figure 2. (A) Serum anti-livin antibodies of healthy volunteers and RCC patients. (B) Serum anti-livin antibody titers and categories of immunohistochemistry (IHC) in RCC tissues. (C) Western blot analysis for immunoreactivity of serum to livin protein in 293T cell lysates. Lane 1, positive control of polyclonal anti-livin antibody. Lanes 2 and 3, serum from an RCC patient. Lanes 4 and 5, serum from a healthy volunteer. pAb = polyclonal antibody; T = livin-transfected 293T cell lysate; W = wild-type 293T cell lysate.

livin protein expressed in the livin cDNA-transfected 293T cells. There was no immunoreactivity in serum from a healthy volunteer (Fig. 2B).

The mean ($\pm$ SD) absorbance value of the anti-livin antibody in patients with livin-positive RCC and in patients with livin-negative RCC was 0.20 ± 0.10 and 0.13 ± 0.08 , respectively. There was a significant difference in the values between the two groups ($P = 0.013$).

Association of Livin Expression or Anti-livin Antibody with Disease-Specific Survival

The 5-year disease-specific survival was 81.6% for all patients. Univariate analysis revealed that pathologic T stage, TNM stage, and tumor diameter were significant factors influencing disease-specific survival of patients with RCC (Table 2). Multivariate analysis revealed no significant factor that affected disease-specific survival. In both analyses neither livin expression nor the anti-livin antibody was a significant factor. The 5-year survival rate was 81.7% and 81.2% in the livin-positive and livin-negative arms, respectively (Fig. 3A). The 5-year survival rate was 81.1% and 82.3% in the anti-livin Ab-positive and anti-livin Ab-negative arms, respectively (Fig. 3B). There was no difference between the two groups in either comparison. Moreover, there was no difference in survival among the four groups stratified according to immunohistochemistry scores.

Table 2. Results of univariate Cox regression analysis for disease-specific survival

Factors	Risk Ratio Label (95% Confidence Interval)	P Value
Clinical stage	1.78 (1.05–3.11)	0.033
Fuhrman grade	2.07 (0.78–6.12)	0.147
Tumor diameter	3.99 (1.05–18.0)	0.042
Pathologic T stage	1.70 (1.08–2.72)	0.022
Tumor necrosis	0.79 (0.12–3.27)	0.762
Livin expression	1.91 (0.47–7.25)	0.347
Anti-livin antibody	1.02 (0.27–4.13)	0.975

COMMENT

To the best of our knowledge this is the first report demonstrating livin expression in RCC. Fifty-eight percent of the RCC cases expressed livin immunohistochemically, but there was no relationship between livin expression in tumors and any clinicopathologic parameter or disease-specific survival. In other words, livin may not be a prognostic marker for RCC. In contrast, Parker *et al.*¹¹ have reported that expression of survivin, another IAP family member, is an independent predictor of clear cell RCC progression. Although there are limitations due to the small sample size and the relatively better prognosis in this study, our results suggest that the characteristics and role of livin in RCC progression might be different from those of survivin.

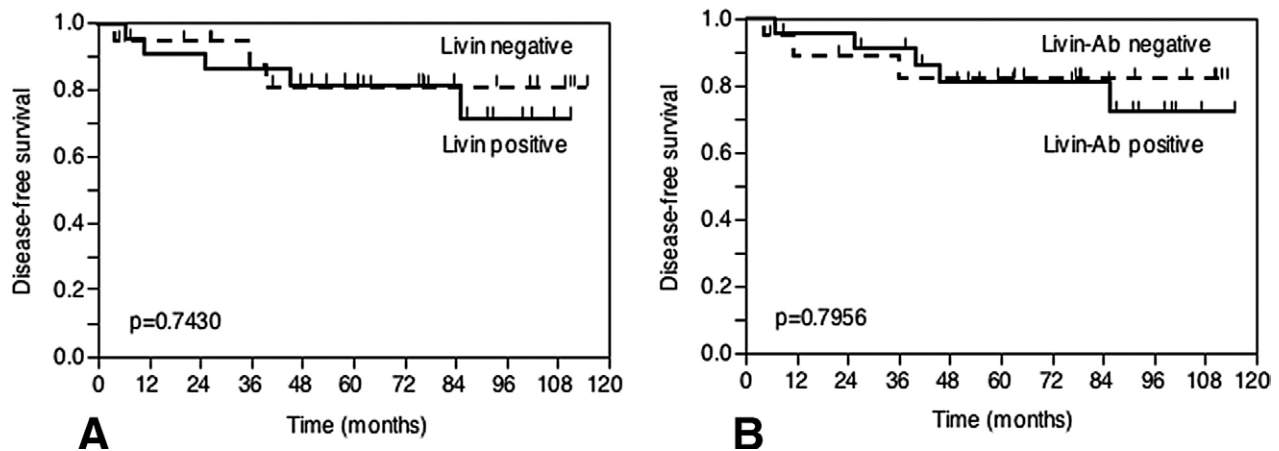


Figure 3. Disease-specific Kaplan-Meier survival curves of 45 patients stratified by livin expression in RCC tissues (**A**) and serum anti-livin antibodies (**B**).

We also demonstrated that 56% of RCC patients had high anti-livin autoantibody titer in their sera. There was a significant relationship between livin expression in RCC tissues and the titer of the anti-livin Ab, but the survival of patients with high anti-livin Ab titers was not different from that of patients with low titers. Moreover, 7 patients (16%) had low anti-livin Ab titers although their RCC tissues expressed livin. Those results suggest that the amount of the anti-livin Ab produced during the natural history of RCC did not reflect antitumor responses. However, Schmollinger *et al.*⁴ reported that vaccination with irradiated autologous melanoma cells engineered to secrete granulocyte macrophage colony-stimulating factor by retrovirus-mediated gene transfer induced anti-livin Abs, and the reactions were associated with cellular immune responses, including livin-specific CD4+ and CD8+ T cells. We plan to evaluate livin-specific cellular immune reactions in RCC patients and start a clinical trial of vaccination targeting livin, evaluating the relationship between the anti-livin Ab titer and the cellular immune and clinical responses. Furthermore, it would be interesting to evaluate whether RCC patients with high anti-livin Ab titers show good responses to immunotherapies.

There were 6 patients (13%) with the anti-livin Ab in serum despite having score 0 for livin expression in the RCC tissue. It is possible that less than 10% of livin-positive cells might affect the immune system in patients because livin is not expressed in normal tissues.^{1,3,5} It is also speculated that livin-positive RCC cells might be rejected immunologically in the early phase of progression and that only livin-negative ones might remain and progress in such patients. In any case there was no healthy volunteer with a high anti-livin Ab titer. Therefore, it seems that tumor-expressed livin can be recognized by the host immune system in patients with high anti-livin Ab titers.

CONCLUSIONS

We have demonstrated that livin is expressed in approximately 60% of RCC cases. Livin expression may not

provide predictive information, but the anti-livin Ab titer in the sera of RCC patients was significantly higher than that in healthy volunteers. Although the immune responses without immunotherapeutic stimulation did not affect patient survival, livin may be recognized as a tumor antigen by the immune system in RCC patients. These findings provide a rationale for further evaluation of immune responses by a tumor vaccine targeting livin.

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References

1. Kasof GM, and Gomes BC: Livin, a novel inhibitor of apoptosis protein family member. *J Biol Chem* **276**: 3238–3246, 2001.
2. Hussein MR, Haemel AK, and Wood GS: Apoptosis and melanoma: molecular mechanisms. *J Pathol* **199**: 275–288, 2003.
3. Lin JH, Deng G, Huang Q, *et al*: KIAP, a novel member of the inhibitor of apoptosis protein family. *Biochem Biophys Res Commun* **279**: 820–831, 2000.
4. Schmollinger JC, Vonderheide RH, Hoar KM, *et al*: Melanoma inhibitor of apoptosis protein (ML-IAP) is a target for immune-mediated tumor destruction. *Proc Natl Acad Sci U S A* **100**: 3398–3403, 2003.
5. Hariu H, Hirohashi Y, Torigoe T, *et al*: Aberrant expression and potency as a cancer immunotherapy target of inhibitor of apoptosis protein family, Livin/ML-IAP in lung cancer. *Clin Cancer Res* **11**: 1000–1009, 2005.
6. Ashhab Y, Alian A, Polliack A, *et al*: Two splicing variants of a new inhibitor of apoptosis gene with different biological properties and tissue distribution pattern. *FEBS Lett* **495**: 56–60, 2000.
7. Altieri DC: The molecular basis and potential role of survivin in cancer diagnosis and therapy. *Trends Mol Med* **7**: 542–547, 2001.
8. Mahotka C, Krieg T, Krieg A, *et al*: Distinct in vivo expression patterns of survivin splice variants in renal cell carcinomas. *Int J Cancer* **100**: 30–36, 2002.
9. Shariat SF, Lotan Y, Saboorian H, *et al*: Survivin expression is associated with features of biologically aggressive prostate carcinoma. *Cancer* **100**: 751–757, 2004.
10. Yagihashi A, Asanuma K, Tsuji N, *et al*: Detection of anti-livin antibody in gastrointestinal cancer patients. *Clin Chem* **49**: 1206–1208, 2003.
11. Parker AS, Kosari F, Lohse CM, *et al*: High expression levels of survivin protein independently predict a poor outcome for patients who undergo surgery for clear cell renal cell carcinoma. *Cancer* **107**: 37–45, 2006.