2 がん抗原のカテゴリー

がん抗原の多くは腫瘍を認識するCTLを用いたcDNA発現クローニング，DNAマイクロアレイ，RDA法などのDNAサブトラクション法，患者抗体が認識する抗原を解析するSEREX法，マウスを用いて抗原がCTLに認識され得ることを証明するreverse-immunogenetical approachなどで同定される[2, 4, 5]。同定された分子の分類方法は様々であるが，前述したMAGEファミリー遺伝子を同定したBoonらのグループは腫瘍と正常組織での発現形式から，①がん共通特異抗原，②過剰発現抗原，③臓器特異的抗原，④遺伝子変異，という分類を提唱している[6~9]。この分類は，より腫瘍細胞に特異的で，より汎用な，免疫療法による副作用の少ない（自己免疫疾患様症状や正常組織への傷害性が少ない）抗原を予測する上で有用である。これまでに同定された腫瘍抗原の中には，がんの維持・増殖・浸潤・転移・再発などと関わりのある機能を持った分子も存在する。がん化に関して機能を持たない分子よりは，がんとしての形質を発揮する機能的分子を免疫療法の標的とする方が効率的といえる。前述の発現形式からの分類で副作用などを予測しつつ，機能的側面からアプローチすることは免疫療法の戦略を練る上で重要である。

この機能的な分類としてWeinbergらのグループは，このがんとしての形質を発揮するのに基本となる遺伝子群を次の6つに分類している[10]。

(A) 細胞増殖に関わる遺伝子群
(B) 成長抑制シグナルに対する不応答性に関わる遺伝子群
(C) アポトーシス耐性能に関わる遺伝子群
(D) 無限増殖に関わる遺伝子群
(E) 血管新生に関わる遺伝子群
(F) 組織浸潤に関わる遺伝子群

これに加え，近年特に注目されているがん幹細胞の概念も取り入れ（(G) 幹細胞性に関わる遺伝子群），7つのカテゴリーに分類し（図1），各々についてそのカテゴリーに含まれるいくつかの分子について概説する（表1）。

(A) 細胞増殖に関わる遺伝子群

細胞増殖時，正常細胞は分裂促進に働く成長シグナルが必要であり，このシグナルは必要時にのみ活性化する。一方，がん細胞ではある種のがん遺伝子が分裂促進シグナルのように作動し，細胞増殖を促している。これらの遺伝子群はしばしば正常組織にも発現しているが，がん細胞で過剰発現している。これまでの報告から，これらの抗原遺伝子もがん患者のCTLで認識され得ることが判明しており，がん患者生体内では不耐性抗原であることが推測される。このカテゴリーに属する遺伝子はさらに，成長因子受容体に関する抗原，細胞周期に関連する抗原の2つのグ

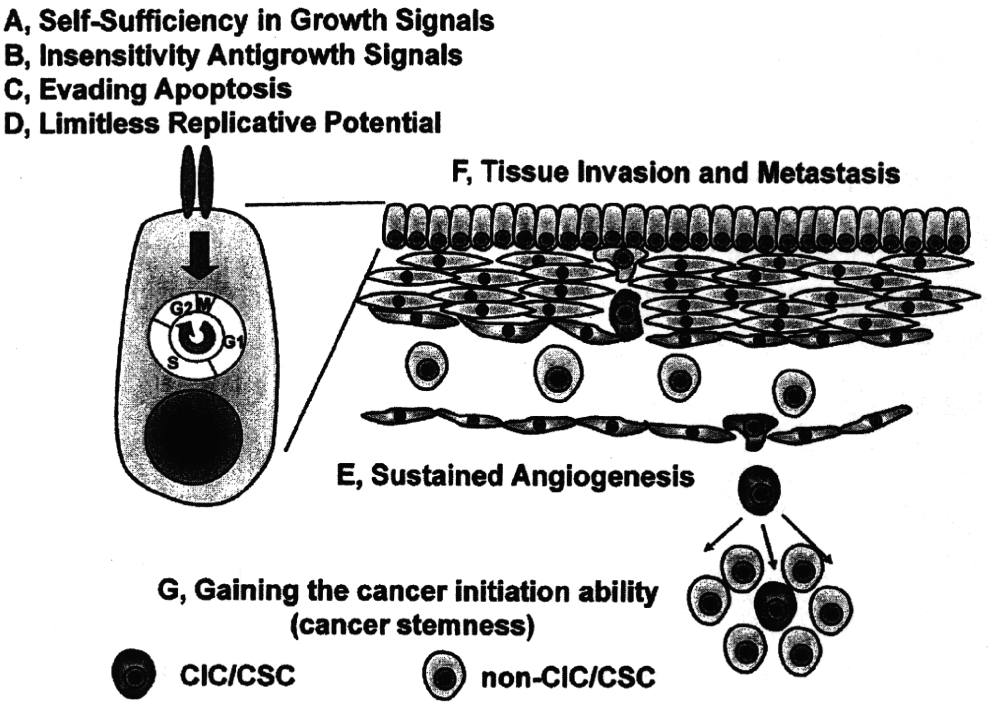

図1　がん抗原の機能的側面に基づいて分類した7つのカテゴリー（文献1)より転用）

表1　抗原ペプチドが同定されているカテゴリー別の代表的抗原

カテゴリー	抗原名
A. 細胞増殖に関わる遺伝子群	$p185^{HER2/neu}$, EGFR, EphA3, FGF5, c-met, cyclin D1, cyclin B1, Aurora-A kinase, Cep55/c10orf3, plk1, STAT1, survivin, survivn 2B
B. 成長抑制シグナルに対する不応答性に関わる遺伝子群	MDM2, Aurora-A kinase
C. アポトーシス耐性能に関わる遺伝子群	Bcl-2, $Bcl\text{-}_{XL}$, BAX-delta, Mcl-1, survivin, survivin 2B, ML-IAP/Livin
D. 無限増殖に関わる遺伝子群	hTERT
E. 血管新生に関わる遺伝子群	VEGF, VEGF-R1, VEGF-R2, RGS5, survivin, survivin 2B
F. 組織浸潤に関わる遺伝子群	MMP2
G. 幹細胞性に関わる遺伝子群	SOX2, SOX10

ループに分けられる。前者には$p185^{HER2/neu}$, EGFRなどが挙げられ，後者にはcyclin D1, Cep55/c10orf3などが挙げられる。

$p185^{HER2/neu}$, EGFRはErbBファミリーに属する受容体型チロシンキナーゼであり，細胞のがん化やがんの進展に関わっているがん遺伝子である[11, 12]。いずれも正常組織でも低レベルではあるが広範に発現しているが，$p185^{HER2/neu}$は乳癌，卵巣癌，大腸癌，胃癌などで高発現しEGFRは多くの悪性腫瘍で高発現している。どちらの分子も抗体療法の標的としても知られているが，CTLの標的となるHLA拘束性抗原ペプチドも多く同定されている[13～20]。ただし，

$p185^{HER2/neu}$抗原配列は免疫原性が低い傾向にあるとの報告もあるため，同時に免疫寛容を克服するような免疫療法デザインが必要と思われる。

Cyclin D1はいくつかのcyclin-dependent kinases（以下Cdk）と結合して細胞周期をS期に誘導する蛋白であり[21]，マントル細胞リンパ腫など一部の血液腫瘍で過剰発現しているのは有名であるが，大腸癌・胃癌・肺癌・乳癌などの固形腫瘍でも高発現している。最近，HLA-A2拘束性抗原ペプチドが同定され，CTLの誘導に成功している[22]。また，Cep55/c10orf3は中心体に局在し，細胞分裂に必須の分子であることが証明され[23]，大腸癌で高発現している遺伝子として同定された[24]。最近，当教室ではマイクロアレイを用いたスクリーニングで乳癌においてCep55/c10orf3高発現していることを見出した。さらにHLA-A24拘束性抗原ペプチドを同定し，この抗原ペプチドを認識する乳癌患者からのCTLを誘導できた[25]。免疫原性が非常に高い分子であり，誘導されたCTLを用いた実験では細胞分裂期以外のCep55陽性細胞をも障害できることから，免疫治療の候補分子として有望だと考えている。

(B) 成長抑制シグナルに対する不応答性に関わる遺伝子群

正常組織では遺伝子変異などを起こした異常細胞が増殖しないようにいくつかの成長抑制シグナルが働いている。細胞周期制御のブレーキの役割を果たすものとして，Cdk阻害蛋白質群（p21・p27など）が知られている。正常細胞ではp21遺伝子の上流にp53の結合配列があり，DNA損傷時にはp53の活性化によりp21が転写され細胞周期停止・細胞増殖抑制に向かう。このp53の機能を抑制している分子の1つにmurine double mutant 2（MDM2）がある。MDM2はいくつかの悪性腫瘍で高発現していることが知られており[26]，細胞増殖抑制に関して理にかなう治療標的といえる。MDM2のHLA-A2拘束性抗原配列に対するCTLを誘導した報告があるがん患者からのCTL誘導が困難であったり[27]，細胞免疫に耐性であるとの報告がある[28, 29]。

(C) アポトーシス耐性能に関わる遺伝子群

正常細胞では不要になった細胞や異常になった細胞をアポトーシスによって排除し，臓器・組織内での規律を保つ機能を有している。正常細胞ではdeath ligandの刺激を受けると，アダプター分子を介してcaspase-8が活性化し，その下流にあるcaspase-3などのエフェクターカスパーゼを活性化してアポトーシスを引き起こしたり，Bidを限定分解することによりミトコンドリアからシトクロムCなどの分子を誘導しミトコンドリア依存性のアポトーシスを引き起こす。細胞内アポトーシスシグナル抑制分子としては，Bcl-2・Bcl-x_Lががん細胞で過剰発現しミトコンドリア依存性のアポトーシスを抑制したり[30]，survivinなどのIAP（inhibitor of apoptosis protein） familyが過剰発現しcaspase-3などを阻害することによりアポトーシスを抑制することが知られている[31]。

Bcl-2，Bcl-x_Lは造血器腫瘍で高発現しているが固形腫瘍でも高発現しており，いずれも

表2　当教室で施行した大腸癌患者のsurvivin 2Bペプチドワクチン療法の第1相試験結果（文献1)より転用）

protocol	Adverse events	Tumor marker (not increased)	CT imaging (SD, PR)	CTL detection (tetramer)
Peptide alone	Anemia, Fever, General malaise	2/10 (20%)	4/10 (40%)	2/10 (20%)
Peptide＋IFA	Induration, Itching, Fever	0/5 (0%)	1/5 (20%)	0/5 (0%)
Peptide＋IFA＋IFN-α	Induration, Itching, Fever, Leucopenia	4/6 (67%)	4/6 (67%)	3/6 (50%)

CTL, cytotoxic T lymphocyte ; IFA, incomplete Freud's adjuvant ; IFN, interferon.

HLA-A2拘束性抗原によるCTL誘導が確認されている[32, 33]。

Survivinはがん細胞では高発現していて正常組織でごく微量に発現しているが，免疫原性が比較的高い遺伝子と考えられている[34]。SurvivinのCTLを誘導するHLA拘束性抗原ペプチドは多数報告がある[35～40]。また，我々はsurvivinのsplicing variantであるsurvivin 2BにコードされるHLA-A24拘束性抗原ペプチドを同定し，消化器癌，口腔癌，尿路上皮癌などで臨床試験を施行している[41]。大腸癌患者で施行した第1相試験の結果を表2に示すが，survivin 2Bペプチドワクチン単独では，腫瘍マーカーの頭打ちが見られた症例は2/10症例（20%），画像上腫瘍縮小（partial responce）・普遍（stable disease）が見られた症例は4/10症例（40%）であったが，interferon-α（以下IFN-α）を併用することにより腫瘍マーカーの評価で4/6症例（67%），画像上の評価で4/6症例（67%）の効果が得られた。また，HLAテトラマー（腫瘍抗原ペプチド・MHC複合体の4量体）を用いた抗原認識T細胞の評価でも，ペプチド単独よりもIFN-αを併用したほうが効果が高い傾向にあった[1]。

(D)　無限増殖に関わる遺伝子群

通常の体細胞では分裂するたびに末端構造のテロメア（(TTAGGG)n）が短くなり，極端に短縮してしまうと染色体構造が不安定となる。染色体構造の安定化のために，テロメアの短縮がある閾値を超えた時点で正常細胞では細胞分裂が停止してしまう。分裂停止した細胞はその後DNA損傷などの傷害を受け，最終的にはアポトーシスが起こり，排除されてしまう。しかし，がん細胞ではテロメラーゼにより短縮したテロメアを伸張する機構が働いており，テロメア長を保っている。テロメラーゼ（ヒトではhuman telomerase reverse transcriptase catalytic subunit：以下hTERT）は正常組織では生殖細胞など一部にしか発現していないが，がん組織においては高頻度に高発現しており[42]，複数のHLA拘束性抗原ペプチドが同定され[43～48]，臨床試験も進行している。

(E) 血管新生に関わる遺伝子群

低酸素部・低栄養部に酸素・養分を供給するために血管形成（血管新生）が引き起こされる。低酸素状態がtriggerとなり転写因子であるHypoxia-induced factor（HIF）が活性化されvascular endothelial growth factor（以下VGEF）が転写・発現亢進し，血管内皮細胞に発現している受容体型チロシンキナーゼであるVEGF受容体（以下VEGF-R）を介して血管内皮細胞の増殖・遊走を引き起こす。VEGF-Rファミリーの中でもこのシグナル伝達の中心的な役割を果たしているのがVEGF-R2であるが，VEGF-R1を介したシグナルも協調して血管新生促進に作用する。がん組織ではこのVEGF・VEGF-Rを介した血管新生が誘導されており，腫瘍の進展に寄与している。VEGFは抗体療法の標的としても有名であるが，最近HLA-B27拘束性抗原ペプチドが同定された[49]。またVEGF-R1・R2ともに，HLA-A2/Kbトランスジェニックマウスを用いた実験系でHLA-A2拘束性抗原ペプチドが同定されている[50, 51]。

(F) 組織浸潤に関わる遺伝子群

がんが組織浸潤するために，細胞外基質を分解する蛋白分解酵素群としてMatrix metalloproteases（以下MMP）遺伝子群が知られている。MMPファミリーには23個の遺伝子が知られているが，その中でもMMP-2，MMP-9はがん細胞での発現が高く，腫瘍の浸潤・転移に関わっていると推測されている。MMPのがん組織での高発現は，予後不良患者，進行がんの患者で見られ，転移の危険性が高いとの報告がある[52]。MMPが患者の予後に影響を与える分子であるため，免疫療法の標的分子として大変魅力的であると思われる。しかし，MMPは正常組織でも発現し免疫寛容が誘導されていることが予想されるため，免疫療法に応用するためには工夫が必要である。MMP-2がHLA-A2拘束性にCTLの標的となることが報告されているが，この報告で同定された抗原ペプチドは$\alpha v\beta 3$陽性細胞にcross presentationされることによって初めてCTLに認識される[53]。全身に発現する自己抗原分子でありながら，腫瘍特異的に反応する機序が解明された貴重な報告である。

(G) 幹細胞性に関わる遺伝子群

近年，がんにも正常組織で見られるような自己複製能・多分化能を有する少数の幹細胞を頂点としたヒエラルキーが存在することが証明されてきている。白血病で研究が先行しているが，固形腫瘍でも盛んに研究が行われている。がん幹細胞（Cancer initiating cells/Tumor initiating cells：以下CIC）は，自己複製能・多分化能を持ち，腫瘍形成能が高いと想定される。CICはがん細胞全体の数%以下であるが，腫瘍の形成・維持に必須であり，がんの再発・転移にも関与していると考えられている。CICを標的とした治療はがん克服のために重要と考えられるが，従来の化学療法や放射線療法に抵抗性であることが知られている。CICに対する免疫療法に関しては未だ解明されておらず，標的となり得るかについての解析は大変興味深い。

SRY（sex determining region Y）-box 2（SOX2）は神経幹細胞の自己複製能を維持するのに必須な遺伝子として同定され，SOX2・Oct-3/4・c-Myc・Klf4を線維芽細胞に遺伝子導入することによりiPS細胞を作成できる[54]。このことはSOX2遺伝子が幹細胞の形質発現に非常に重要な役割を果たしていると考えられる。このSOX2はすでに神経系腫瘍関連抗原としてHLA拘束性抗原ペプチドが同定されている[55]。我々は肺癌からのCICの抗原分子候補としてSOX2を同定し，HLA-A24拘束性抗原ペプチドを同定している（データ未発表）。SOX2はCICを傷害する免疫療法の標的として有望であるが，正常幹細胞に発現していて生体内では免疫寛容を受けている可能性や正常幹細胞を障害することから予測不能な副作用が出る可能性がある。このことから，正常幹細胞には発現していないが幹細胞性維持に必須な遺伝子を同定・標的にしたり，SOX2などの幹細胞性を司る遺伝子の上流で制御するような分子でしかも癌細胞のみに発現している遺伝子を同定・標的にするなど工夫が必要と考えられる。

また，前述したCep55/c10orf3は大腸癌細胞株においてCICにも発現し，同定したHLA-A24拘束性ペプチドを認識するCTLは非CICと同様に，CICも傷害することを掴んでいる（現在投稿中）。

3　結語

がん免疫療法の標的抗原について，機能的側面に基づいたカテゴリーに分類して概説した。個々に列挙した各々に分類される分子は，紙面に限りがあるため，ごく一部しか紹介していない。この機能的側面に即した分類は，がんの生物学的側面を理解する上で有用であるばかりでなく，他の治療法を併用する場合において有用であると考えられる。

化学療法や放射線療法などの既存の治療法は，副作用が強い割には，固形腫瘍では生存率・QOLなどの飛躍的な改善といった大きな成果が得られていないのが現状である。免疫療法はこれらの治療に比べると副作用も比較的弱く，がん細胞にとって「致命的」で「直接的」な作用を期待できる抗原分子をデザインすることも可能である。度重なる化学療法や放射線療法により宿主の免疫能力が低下してしまう前に，これらの既存の治療と相補的な関係で治療デザインすることも可能である。ただ，現状では既存の治療を凌駕する成績は残念ながら得られていない。より効果的な免疫療法が開発できれば，既存の治療とともにスタンダードながん治療として確立されると考えられる。

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Biological Heterogeneity of the Peptide-binding Motif of the 70-kDa Heat Shock Protein by Surface Plasmon Resonance Analysis*

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70-kDa heat shock protein family is a molecular chaperone that binds to a variety of client proteins and peptides in the cytoplasm. Several studies have revealed binding motifs between 70-kDa heat shock protein family and cytoplasmic proteins by conventional techniques such as phage display library screening. However, little is known about the binding motif based on kinetic parameters determined by surface plasmon resonance analysis. We investigated the major inducible cytosolic 70-kDa heat shock protein (Hsp70)-binding motif with the human leukocyte antigen B*2702-derived peptide Bw4 (RENLRIALRY) by using a Biacore system based on surface plasmon resonance analysis. The K_D value of Hsp70-Bw4 interaction was 1.8×10^{-6} M. Analyses with truncated Bw4 variant peptides showed the binding motif of Hsp70 to be seven residues, LRIALRY. To further study the characteristics of this motif, 126 peptides derived from Bw4, each with single amino acid substitution, were synthesized and analyzed for Hsp70 binding affinity. Interestingly, the Hsp70 binding affinity was abrogated when the residues were substituted for by acidic (Asp and Glu) ones at any position. In contrast, if the substitute residue was aromatic (Trp, Tyr, and Phe) or an Arg residue at any position, Hsp70 binding affinity was maintained. Thus, this study presents a new binding motif between Hsp70 and peptides derived from the natural protein human leukocyte antigen B*2702 and may also elucidate some characteristics of the Hsp70 binding characteristic, enhancing our understanding of Hsp70-binding determinants that may influence diverse cellular and physiological processes.

Molecular chaperones of the 70-kDa heat shock protein family perform numerous functions in the quality control of cells that mediate protein folding, translocation, assembly/disassembly, and repair of unfolded proteins damaged by environmental stress (1). Chaperone activity of this protein family is necessary to recognize binding sites of a target native protein, referred to as the binding motif. It has been reported that the endoplasmic reticulum 70-kDa heat shock protein Bip (Grp78) binds to a peptide containing at least seven residues with maximal affinity in the presence of ATPase activity (2, 3). It was also suggested that peptide-binding sites of Bip show a preference for sequences rich in aliphatic residues. Subsequently, from a study employing a phage display library, the binding motif for Bip was revealed to be a heptapeptide with high contents of aromatic and hydrophobic residues (4).

On the other hand, the bacterial cytoplasmic 70-kDa heat shock protein homolog DnaK has a substrate-binding region in the C terminus, and repeated binding/release to substrates is dependent on the ATPase cycle at an ATPase domain of the N terminus (5, 6). Zhu *et al.* (7) reported a crystal structure of the C-terminal substrate-binding domain of DnaK, which was located in a hydrophobic substrate-binding cleft with a central pocket placed between a β sheet and two α helices. The consensus motif recognized by DnaK consisted of peptides comprising a hydrophobic core of 4–5 residues flanked by basic residues (8). Moreover, in virus-infected cells, it is well known that a 60-kDa heat shock protein (Hsp60)[2] and a 90-kDa heat shock protein (Hsp90) bind to hepatitis B virus-derived proteins such as polymerase and transcriptase (9, 10). Recently, it was reported that the host major inducible cytosolic 70-kDa heat shock protein (Hsp70)-binding motif interacted with a heptapeptide from a nucleoprotein of measles virus (11) and stimulated transcriptional activities of this virus (12, 13). Thus, the 70-kDa heat shock protein family is capable of recognizing and binding not only to the structural region of a protein but also to a specific localized peptide motif.

Although 70-kDa heat shock protein family-binding motifs derived from observations of interactions with its many substrate proteins have been proposed, little is known about the binding motif based on kinetic parameters determined by surface plasmon resonance (SPR) analysis. Because target molecules basically bind to 70-kDa heat shock protein in low affinity interactions, it might be difficult to measure binding interaction by conventional methods such as competitive immunoprecipitation.

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[2] The abbreviations used are: Hsp, heat shock protein; Hsc, heat shock cognate protein; SPR, surface plasmon resonance; HLA, human leukocyte antigen; RU, resonance unit.

The use of SPR technology, on which bimolecular interaction analysis (Biacore) is based, provides us with the most expedient approach for measurement of low affinity protein interactions in real time. With the Biacore system, one interaction partner is conjugated on the surface of a sensor chip (ligand), and other binding partners flow over the surface (analyte), facilitating analysis of binding differences between multiple analytes and a single ligand. In this study, we investigated the interaction of Hsp70 with the HLA-B*2702-derived peptide Bw4 (RENLRIALRY, HLA-B*2702 amino acids 75–84) and its various truncated and substituted peptides using a Biacore system. Nössner *et al.* (14) reported that Bw4 can bind to Hsp70 and 70-kDa heat shock cognate protein (Hsc70) and elicit a suppressive effect on T cell function. Because the Hsp70-binding motif basically is a heptapeptide, the aim of this study was to find a new Hsp70-binding motif and its biologic characteristics, if present, using the Biacore system.

Consequently, we demonstrated that Hsp70 bound to Bw4 with $K_D = 1.8 \times 10^{-6}$ M. Using truncated and Ala-substituted variants of Bw4, the Hsp70-binding motif of Bw4 was newly found to be heptapeptide LRIALRY. Moreover, we found that if residues in this motif were substituted for by an aromatic (Trp, Tyr, and Phe) or Arg residue at any position, Hsp70 binding affinity was maintained, whereas with acidic residue substitutions, binding affinity to Hsp70 was markedly decreased. The present study suggests a new binding motif between human Hsp70 and peptides derived from natural protein HLA-B*2702 and also discloses characteristics of the Hsp70-binding motif structure. Our findings may contribute to enhanced understanding of 70-kDa heat shock protein family binding determinants that may influence diverse cellular and physiological processes.

EXPERIMENTAL PROCEDURES

70-kDa Heat Shock Proteins and Synthetic Peptides—Recombinant human Hsp70 protein (NSP-555), the endoplasmic reticulum 70-kDa heat shock protein Bip (Grp78) (SPP-765), and *Escherichia coli* DnaK protein (SPP-630) were purchased from StressGen (Victoria, Canada). All of the peptides were synthesized by Sigma-Aldrich. The peptides were dissolved with 5% Me_2SO (Merck) in phosphate-buffered saline at the indicated concentrations in each experiment.

Surface Plasmon Resonance Analysis using the Biacore System—SPR experiments were performed with a Biacore 3000 system (Biacore, Inc., Uppsala Sweden). With this system, molecules of interest (ligands) are immobilized on a sensor surface, and binding partners (analytes) can then be passed over it in a mobile aqueous phase. Their interaction on the sensor surface can subsequently be monitored in real time without the use of labels. To investigate Hsp70-, Bip-, and DnaK-peptide interactions, we employed Hsp70, Bip, and DnaK proteins as ligands that were immobilized on the sensor surface, and Bw4 (RENLRIALRY, HLA-B*2702 amino acids 75–84) and Bw6 (RESLRNLRGY, HLA-B*0701 amino acids 75–84) peptides as the analytes. It is known that Bw4, but not Bw6, can bind to Hsp70/Hsc70 and elicit a suppressive effect on T cells (14). In addition, truncated and amino acid-substituted peptides of Bw4 also were used as analytes.

Hsp70, Bip, and DnaK were immobilized by amine coupling methods, according to the instruction manual for the Biacore 3000, which utilizes a primary amino group of proteins for covalent attachment to the matrix. Briefly, a dextran layer on a sensor chip that is covalently attached to a carboxymethylated dextran gold surface (CM5 sensor chip; Biacore, Inc.) was activated by injection of a mixture of *N*-hydroxysuccinmide and carbodiimide, creating a reactive ester on the surface for 10 min. Subsequently, 100 μl of 20 μg/ml ligand in 10 mM sodium acetate (pH 5.5) was injected for 10 min and loaded with 1 M ethanolamine for 10 min to block binding to remaining nonreacted ester groups. Finally, the chip surface was washed with running buffer (5% Me_2SO, 2.5 mM magnesium acetate, 2.5 mM ATP (disodium salt; Sigma) in phosphate-buffered saline) to remove any loosely bound ligands. The amounts of immobilized Hsp70, Bip, and DnaK as ligands were 19,000, 14,347, and 7,843 resonance units (RU), respectively, corresponding to sensor surface areas of ~19, 14.3, and 7.8 ng/mm^2, respectively.

To estimate these ligand-peptide interactions, the affinity of the interaction, *i.e.* the equilibrium dissociation constant (K_D), was determined from the level of binding at equilibrium as a function of the sample concentrations by BIAevalution version 4.1 software (Biacore, Inc.). All of the peptides were dissolved with running buffer including 5% Me_2SO, and binding experiments were performed at 25 °C in running buffer with a flow rate of 20 μl/min. To reduce errors in reference subtraction, solvent effects were compensated for by a Me_2SO calibration procedure according to the BIAcore manufacturer's instructions. All of the peptide responses were corrected for Me_2SO bulk differences via the Me_2SO calibration curves that were obtained by the Me_2SO calibration procedures performed at the beginning and end of each experiment.

RESULTS

The Biacore assay based on SPR was used to monitor the Hsp70-, Bip-, and DnaK-peptide interactions. SPR detects molecular interactions because there is a corresponding change in the refractive index if binding occurs as a peptide passes over a prepared sensor surface. The responses on the sensorgram are designated RU. We initially attempted to estimate the binding affinity between Hsp70 and the Bw4 or Bw6 peptide. Each peptide was injected at a concentration of 50 μM for 2 min at a flow rate of 20 μl/min to detect the association phase and loaded with running buffer alone on the sensor chip for 2 min at a flow rate of 20 μl/min to detect the dissociation phase. As shown in Fig. 1*A*, the immobilized Hsp70 on the sensor chip bound to Bw4 peptide, whereas Bw6 exhibited low binding compared with the Hsp70-Bw4 interaction. Bw4 binding to Hsp70 tended to be extremely fast, reaching equilibrium immediately after Bw4 peptide injection, and the complex dissociated just as quickly when the flow was switched to a running buffer, with RU returning to the basal level. Therefore, it was difficult to calculate the kinetic parameters (K_a and K_d) directly. The affinity of interaction was determined from the level of binding at equilibrium as a function of the sample concentration, which is referred to as the equilibrium dissociation constant (K_D).

We attempted to estimate the binding affinity in the Hsp70-Bw4 interaction. Bw4 peptide in concentrations ranging from

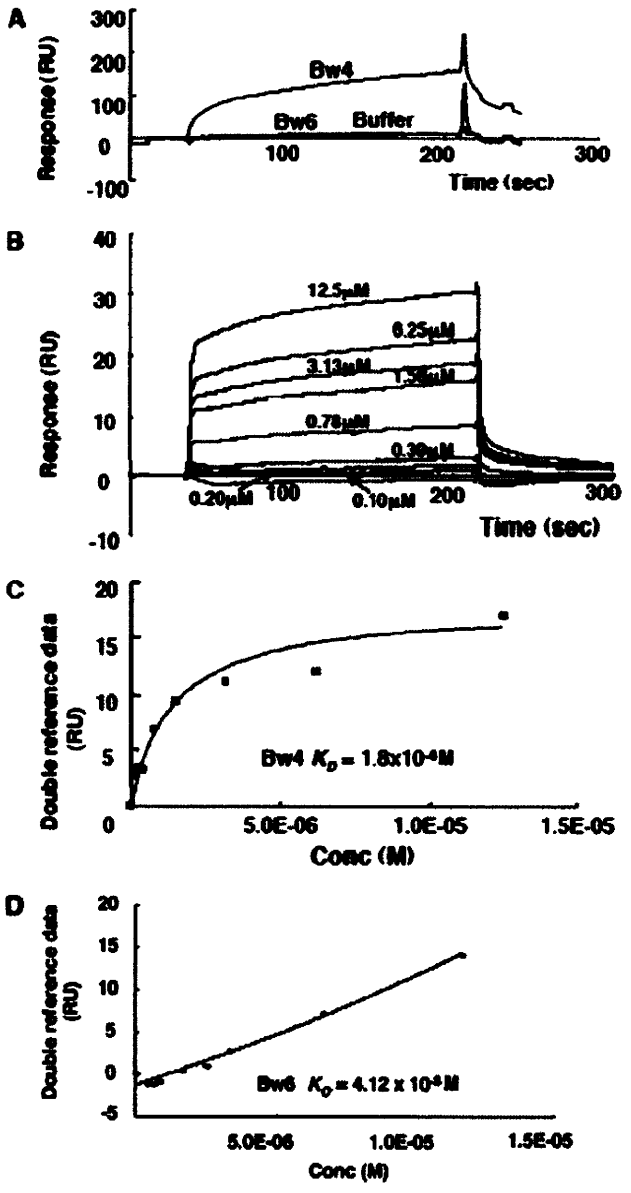

FIGURE 1. **The binding of human Hsp70 to peptides Bw4 and Bw6.** *A*, Biacore 3000 sensorgrams illustrating binding of synthetic peptides Bw4 and Bw6 to immobilized Hsp70. Each 50 μM peptide solution was passed over sensor chips on which 19,000 RU of Hsp70 was immobilized. Retention of a peptide on a sensor chip was indicated by a change in RU over the course of the 200-s injection interval. *B*, overlay plot of sensorgrams of interaction of Bw4 peptide with Hsp70. Bw4 peptides in concentrations (*Conc*) of 0.1, 0.2, 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 μM were injected over the Hsp70 sensor chip, and the magnitude of Bw4 binding to immobilized Hsp70 was analyzed. *C* and *D*, a kinetic plot and binding isotherm for binding of Bw4 or Bw6 to the Hsp70 sensor chip in peptide concentrations ranging from 0.1 to 12.5 μM. Binding affinity was determined from the level of binding at equilibrium as a function of the sample concentration using BIAevaluation version 4.1 software and referred to as the equilibrium dissociation constant (K_D).

0.1 to 12.5 μM was injected over an Hsp70 sensor chip for 2 min at a flow rate of 20 μl/min and loaded with running buffer alone for 2 min at the same flow rate. Association and dissociation phases of the Hsp70-Bw4 interaction at each concentration were shown to occur in a dose-dependent manner (Fig. 1*B*). Subsequently, kinetic analysis based on these data were performed, and the equilibrium dissociation constant (K_D) was determined using BIAevalution (version 4.1) software. As shown in Fig. 1*C*, a specific binding response between Hsp70 and Bw4 was observed, and the estimated K_D for binding was 1.8×10^{-6} M. On the other hand, as shown in Fig. 1*D*, the estimated K_D for binding between Hsp70 and Bw6 in the same manner was found to be 4.12×10^{-3} M, indicating that this binding affinity was ~440-fold lower than the Hsp70-Bw4 interaction.

We also tried to determine whether peptide binding affinity to isotypes of the 70-kDa heat shock protein family such as mammalian endoplasmic reticulum-resident Bip and prokaryotic DnaK molecules was different from Hsp70-Bw4 or -Bw6 interactions. As shown in Fig. 2 (*A* and *B*), it was somewhat surprising that the estimated K_D values for binding of Bw4 to Bip and DnaK were 3.72×10^{-3} M and 1.85×10^{-4} M, respectively, indicating that Bip and DnaK had low binding affinity as compared with Hsp70. In terms of interactions with Bw6, the estimated K_D values for binding to Bip and DnaK were 9.0×10^{-4} M and 4.83×10^{-3} M, respectively (Fig. 2, *C* and *D*).

To investigate characteristics of the binding motif of Hsp70-Bw4 interaction, truncated Bw4 variant peptides were employed. The truncated Bw4 peptides presented in Table 1 were injected over an Hsp70 sensor chip in a similar manner. Truncated 9-, 8-, and 7-mer Bw4 peptides from which N-terminal Arg, Arg-Glu, and Arg-Glu-Asn residues were deleted, respectively, interacted with Hsp70 with high affinity ($K_D < 5 \times 10^{-6}$ M), as did the wild type, but 6-mer (or fewer) peptides could not bind ($K_D > 1 \times 10^{-3}$ M) to Hsp70 (Table 1). In contrast, truncated Bw4 peptides with deletion of only a C-terminal Tyr did not show interaction with Hsp70 ($K_D > 1 \times 10^{-3}$ M), indicating that a C-terminal Tyr residue was critical for binding to Hsp70 and that the end of the binding motif of the C terminus seemed to be Tyr-84 of HLA-B*2702. Therefore, the Hsp70-binding motif interacting with Bw4 was 7-mer amino acid residues, LRIALRY, designated N3-Bw4. We also determined the K_D value of this peptide. As shown in Fig. 3, the K_D for binding between Hsp70 and N3-Bw4 peptide was estimated to be 2.6×10^{-6} M.

Next, key amino acid residues in the motif of Bw4 binding to Hsp70 were studied using synthetic alanine-substituted peptides. Nine of these peptides were loaded over an Hsp70 sensor chip as above. We found that R1A, E2A, and N3A peptides that substituted for alanine at Arg-1, Glu-2, and Asn-3, respectively, maintained high affinities ($K_D < 5 \times 10^{-6}$ M), like the wild type (Table 2), indicating that three N-terminal residues without binding motifs could not influence Hsp70 binding affinity. However, other alanine substitutions at L4A, R5A, I6A, L8A, R9A, and Y10A resulted in loss of binding ($K_D > 1 \times 10^{-3}$ M) to Hsp70 (Table 2). Thus, it was demonstrated that each amino acid residue in the binding motif LRIALRY was critical for binding of Hsp70-Bw4 peptides.

Subsequently, characteristics of the Hsp70-peptide-binding motif were investigated using a peptide array. An array of single amino acid-substituted peptides, which consisted of 126 peptides, of Bw4 wild peptide (10-mer peptide), was synthesized. For example, in the case of substituted peptides at position 8 of the N terminus, a Leu residue was substituted for by 18 amino acid residues: L8W, -Y, -F, -R, -K, -H, -M, -I, -V, -T, -A, -Q, -G, -P, -N, -S, -D, and -E. Cys substitutions were not performed in

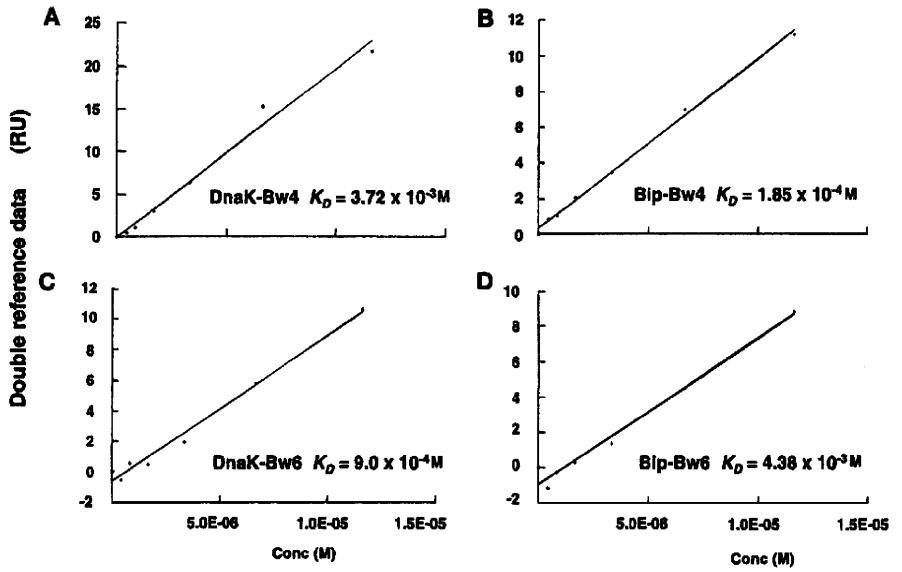

FIGURE 2. **A kinetic plot and binding isotherm for binding of Bw4 to Bip (*A*) and DnaK (*B*) sensor chips and of Bw6 to Bip (*C*) and DnaK (*D*) sensor chips in concentrations ranging from 0.1 to 12.5 μM.** The K_D value was determined using BIAevaluation version 4.1. *Conc*, concentration.

TABLE 1
Binding assay of truncated Bw4 peptides

Name	Sequence of truncated peptide	Binding affinity (K_D)
		M
Bw4	RENLRIALRY	1.8×10^{-6}
N1-Bw4	ENLRIALRY	1.9×10^{-6}
N2-Bw4	NLRIALRY	1.3×10^{-6}
N3-Bw4	LRIALRY	2.6×10^{-6}
N4-Bw4	RIALRY	$>10^{-3}$
N5-Bw4	IALRY	$>10^{-3}$
C1-Bw4	RENLRIALR	$>10^{-3}$
C2-Bw4	RENLRIAL	$>10^{-3}$
C3-Bw4	RENLRIA	$>10^{-3}$
C4-Bw4	RENLRI	$>10^{-3}$
C5-Bw4	RENLR	$>10^{-3}$

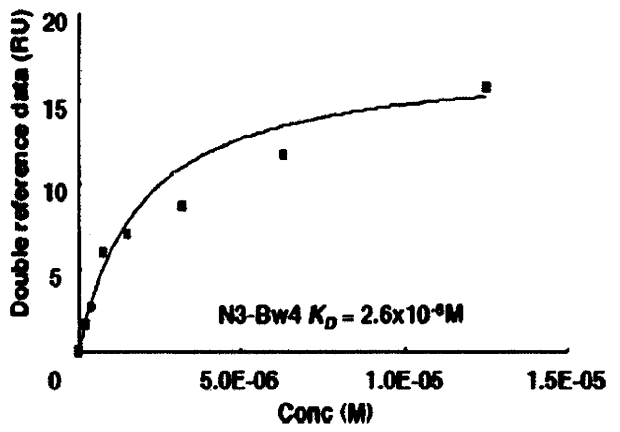

FIGURE 3. **A kinetic plot and binding isotherm for binding of the 7-mer peptide variant derived from Bw4 to the Hsp70 sensor chip in concentrations ranging from 0.1 to 12.5 μM.** The K_D value was determined using BIAevaluation version 4.1. *Conc*, concentration.

this study to avoid multimerization among substituted peptides by S-S binding. All of the peptides were loaded over an Hsp70 sensor chip in a similar manner. As shown in Fig. 4, single amino acid-substituted peptides in white squares represent the high affinity with Hsp70 ($K_D < 5 \times 10^{-6}$ M), whereas peptides in *blue squares* represent decreased binding affinity ($K_D > 1 \times 10^{-3}$ M). Peptides, except for R5Y, with aromatic residues (Trp, Tyr, and Phe) and Arg substitution showed high affinity with Hsp70, like the wild type, at any position, whereas acidic residue (Asp and Glu)-substituted peptides lost their binding affinity ($K_D > 1 \times 10^{-3}$ M).

The estimated K_D values for binding between the Hsp70 and five peptides with aromatic Ile or Arg substituted at position 8 are shown in Fig. 5. These peptides in concentrations ranging from 0.1 to 12.5 μM, were injected over an Hsp70 sensor chip for 2 min at a flow rate of 20 μl/min and loaded with running buffer alone for 2 min at the same flow rate. Specific binding affinity interaction between Hsp70 and these peptides was observed, and the estimated K_D values for binding of L8Y, -W, -F, -R, and -I were 6.7, 1.1, 1.4, 1.4, and 1.8×10^{-6} M, respectively, indicating binding affinity mostly similar to the wild type. Thus, as shown in Fig. 6, it appears that aromatic and Arg residues are favored residues for interaction with Hsp70, whereas acidic residues are not favored.

DISCUSSION

The present study demonstrated, by SPR analysis, a new HLA-derived heptapeptide-binding motif, LRIALRY, interacting with human Hsp70. Nössner *et al.* (14) reported that the Bw4 peptide bound to Hsp70/Hsc70 and elicited a suppressive effect on T cell function. Bw4, consisting of a 10-mer peptide, is derived from the most polymorphic region in the α1 domain of HLA-B*2702, where it is important for self and nonself recognition by T cell receptors. We focused on the interactions of human Hsp70 with native Bw4 and its truncated and amino acid-substituted variants, because peptides bound to Hsp70 are postulated to have immunomodulatory effects such as T cell tolerance or activation in transplantation and tumor immunity. For example, it was reported that the 70-kDa heat shock proteins, Hsp70/Hsc70 bound to HLA-DRB1-derived peptide sequences comprising the shared epitope, such as HLA-DRB1*0401$^{65-77}$(KDLLEQKRAAVDT) and -DRB1*0101$^{65-77}$(KDLLEQRRAAVDT), which are considered to be associated with an increased risk for rheumatoid arthritis. This might be attributed to the enhanced activation of $CD4^+$ T cells against Hsp70/Hsc70-chaperoned antigen peptides via autophagy (15–17). Thus, the interaction between 70-kDa heat shock protein family and HLA molecules seems to provide some biological insights (18–21).

In searching for the peptide-binding motifs for the 70-kDa heat shock protein family, it was first reported that an endoplasmic reticulum protein, Bip, could bind to peptides containing at least seven residues with maximal affinity in the presence of ATPase activity (2, 3). It was also indicated that the peptide-

TABLE 2
Binding assay of Ala-substituted peptides

Name	Sequence of alanine substitution	Binding affinity (K_D)
		M
Bw4	RENLRIALRY	1.8×10^{-6}
Bw4-R1A	AENLRIALRY	1.1×10^{-6}
Bw4-E2A	RANLRIALRY	1.4×10^{-6}
Bw4-N3A	REALRIALRY	2.2×10^{-6}
Bw4-L4A	RENARIALRY	$>10^{-3}$
Bw4-R5A	RENLAIALRY	$>10^{-3}$
Bw4-I6A	RENLRAALRY	$>10^{-3}$
Bw4-L8A	RENLRIAARY	$>10^{-3}$
Bw4-R9A	RENLRIALAY	$>10^{-3}$
Bw4-Y10A	RENLRIALRA	$>10^{-3}$

N3W	L4W	R5W	I6W	A7W	L8W	R9W	Y10W
N3Y	L4Y	R5Y	I6Y	A7Y	L8Y	R9Y	*
N3F	L4F	R5F	I6F	A7F	L8F	R9F	Y10F
N3R	L4R	*	I6R	A7R	L8R	*	Y10R
N3K	L4K	R5K	I6K	A7K	L8K	R9K	Y10K
N3H	L4H	R5H	I6H	A7H	L8H	R9H	Y10H
N3M	L4M	R5M	I6M	A7M	L8M	R9M	Y10M
N3I	L4I	R5I	*	A7I	L8I	R9I	Y10I
N3L	*	R5L	I6L	A7L	*	R9L	Y10L
N3V	L4V	R5V	I6V	A7V	L8V	R9V	Y10V
N3T	L4T	R5T	I6T	A7T	L8T	R9T	Y10T
N3A	L4A	R5A	I6A	*	L8A	R9A	Y10A
N3G	L4G	R5G	I6G	A7G	L8G	R9G	Y10G
N3P	L4P	R5P	I6P	A7P	L8P	R9P	Y10P
*	L4N	R5N	I6N	A7N	L8N	R9N	Y10N
N3S	L4S	R5S	I6S	A7S	L8S	R9S	Y10S
N3Q	L4Q	R5Q	I6Q	A7Q	L8Q	R9Q	Y10Q
N3D	L4D	R5D	I6D	A7D	L8D	R9D	Y10D
N3E	L4E	R5E	I6E	A7E	L8E	R9E	Y10E

FIGURE 4. **Illustration of the Hsp70-peptide-binding motif.** An array of 126 single amino acid-substituted peptides was synthesized based on the Bw4 sequence (10-mer peptide). For example, in the case of substituted peptides at position 8 of the N terminus, Leu was substituted for by 18 different amino acid residues. These are represented as L8W, -Y, -F, -R, -K, -H, -M, -I, -V, -T, -A, -G, -P, -N, -S, -Q, -D, and -E in this figure. *White blocks* represent amino acids with preserved binding affinity for Hsp70 interaction ($K_D < 5 \times 10^{-6}$ M), whereas *blue blocks* indicate loss of binding affinity ($K_D > 1 \times 10^{-3}$ M). The *blocks* with *asterisks* indicate sequential peptides the same as the wild type.

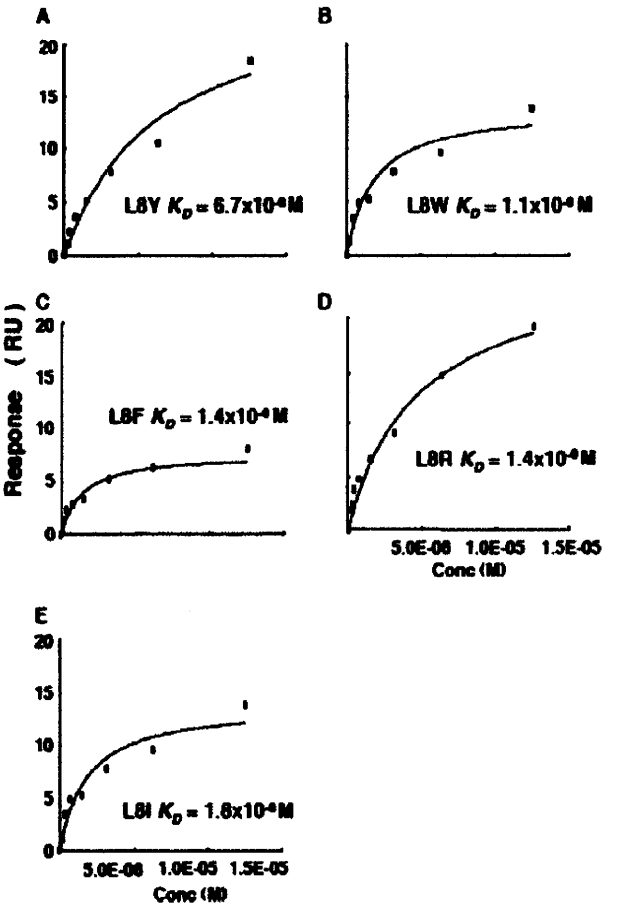

FIGURE 5. **A kinetic plot and binding isotherm for binding of five substituted peptides: L8Y (*A*), L8W (*B*), L8F (*C*), L8R (*D*), and L8I (*E*) to on Hsp70 sensor chip in concentrations ranging from 0.1 to 12.5 μM.** The K_D value was determined using BIAevaluation version 4.1. *Conc*, concentration.

binding site of Bip showed a preference for sequences rich in aliphatic residues. Subsequently, from a study employing phage display library screening, the binding motif for Bip was revealed to be a heptapeptide with high contents of aromatic and hydrophobic residues (4). In the heptapeptide sequence, it was suggested that aromatic and hydrophobic residues occupied alternating positions. On the other hand, bacterial cytoplasmic 70-kDa heat shock protein homolog DnaK has a substrate-binding region in the C terminus, and repeated binding/release to substrates is dependent on the ATPase cycle at the ATPase domain of the N terminus (5, 6). Zhu *et al.* (7) reported the crystal structure of the C-terminal substrate-binding domain of DnaK, which was located in a hydrophobic substrate-binding cleft with a central pocket placed between a β sheet and two α helices. Basically, this domain is considered to bind to substrates by making hydrogen bonds (22). In addition, it was reported that a consensus motif recognized by DnaK consisted of peptides comprising a hydrophobic core of 4–5 residues flanked by basic residues (8). In the current study, the binding motif of the HLA-derived protein LRIALRY consisted of a hydrophobic core of 3 residues flanked by basic Arg residues.

Although such characteristics appeared to be similar to the consensus motif of DnaK, it was interesting that DnaK-Bw4 interaction was ~500-fold lower than Hsp70-Bw4 interaction. The substrate-binding domain of Hsp70 shares ~50% homology in its amino acid sequence with DnaK (18, 23). These structural differences might result in differences of binding affinity. For example, Fourie *et al.* (24) investigated the binding of 36

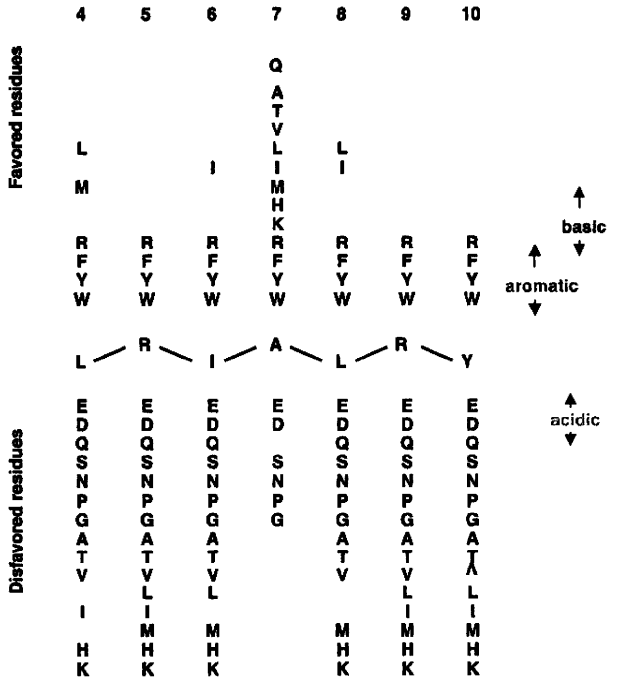

FIGURE 6. **Schematic illustration of the peptide-binding motif to human Hsp70.** Aromatic and Arg residues are substitutable residues that could preserve high affinity to Hsp70, suggesting that they are favored residues. In contrast, substitutions with acidic residues resulted in loss of binding affinity, suggesting that they are disfavored residues.

peptides to Hsc70, Bip, and DnaK by competition assay, demonstrating that there was little common peptide bound to these three proteins with similar binding affinity. It was also reported that mutant p53 protein-derived V10 peptide (substitution of Cys-126 by Tyr) was bound to Bip and DnaK but that V10-Hsc70 interaction was weaker than Bip and DnaK interaction (24). In our Biacore study, this appeared to be true, because the K_D values for binding of Bip and DnaK to V10 peptide were estimated to be 4.28×10^{-5} M and 2.26×10^{-5} M, respectively. In contrast, that of Hsp70-V10 peptide was markedly lower ($K_D > 1 \times 10^{-3}$ M) than the Bip-and DnaK-V10 interaction (data not shown), which was consistent with their data (24). It is known that Hsp70/Hsc70 have very similar biochemical functions and are highly homologous proteins in their primary amino acid sequence except for a short heterologous C-terminal portion with an unknown biochemical function (18, 23, 25–27). Therefore, although we did not confirm the interaction between Hsc70 and V10 peptide, it is highly likely that this interaction could occur similarly to that of Hsp70 and V10 peptide in our current Biacore system.

In the peptide array experiments, when a residue in the LRIALRY motif was substituted for by an acidic residue, the binding affinity to Hsp70 was reduced in all cases. In the case of DnaK, acidic residues were excluded from cores and were found to be disfavored in the flanking region when 4360 peptides derived from 37 biologically relevant proteins were screened (8). In addition, Zhang and Oglesbee (13) reported similar results in which substitutions with acidic residues markedly reduced binding affinity to Hsp70. Thus, acidic residues appear to be disfavored in binding to the human 70-kDa heat shock protein family.

Meanwhile, it was reported that Hsp70 and Hsp90 could transfer peptides produced by the ubiquitin-proteasome system from the cytoplasmic milieu to the endoplasmic reticulum via transporters associated with antigen processing but not by natural diffusion (28, 29). These peptides were then bound to an endoplasmic reticulum-resident chaperone, GP96, and loaded onto major histocompatibility complex class I molecules in the endoplasmic reticulum (30–32). It was also reported that peptides bound to Hsp70 in tumor cells were identified as tumor antigens and that the purified tumor-derived Hsp70-peptide complex elicited tumor-specific cytotoxic T cell immunity *in vitro* and *in vivo* (33–36). We showed here that the new Hsp70-binding motif was a heptapeptide, LRIALRY. Interestingly, in this motif, aromatic residues or an Arg residue could be substituted at any position. These data might help elucidate the interaction of this motif with tumor antigens and suggest the possibility of construction of immunomodulatory cancer vaccines (35, 36).

Our study may also have several biologic implications. When we searched for homologous proteins with the Swiss-Prot data base, there were many such proteins with the same sequence in their primary amino acids as the LRIALRY motif. The majority of these proteins included HLA class I heavy chain molecules such as A*23, A*24, A*25, A*32, B*38, B*49, B*51, B*52, B*53, B*57, B*58, and B*59. When we extended the search for homologous sequences of proteins with residues favored in the motif as illustrated in Fig. 6, there were many human housekeeping proteins such as Hsp40 homologs, α adrenergic receptors, adenylate cyclase, protein ariadne-2, collagen α-3 chain receptor, acetyl-CoA carboxylases, HLA class IIDQ4, DQ-DRW9, tyrosine-protein kinase JAK2, and many other proteins, and the number of such molecules was at least more than 317. In case of Hsp40 homologs among these 317 molecules, recent studies suggested that these protein-derived peptides efficiently activated $CD4^+CD25^+$ regulatory T cells (37–39). Therefore, one explanation for these data is that successful induction of immunosuppressive regulatory T cells might be attributable to Hsp40-derived peptides that could bind to Hsp70 with high affinity. Although the biologic significance of the current peptide-binding motif to Hsp70 is not yet clear, one immunological speculation is intriguing. There is a possibility that Hsp70-chaperoned peptides with our current motif may preferentially enter into the endoplasmic reticulum, make complexes with peptide-HLA class I, and subsequently be expressed on the cell surface. If these interactions happen in the thymus, our current observations might be compatible with the efficient induction of the negative selection of T cells, because peptides with this motif can be derived from not only self HLA class I heavy chains themselves but also many sets of self-housekeeping proteins.

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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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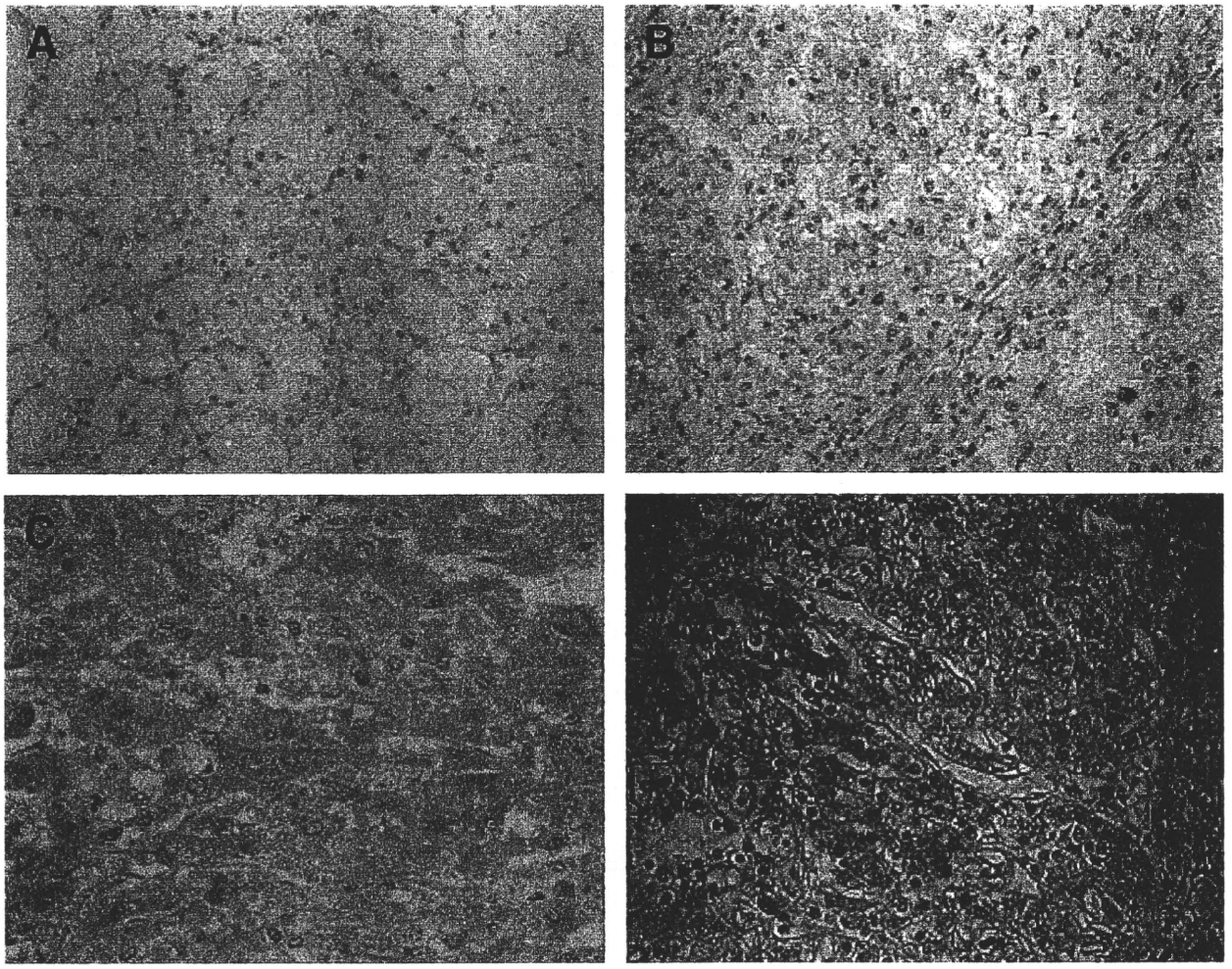

Figure 1. Representative pictures of immunohistochemical staining with a monoclonal antibody reacting to livin in RCC. **(A)** Score 0; **(B)** score 1; **(C)** score 2; **(D)** score 3.

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-μm-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 ×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 diflouride 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 considered 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

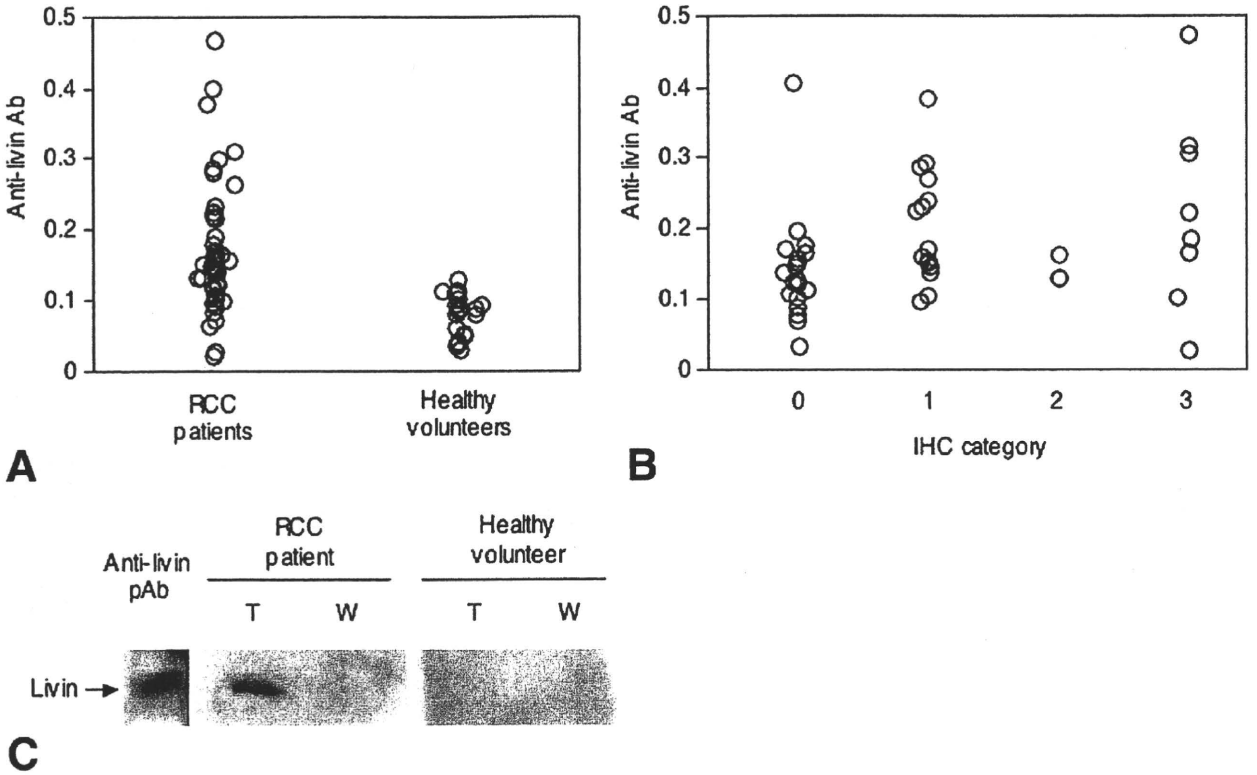

Figure 2. (A) Serum anti-livin antibodies of healthy volunteers and RCC patients. **(B)** Serum anti-livin antibody titers and categories of immunohistochemistry (IHC) in RCC tissues. **(C)** Western blot analysis for immunoreactivity of serum to livin protein in 293T cell lysates. Lane 1, positive control 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 (± 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.

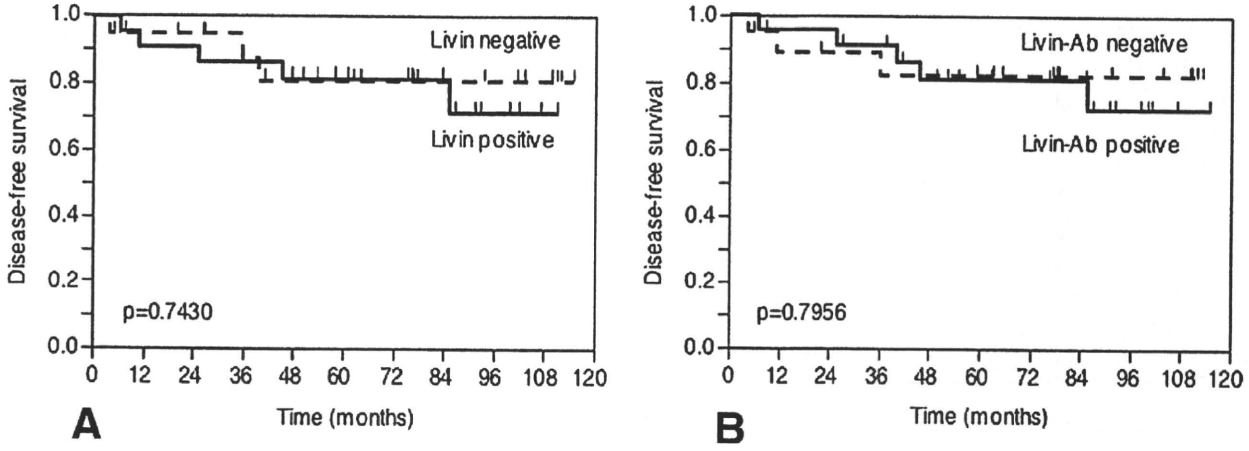

Figure 3. Disease-specific Kaplan-Meier survival curves of 45 patients stratified by livin expression in RCC tissues **(A)** and serum anti-livin antibodies **(B)**.

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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