Identification of autoantibody against fatty acid synthase in hepatocellular carcinoma mouse model and its application to diagnosis of HCC

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Abstract. Autoantibodies, which are generated by immune system recognizing the presence of the abnormal tumor-associated antigens, are promising biomarkers for early detection of tumors. Recently, we established a B cell hybridoma pool derived from H-ras12V transgenic mouse, a typical hepatocellular carcinoma model, as a source of tumor-associated autoantibodies without using any extracellular antigens and have characterized the specific target antigens against them. K1 autoantibody, one of them, was investigated in this study and its target antigen was identified by mass spectrometric analysis as fatty acid synthase (FASN), an important oncogenic protein. Moreover, a specific mimotope against K1 autoantibody was screened from the cyclic random hepta-peptide phage library and, using it as a coating antigen for ELISA, we could distinguish patients with hepatocellular carcinoma (HCC) vs. normal subjects with 96.55% sensitivity and 100% specificity. These results imply that anti-FASN autoantibody is induced in patients with HCC and detection of anti-FASN autoantibody can be used for the diagnosis of HCC.

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Introduction

Currently available serum markers are based on the measurement of tumor antigens, such as α-fetoprotein (AFP), prostate-specific antigen (PSA), and cancer antigen CA125. However, no single marker has been recognized as a true cancer marker due to lack of sensitivity and specificity. To overcome the limitations of these tumor antigen biomarkers, alternative attempts are sought for the development of cancer biomarkers based on the detection of circulating autoantibodies against tumor-associated antigens in patient sera (1).

A variety of techniques to discover novel tumor-associated autoantigens have been developed, such as SEREX (serological analysis of recombinant cDNA expression libraries), two-dimensional electrophoresis/Western blotting of tumor cell lysates, panning of a phage display library constructed using tumor cell cDNA library, and protein microarray (2). These techniques were expected to be powerful, which present thousands of candidate antigens at once to allow analysis of many kinds of autoantibodies in patient sera. But patient sera are mixtures of hundreds of autoantibodies and the amount of each autoantibody is not comparable to another, which makes the discovery of autoantigens biased toward the most abundant ones. For these reasons, tumor-associated antigens discovered by these techniques are not so diverse and useful, despite the expectation that hundreds of tumor-associated antigens and specific autoantibodies against them are present in patient sera.

To identify tumor-associated autoantibodies of hepatocellular carcinoma (HCC), we constructed B-cell hybridoma pool using splenocytes derived from H-ras12V HCC mouse model (3) and the stable hybridoma clones that produce antibodies reactive to HCC cells were selected. Several hundreds of autoantibody-secreting B-cell lines were established and we have analyzed the specific antigens against these antibodies. In

this study, the target antigen against K1 autoantibody was analyzed and revealed as fatty acid synthase (FASN), a well-known oncogenic protein (4). In addition, a specific mimotope of K1 autoantibody was screened from a cyclic random hepta-peptide phage library and, when using it as a coating antigen for ELISA, we could distinguish patients with HCC from normal subjects with 96.55% sensitivity and 100% specificity. These results indicate that anti-FASN autoantibody is induced in HCC patient sera and the detection of anti-FASN autoantibody can be useful for the diagnosis of HCC.

Materials and methods

Cell lines and serum samples. The cell lines were obtained from American Type Culture Collection (ATCC) and cultured in DMEM or RPMI-1640 medium containing 10% fetal bovine serum. All cell lines originated from humans, except Hepa-1c1c7, which is a mouse hepatoma cell line. Human HCC serum samples were collected from Catholic Hospital (Seoul). Normal serum samples were collected from volunteers or patients without cancer. Serum samples were kept at -70°C until use.

Preparation of monoclonal autoantibodies reactive to hepatoma cells. Splenocytes from H-ras12V transgenic mice (3) at about 10 months old were used for the construction of B-cell hybridoma cell pool producing tumor-associated autoantibodies. Cell fusion and selection of B-cell hybridoma were performed as described previously (5). The reactivity of antibodies produced by these B-cell clones was determined by intracellular staining of HepG2 or Hepa-1c1c7 cells with each autoantibody, followed by flow cytometric analysis as described below. The isotypes of each autoantibody were determined using an isotyping kit (Pierce). For preparation of K1 autoantibody, ascites fluid was produced and antibody was purified using protein L-agarose (Pierce).

Flow cytometric analysis of autoantibodies. Cells were fixed and permeabilized with BD cytoperm/cytofix solution (BD), followed by a incubation with primary antibody solution (hybridoma cell cultured media or purified antibody) at 4°C for 40 min. Cells were washed and stained with anti-mouse Ig goat $(Fab')_2$-RPE (Dako). The stained cells were analyzed by FACScalibur (BD) and obtained data were analyzed using CellQuest software (BD). When determining whether the auto-antibody-mimotope phage can compete with target cellular antigen for the antibody binding, primary antibodies were pre-incubated with each phage at room temperature for 60 min.

Preparation of whole cell lysates and Western blot analysis. Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS) supplemented with 1 mM EDTA, protease inhibitors (Roche), and phosphatase inhibitors (Roche). Equal amounts of protein (50 μg) were resolved by SDS-PAGE and transferred onto a polyvinylidenedifluoride membrane (Millipore). The membranes were probed with K1 antibody and HRP-linked anti-mouse Ig GAM antibody (Abcam). Positive bands were detected by chemiluminescence (GE).

Immunofluorescence microscopy. Cells plated on 18x18-mm glass coverslips in 6-well plates were treated with BD cytofix/cytoperm solution at 4°C for 40 min. Fixed/permeabilized cells were incubated with K1 antibody diluted in BD cytoperm/wash solution (5 μg/ml). After an 1-h incubation at 4°C, cells were washed and incubated with FITC-conjugated anti-mouse immunoglobulin $F(ab')_2$ antibody at 4°C for 1 h. Coverslips were mounted with Vectshield medium containing DAPI (Vector Laboratories) and analyzed using a Zeiss LSM510 Meta microscope (Carl Zeiss MicroImaging).

Panning of the phage library against K1 antibody. For the selection of the mimotope specific to K1 autoantibody, the phage display random cyclic peptide library, Ph.D. - C7C™ (New England Biolabs), was used following the manufacturer's instructions. Briefly, $2x10^{11}$ phage virions were incubated with 300 ng K1 antibody, followed by affinity capture of antibody-phage complexes onto protein L-agarose beads. Bound phages were eluted and used for determination of the phage titer or amplification. The amplified phages were precipitated using 20% PEG/2.5 M NaCl and used for the next round of panning. Panning was repeated four times and sequencing of selected mimotope phages was performed following the manufacturer's instructions.

Phage ELISA. The ELISA plate (Maxisorp; Nunc) was coated with selected phage (10^{10} pfu/well) in 0.1 M sodium carbonate buffer (pH 8.6). After the wells were blocked with protein-free blocking buffer (Pierce), primary antibody solution (100 ng purified mAb/100 μl blocking buffer) was added and incubated at room temperature for 2 h. For the secondary reagent, HRP-linked anti-mouse immunoglobulin GAM antibody (Sigma; 1:2000 diluted in blocking buffer) was used. Tetramethylbenzidine-based substrate solution (Pierce) was used for the color development. For evaluation of the effect of reducing disulfide bonds on peptide antigenicity, cyclic phages were reduced and alkylated and then coated onto ELISA plates following the methods of Rojas *et al* (6). For the detection of reactivity of patient's sera to specific phages, human sera (1:1000 diluted in protein-free blocking buffer) pre-adsorbed with empty phage (10^{10} pfu/100 μl) and ER2738 extract (20 μg/100 μl) were used and detected by HRP-conjugated anti-human immunoglobulin GAM antibody (1:2000 diluted in protein-free blocking buffer). Empty M13 phage without a peptide insert was used as control coating antigen. ELISA results were evaluated by a receiver operating characteristics (ROC) curve using Prism 5 (GraphPad Software).

Immunoprecipitation of target protein and mass analysis. For the immunoprecipitation of target protein against K1 auto-antibody, purified K1 antibody was conjugated to AminoLink plus coupling resin (Pierce) following the manufacturer's instructions. Then, Hepa-1c1c7 cell lysates prepared in RIPA buffer (2 mg/ml) were incubated with K1 antibody-conjugated beads overnight at 4°C. Elution was performed twice with 0.5 mM K1p7 peptide (Table I; Peptron) dissolved in PBS and eluate was concentrated using Speedvac. The concentrate was run on 8-10% SDS-PAGE and subjected to immunoblotting or Coomassie staining. The bands corresponding to those probed by K1 antibody were excised and used for in-gel digestion. Protein identification was performed by using Nano-LC/ESI-MS/MS as described by Lee *et al* (7).

siRNA transfection. HepG2 cells were transfected with siRNA against FASN (Bioneer) by using Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer's instructions. RT-PCR or Western blotting was performed at 72 h after transfection. The sequences of siRNA against FASN are as following; sense: 5'-CUGUAACUGUCAGUGUACA (dTdT)-3', antisense: 5'-UGUACACUGACAGUUACAG (dTdT)-3'.

RT-PCR analysis. Total RNA was extracted from cells using Qiagen RNA extraction kit (Qiagen) and the first-strand cDNA was synthesized using Superscript III (Invitrogen). RT-PCR for FASN was performed using the following primer pairs: forward primer: 5'-CCCCTGATGAAGAAG GATCA-3', backward primer: 5'-ACTCCACAGGTGGGAA CAAG-3'.

Immunohistochemical staining of HCC tissue. The immunohistochemical studies were performed in formalin-fixed and paraffin-embedded 4-μm thick tissue sections, using K1 autoantibody (5 μg/ml) and the streptavidin-biotin peroxidase complex method (3).

Results

B-cell hybridoma clones producing tumor-associated autoantibody generated from H-ras12V transgenic HCC mouse model. To overcome the limitations of previous studies about tumor-associated autoantibodies, we intended to separate each tumor-associated autoantibody by separating each autoantibody-producing B cells, which was possible by construction of B cell hybridoma pool using splenocytes derived from HCC mouse model and clonal selection of B cell hybridoma which produce tumor-associated autoantibodies.

Wang and colleagues established H-ras transgenic mice using H-ras12V gene under the control of the mouse albumin enhancer/promoter (3). Ras and related signal pathways play important roles in hepatocarcinogenesis (8). Ras/ERK upregulation is highest in human HCC with a poor prognosis and positively correlates with tumor proliferation, genomic instability, and microvascularization. Although the mutational activation of RAS protein occurs in hepatic tumors with an incidence of 5-58% (9,10), the receptor-mediated hyperactivation of RAS-dependent signal transduction pathway is a frequent event in human hepatocarcinogenesis (11,12). Hepatic tumor formation in H-ras transgenic mice showed a significant bias toward the males, which is consistent with the male prevalence of hepatic tumors in humans. Moreover, the MEK/ERK pathway is more strongly activated in male H-ras12V transgenic mice than it is in females. These results indicate that the H-ras12V transgenic mouse is a valuable animal model for use in studies on the molecular mechanisms associated with hepatocellular oncogenesis.

We obtained the splenocytes from these H-ras12V transgenic mice (nine male mice about 10 months old) and constructed B-cell hybridoma clones producing HCC-related autoantibodies without immunization of any other exogenous antigen or tumor cells. HCC formation was shown in seven mice (A, C, F, G, E, K and M), from which many more B-cell hybridoma clones were produced than the other two mice (B and D) which did not develop HCC (Fig. 1A). Hundreds of autoantibodies generated by these B-cell hybridoma clones were examined separately by flow cytometric analysis of intracellular stained-tumor cells. The reactivity of autoantibodies which were generated from HCC-bearing mice was higher than that in the other two non-tumor mice, which indicates that generation of tumor-associated autoantibodies is correlated with the extent of tumorigenesis (Fig. 1A). B-cell hybridoma clones, which produce highly reactive autoantibodies, were selected for further studies.

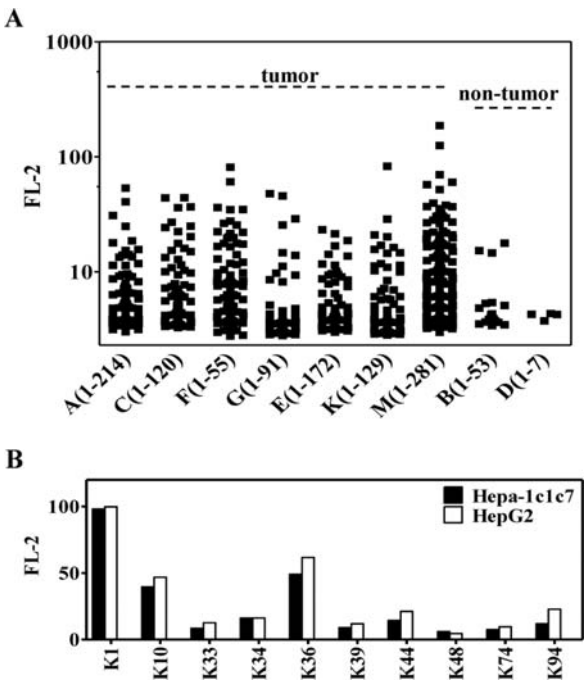

Figure 1. HCC-associated autoantibody, K1, was obtained from H-ras12V transgenic HCC mouse model. (A) Construction of autoantibody-generating B-cell hybridoma pool using splenocytes obtained from H-ras12V HCC mouse model. Immortalized B-cell hybridomas were constructed and autoantibodies produced by these cells were examined by the reactivity against fixed and permeabilized HepG2 cells. Each spot reveals the reactivity of an individual autoantibody. (B) Among nine groups of autoantibody-generating B-cell hybridoma, K mouse-derived cells were analyzed first and the reactivity of K1 autoantibody was predominant.

For the first step of the characterization of these autoantibodies, the isotypes of about 40 autoantibodies were determined. Interestingly, most of the autoantibodies were IgM (27 of 42) and the rest were IgA (11 of 42) or IgG (4 of 42), which may be related to the function or status of these antibodies in the process of tumorigenesis. We expected that the autoantibodies generated in the early stage of HCC formation would be more valuable as early biomarkers. Therefore, we started the analysis of autoantibodies from K mouse which had HCC in small size. Ten of the B-cell hybridoma clones from K mouse were selected and the reactivity of these autoantibodies was compared. As shown in Fig. 1B, the reactivity of K1 autoantibody was much higher than the other autoantibodies, which urged us to analyze it first.

K1 autoantigen expressed in tumor cells. The isotype of K1 autoantibody was IgM, which was purified from cell-cultured media or ascites fluid of K1 B cell hybridoma clone by protein

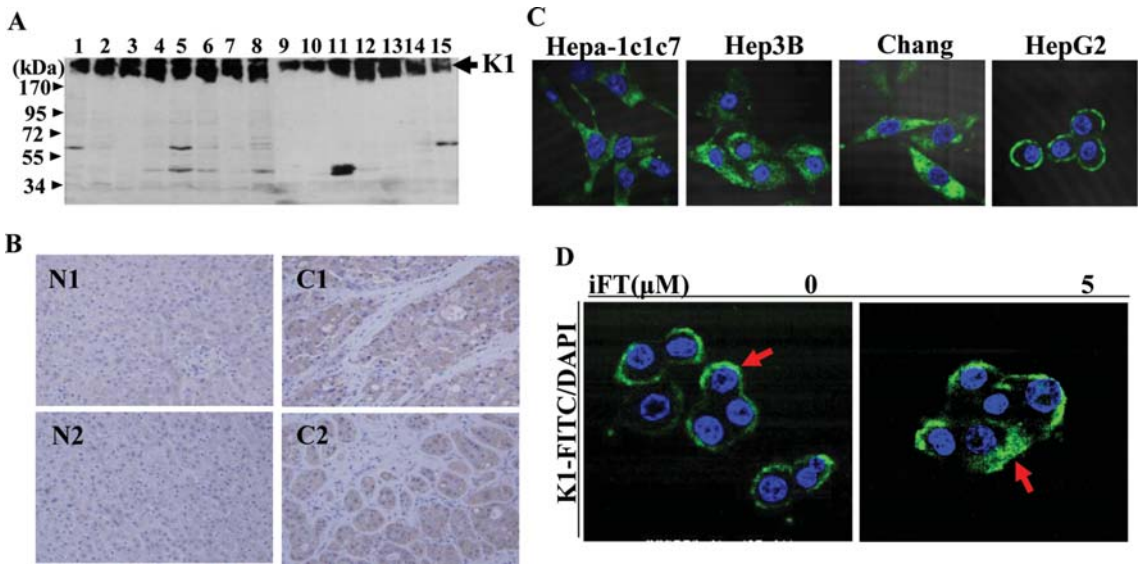

Figure 2. K1 autoantigen expressed in various tumor cells. (A) Western blot analysis on the target antigen of K1 autoantibody. Total cell lysates (50 μg) were separated on 8-10% SDS-PAGE gel, followed by blotting and probing with K1 autoantibody (1, Hepa1c1c7; 2, HepG2; 3, Hep3B; 4, Huh7; 5, PLC/PRF/5; 6, SK-Hep1; 7, Chang; 8, HeLa; 9, SNU601; 10, MCF7; 11, HT29; 12, A549; 15, HT22). (B) Immunohistochemical staining using K1 autoantibody in liver tissue from patients with HCC (C1 and C2) or normal subjects (N1 and N2). (C) Confocal microscopic analysis of intracellular localization of K1 autoantigen in liver cell lines (Hepa-1c1c7, Chang, Hep3B and HepG2). (D) Inhibition of Ras activity induced translocation of K1 autoantigen from plasma membrane to cytoplasm. Farensyltransferase inhibitor was treated on HepG2 cells for 16 h to a final concentration of 5 μM.

L-agarose. Using purified K1 autoantibody, target antigen was analyzed by several methods. As shown in Fig. 2A, K1 autoantibody was able to detect their target antigen by Western blotting. Its target antigen was a protein of a high molecular weight (>200 kDa) and detected in various human tumor cells lines including hepatoma, colon cancer, and breast cancer, which means that its expression itself may not be specific to hepatoma.

To analyze the expression of K1 autoantigen in human hepatoma tissue, immunohistochemical staining using K1 autoantibody was performed. As shown in Fig. 2B, staining of K1 autoantibody was dominant in hepatoma tissue compared to normal. Four cases of hepatoma tissue were stained, of which two were strongly stained and others were stained slightly or similarly to that of normal liver, which might indicate heterogeneity of tumor cells.

Tumor-associated autoantigens, such as ECPKA, GRP78, and PRX, are cytosolic proteins overexpresssed in tumor cells (13-15). However, they are also detected in tumor patient sera (16-18), which induce humoral immune response. Although the mechanism of their secretion from tumor cells is not understood, their post-translational modification seems to be related to their secretion. During the process of secretion, autoantigens might stay in plasma membrane temporarily because tumor-associated antigens must translocate the plasma membrane for their secretion, which might be visualized by microscopic analysis of immunofluorescence-stained tumor cells. When intracellular localization of K1 antigen was analyzed by confocal microscopy, its expression was localized mainly in the cytoplasm of various liver cells (Hepa-1c1c7, Chang, Hep3B), with the exception of HepG2 cell, in which K1 antigen was incorporated in the plasma membrane (Fig. 2C). To examine whether the membrane localization of K1 autoantigen in HepG2 cells is dependent on the activity of Ras in tumor cells, K1 autoantigen was re-examined after treatment of a Ras inhibitor, a farensyltransferase inhibitor (19). As shown in Fig. 2D, treatment of a farensyltransferase inhibitor led K1 autoantigen to translocate from plasma membrane to cytoplasm, which might mean that a post-translational modification on K1 antigen is related to ras activity, which cause temporary association of K1 antigen into plasma membrane.

Table I. Phage-displayed peptides obtained from biopanning with K1 autoantibody.

Clone number	Peptide Sequence[a]
K1-p3	CRMSRRSNC
K1-p7	CMRNRPKRC
K1-p22	CRRRLNRTC
K1-p33	CRMRIRRNC
K1-p34	CHPHPRPRC

[a]Peptide-coding DNA segments from K1 positive clones (20 clones fron Cys-flanked 7-mer library) were sequenced and amino acid sequences were deduced. Five clones of different sequences were identified.

Screening of mimotope against K1 antibody from the cyclic hepta-random peptide phage library. Autoantibody signature captured by using auto-antigenic mimotope-containing phages has been suggested as a potential diagnostic method for prostate cancer (20). Wang and his coworkers screened a phage display cDNA library derived from prostate cancer tissue with patient sera and used their selected mimotope

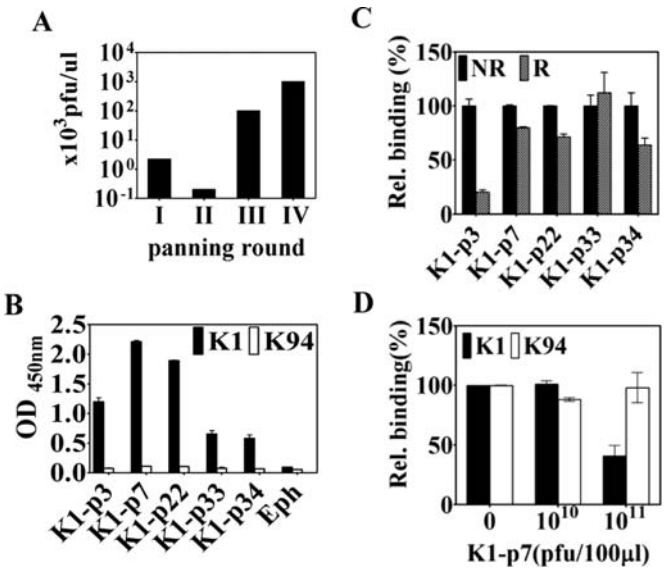

Figure 3. Panning of mimotope phage against K1 autoantibody. (A) For the selection of mimotope specific to K1 autoantibody, biopanning with K1 autoantibody was performed against random cyclic hepta-peptide phage library. (B) The reactivity of selected phages against K1 autoantibody was examined by phage ELISA. Empty phage (Eph) without an insert peptide sequence was used as control antigen and K94, another HCC-derived autoantibody, was used as control antibody. (C) Effect of reducing disulfide bonds on the reactivity of cyclic peptide mimotope. The cyclic mimotope phages, either untreated (NR) or treated with DTT and iodoacetamide (R), were tested for their reactivity against K1 autoantibody. The reactivity of treated phages (%) was calculated compared with untreated controls. (D) Competitive inhibition of K1 antibody binding to HepG2 cells with K1p7 mimotope phage. Fixed and permeabilized HepG2 cells were treated with K1 autoantibody, which was pre-adsorbed with or without K1p7 phages. The reactivity of phage-added reaction was calculated by comparing to the untreated control.

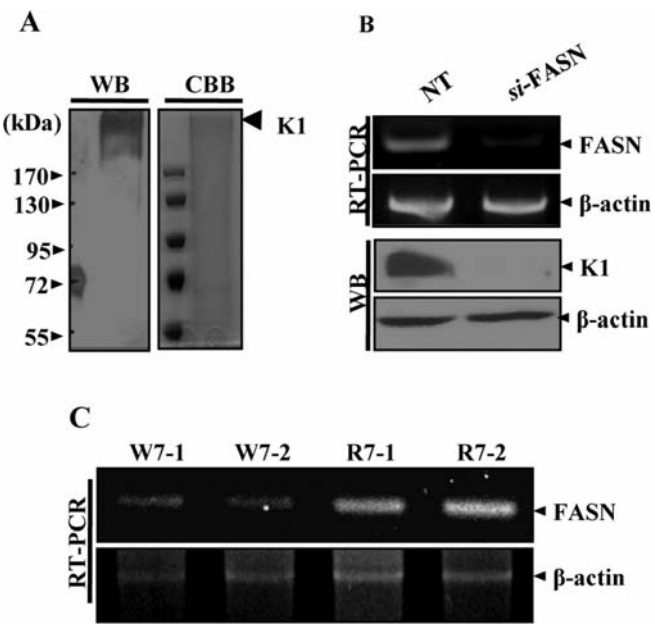

Figure 4. Identification of K1 autoantigen as FASN. (A) Target antigen of K1 autoantibody was purified by using K1 autoantibody-conjugated beads. Purified protein fraction was Western blotted and immunostained with K1 autoantibody or stained with Coomassie Brilliant Blue. The protein band corresponding to K1 autoantigen was identified as FASN by mass spectrometric analysis. (B) Knockdown of FASN using siRNA eliminated the expression of K1 autoantibody-stained protein. Suppression of FASN was analyzed by RT-PCR and the expression of K1 autoantigen was analyzed by Western blotting. ß-actin was used as loading control. (C) RT-PCR analysis on the expression of FASN in liver tissue of H-ras12V HCC mouse model (R7-1 and R7-2). The normal liver tissue (W7-1 and W7-2) was used as a control.

phages for tumor diagnosis successfully. Their results indicate that the antigenicity of autoantigens is restricted to one or two epitopes of a target protein, which makes it possible to detect specific autoantibodies with such defined antigenic structures, without using whole antigenic proteins. The restriction of antigenicity of a certain autoantigen to only one epitope was also shown in the case of GRP78 (17). In this study, we also tried to define antigenic sites against K1 autoantibody using the phage peptide library, which would be convenient 'bait' for the detection of autoantibody in patient sera. We screened the cyclic hepta-random peptide phage library, which is more effective to define the antigenic structure precisely (21). After four rounds of biopanning of the phage library (Fig. 3A), we obtained five phages with different insert peptide sequences (Table I), of which the reactivity against K1 antibody was analyzed by ELISA. As shown in Fig. 3B, K1 autoantibody was reactive to all of these mimotope phages, which have arginine-rich sequences. These phages confer hepta-peptide mimotope as a cyclic form maintained by a disulfide bond. To analyze the conformation-dependency of mimotope-antibody binding, mimotope phages of an intact form or reduced form were prepared and the reactivity was compared. As shown in Fig. 3C, K1 antibody binding was maintained at >50% despite loosening of the cyclic conformation, which means that the binding of K1 antibody is partially dependent on the characteristics of mimotope peptide sequences. Among seven selected phages, the reactivity of K1p7, having an insert peptide sequence of CMRNRPKRC, was highest and it competitively inhibit the binding of K1 antibody to HepG2 cells, which demonstrates that K1p7 phage properly mimics the epitope structure of a specific target antigen (Fig. 3D). Using the insert peptide sequence of K1p7, a candidate protein was searched in protein sequence database, but no appropriate protein was found.

Protein identification of K1 autoantigen as FASN. For the identification of K1 antigen, immunoaffinity chromatography using K1 autoantibody-conjugated beads was performed. Hepa-1c1c7 cell lysate was used as a source of target antigen and specific elution was performed using a synthetic peptide of CMRNRPKRC, the mimotope sequence of K1p7 phage. The eluate was concentrated and separated on 8% SDS-PAGE gel and the protein band corresponding to K1 antigen was excised, in-gel digested with trypsin. The peptide extracts of in-gel digestion were analyzed by Nano-LC-ESI-MS/MS (Fig. 4A; Table II) and, finally, K1 antigen was identified as fatty acid synthase (FASN), one of the well-known metabolic oncogene. To confirm the result of mass spectrometric analysis, HepG2 cells were transfected with siRNA against FASN and total cell lysates were analyzed by Western blotting. As shown in Fig. 4B, the protein band stained with K1 autoantibody in HepG2 cells disappeared when the expression of FASN was shut down.

Table II. Identification of K1 autoantigen by mass spectrometric analysis.

Gene_Symbol=Fasn	Fatty acid synthase	Mass: 272257	Score:80	Queries matched:4
Query	Peptides		Score	Sequence within mouse FASN NP_032014 (1…2504)
1	R.LKEDTQVADVTTSR.C		80	1069-1084
2	R.AKMTPGCEAEAEAEALCFFIK.Q		13	2345-2367
3	K.LGMLSPDGTCRSFDDSGSGYCR.S		12	202-225
4	R.GQCIKDAHLPPGSMAAVGLSWEECK.Q		6	606-632

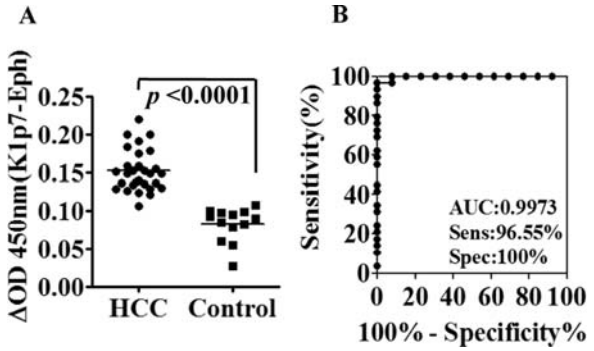

Figure 5. Detection of anti-FASN autoantibody in sera from patients with HCC. (A) Detection of autoantibody against FASN in HCC patient sera using K1p7 phage. The specific binding of autoantibody against K1p7 phage was expressed as the difference between the OD value of K1p7 ELISA and that of empty phage (Eph) ELISA. These experiments were repeated three times. (B) ROC curve of FASN-specific autoantibody ELISA in HCC patient and control sera.

FASN is a large (~265 kDa) homo-dimeric enzyme that is responsible for *de novo* fatty acid synthesis. Normal cells express low endogenous levels of FASN because they obtain significant amount of fatty acid from the diet. In contrast, FASN expression and activity in cancer cells can be extremely high because of increased requirements for long chain fatty acids (22). Also, as mentioned above, the presence of tumor-associated autoantibodies implicates the overexpression of target antigen in tumor tissue. Therefore, we examined the expression level of FASN in H-ras12V mouse model compared to normal subjects by RT-PCR. As shown in Fig. 4C, FASN expression in liver tissue from H-ras12V mice was increased about 2-fold more than in controls. Collectively, these results suggest that FASN was overexpressed in H-ras-mediated hepatocarcinogenesis and induced a specific autoantibody.

Detection of autoantibodies against FASN in HCC patient sera. FASN is mainly expressed in normal hepatocytes and adipocytes, but it is active in cancer cells and the increased expression of FASN has emerged as a phenotype common to most human carcinomas, including colorectal, gastric, mammary, ovary, and prostate adenocarcinomas. Overexpression of FASN in tumor cells is often associated with a poor prognosis. FASN in cancer cells is uncoupled from dietary fat intake and variety of oncogenic changes [(amplification of H-ras, erB-2, EGFR; (24)] result in FASN-catalyzed lipogenesis (24,25). Also soluble FASN in blood is detected in the case of breast or prostate cancer, which is measured by ELISA for cancer diagnosis (26). But until now there were no reports on FASN-overexpression in HCC or tumor-related anti-FASN autoantibody.

Our results showed FASN was overexpressed in Ras-mediated hepatocarcinogenesis and autoantibody against FASN was induced in HCC-bearing model mouse, which implicates that detection of soluble FASN or anti-FASN autoantibody can be used as a biomarker for HCC. In this study, we tried to setup a tumor-diagnostic method by measuring anti-FASN autoantibody in HCC patient sera, because tumor-associated autoantibodies are more efficient as tumor biomarkers than soluble tumor-associated proteins in sera, in the aspects of stability or simplicity (1). We developed indirect ELISA for the detection of autoantibody against FASN using the mimotope phage K1p7 as a coating antigen and human serum as autoantibody source. Human serum is a mixture of thousands of proteins that may induce non-specific antibody-antigen reactions (20). For the correction of the non-specific reaction, pre-adsorption of human sera was performed using ER2738 host cell extracts and empty phage. Empty phage that has no insert mimotope sequence was also used as a control coating antigen. On these conditions, 28 cases of HCC patient sera and 13 cases of normal sera were analyzed and the difference of OD value to K1p7 and empty phage was graphed. As shown in Fig. 5A, the reactivity of HCC patient sera against K1p7 phage was significantly different from that of sera from normal individuals ($p<0.0001$). The sensitivity of this ELISA was 96.55% and specificity was 100% when the cutoff value was 0.114 (AUC, 0.9973; Fig. 5B), which indicates that ELSA composed of K1p7 phage, a mimotope against anti-FASN autoantibody, can be used as an efficient diagnostic method of HCC.

Discussion

In this study, we suggest an anti-FASN autoantibody in patient sera as a novel HCC diagnostic marker. A diagnostic method for HCC was set up by using mimotope phage which could measure anti-FASN autoantibody in patient sera, of which sensitivity was 96.55% and specificity was 100%. This is much more effective for HCC-diagnosis than any other methods measuring tumor-associated autoantibodies or serum markers (27). But, more extensive study with a large

test group is still needed to confirm the utility of this method. Also, the association of anti-FASN autoantibody with other tumors or chronic liver disease must be examined.

Many studies exist on the oncogenic function of FASN, but little information has been appeared regarding extracellular FASN, its secretion mechanism, and induction of autoantibody. FASN in lipid raft was reported in prostate cancer cells (28). FASN is overexpressed in prostate cancer cells, and co-localized with Caveolin-1, a typical palmitoylated lipid-raft protein. FASN has a caveolin-binding motif sequence and co-localization of these proteins is dependent on activation of upstream signaling mediators. In our study, we also observed that FASN is membrane-associated in HepG2 cells and it is dependent on Ras activity. However, until now, the cause of membrane association of FASN was not clearly identified. In our opinion, FASN may be palmitoylated by oncogenic signals, which induces its membrane-association as well as secretion into serum. A study on the relationship between Ras activity and modification of FASN would be an interesting topic, which should be investigated in the future.

K1 HCC-associated autoantibody has been discovered from the B-cell hybridomas constructed from H-ras12V transgenic HCC mouse model. Although sera from patients with tumors are the ideal source for analyzing tumor-associated autoantibodies, a mouse tumor model is a good substitute source of tumor-associated autoantibodies. At first, much concern was voiced about the difference between patients with HCC and the mouse model, but results of analysis on H-ras12V mice removed such worries. Now, hundreds of B-cell hybridoma clones, which were constructed from H-ras12V transgenic mice, are available for the study of tumor-associated autoantibodies. We expect many more tumor marker autoantibodies will be found by studying these autoantibodies and the appropriate combination of these biomarkers could become a more efficient diagnostic method for tumors.

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