

RESEARCH ARTICLE

A protein chip approach for high-throughput antigen identification and characterization

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Proteomics research in humans and other eukaryotes demands a large number of high-quality mAbs. Here, we report a new approach to produce high-quality mAbs against human liver proteins using a combined force of high-throughput mAb production and protein microarrays. After immunizing mice with live cells from human livers, we isolated 54 hybridomas with binding activities to human cells and identified the corresponding antigens for five mAbs *via* screening on a protein microarray of 1058 unique human liver proteins. Finally, we demonstrated that using the five mAbs we could characterize the expression profiles of their corresponding antigens by using tissue microarrays. Among them, we discovered that eIF1A expressed only in normal liver tissues, not in hepatocellular carcinoma in humans.

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1 Introduction

The human liver is the largest organ in the body, and its complexity is probably second only to the brain [1]. It displays various physiological functions involved in metabolism, secretion, biosynthesis, and biotransformation (detoxification). Liver diseases, such as viral hepatitis, liver carcinoma, alcoholic and drug-related liver injuries, and autoimmune

liver disease, pose a great threat to the public health. Understanding of the global molecular networks of liver physiology and pathology will greatly promote the development of diagnostics and therapeutics for these diseases. Thus, the Human Liver Proteome Project (HLPP) was launched in 2002 [2, 3] to address the above issue.

Large-scale proteomic studies require affinity reagents, such as mAbs, to specifically recognize thousands of proteins in a complex sample. Such specific recognition can be used to establish the identity of a given protein. Moreover, mAbs can be used to monitor changes in protein concentration,

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Abbreviations: HCC, hepatocellular carcinoma; HLPP, Human Liver Proteome Project, IHC, immunohistochemical; IP, immunoprecipitation

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localization, and clustering pattern *in situ* in cells and tissues under normal and pathological conditions [4]. The understanding of diseases and protein functions at the proteomic level will be greatly benefited when specific antibodies against each protein are derived and made generally available.

Two major methods have been developed to produce mAbs [5]: the traditional hybridoma fusion [6] and phage antibody display techniques [7]. Although effective and adopted by many companies, the display technologies are not yet user-friendly [8] and still are unable to create high-affinity mAbs without lengthy downstream manipulations [9]. Consequently, most research laboratories use classical hybridoma fusion [10] because, once available, mAbs thus obtained can be produced in relatively large quantity, and they are dimeric, stable, and contain a notably large tag – the Fc domain [8]. Although extremely useful, this technology is not easily adaptable to high-throughput production of the thousands of mAbs required for proteomic studies [4, 5, 8]. One of the major bottlenecks is the need for substantial amounts of highly pure proteins [11].

Here, we report a novel approach to the rapid generation of high-quality mAbs against proteins expressed in human livers and to identification of their corresponding antigens by using protein chips. We further demonstrate that the new mAbs generated in this study are useful in Western blot and immunohistochemical (IHC) analyses and have the potential to be developed into biomarkers for various liver diseases in humans.

2 Materials and methods

2.1 Preparation of immunogens

Human liver tissues from healthy adults were minced and ground into cell suspension in the RPMI-1640 media. The cell suspension was centrifuged at 800 rpm for 10 min, washed three times with 0.01 M PBS, and adjusted to a final concentration of 1×10^8 cells/mL. The remaining tissues were stored in liquid nitrogen for later use. The health human liver tissues were obtained from excised tissues donated by liver injury patients. The study was performed after informed consent being signed and was approved by the Institutional Medical Ethics Committee of FMMU.

2.2 Immunization and generation of hybridomas

Balb/c mice (female, 4–6-wk old) were immunized intraperitoneally with 100 μ L of the liver cell suspension (1×10^8 cells/mL) three times at 2-wk intervals. Three days after the final intraperitoneal immunization, the spleens of the mice were removed, and the spleen cells from the immunized mice were isolated and fused with Sp2/0 myeloma cells at a ratio of 3 Sp2/0 myeloma to 10 spleen cells, using PEG 1500 (Roche). The fused cells were first cultured

in the RPMI-1640 media containing 20% newborn calf serum and 1% hypoxanthine–aminopterin–thymidine (HAT) suspension (Roche).

2.3 Cell immunofluorescent staining

Seven types of cell lines – CCC-HEL-1, QZG, SMMC-7721, ECV304, THP-1, MT4, and Fb – were separately plated at a density of 1×10^5 cells/mL on glass slides and cultured in Petri dishes overnight. Cells were then fixed with cold acetone for 5 min and blocked with 5% nonimmune goat sera for 30 min at 37°C. Appropriate amounts of supernatants of the hybridoma cell culture were added to each slide and incubated for 1 h at 37°C. After several washes with PBS, FITC-conjugated goat anti-mouse IgG diluted 500-fold was added to the cells and incubated for 1 h at 37°C. The hybridomas that showed binding affinity to any of the seven cell lines were scored as positives and selected for further cloning *via* the limiting dilution method.

2.4 Preparation of mAbs

Using the positive hybridoma cells, we prepared mAb using ascites production. 1×10^6 cells *per* mouse were injected intraperitoneally to Balb/c mice. After 8–14 days, ascites were collected and cleaned by centrifuging at 3000 rpm/min for 20 min.

2.5 Cloning of the human full-length ORFs

On the basis of gene annotation and available gene expression data, 3069 pairs of gene-specific primers were designed to PCR-amplify full-length ORFs expressed in human livers. A full-length human liver cDNA library (BD Biosciences, USA) was used as a template in PCR reactions. The products showing the expected sizes were cotransformed with linearized pEGH into yeast cells, and the transformants were selected on SC-URA media [12]. The recombinant plasmids were rescued and sequenced through the upstream junctions between the vector and the ORFs. The resulting sequence traces were batch-blasted against the NCBI database. For those that failed to produce transformants or contained frame-shift mutations, two more attempts were carried out.

2.6 Protein purification and protein chip fabrication

The sequence-verified human ORF constructs were retransformed into yeast, and the yeast transformants were arrayed in a 96-well format and stored as glycerol stocks at –80°C. The recombinant human proteins were purified as described previously [12]. Using a commercial microarrayer, the purified human proteins were spotted onto poly-L-lysine-coated slides at high density.

2.7 mAb identification using protein microarrays

The 54 ascites samples were arrayed in a 7 × 8 format and pooled both vertically and horizontally to form 15 minipools. Each pool of ascites was then diluted 10 000-fold. After blocking, each diluted ascites pool was added to a chip and incubated at room temperature (RT) for 30 min with gentle shaking in humidified chamber. The chips were then washed three times in PBST (1% Tween-20) buffer for 10 min at 42°C with shaking. Anti-mouse IgG antibodies labeled with Cy-3 (Jackson Laboratories, USA, 1:1000 dilution in PBS) were added to the chips and incubated in the dark at RT for 1 h. The chips were then washed three times with prewarmed PBST for 10 min each at 42°C with gentle shaking. After rinsing twice in filter-sterilized, double-deionized water, chips were spun to dryness.

2.8 Data analysis

Each protein microarray was scanned with Scanarray™ Lite scanner (Perkin Elmer, USA). Binding signals were acquired and analyzed using the Genepix Pro 5.1 software (Axon Instruments, USA).

2.9 Western blot

Equal amounts of proteins were separated by SDS-PAGE and transferred onto PVDF membrane by semidry blotting. The membrane was blocked in 5% nonfat milk in PBST for 1 h, incubated with ascites diluted 1:1000 in 5% nonfat milk in PBST for 1 h, and washed three times in PBST. HRP-conjugated anti-mouse IgGs diluted 1:1000 in 5% nonfat milk in PBST was added for a 1-h incubation. After washing three times in PBST, chemiluminescent detection was performed (Pierce, USA).

2.10 Immunohistochemistry

Two kinds of tissue microarrays (Cybrdi, China) were employed to perform IHC staining with mAbs and the Eli-Vision plus method was used for signal visualization. The first type of tissue microarrays contains multiorgan tumor and normal tissues from nine human organs, and the second type contains hepatitis, hepatocirrhosis, hepatoma, and normal tissues from human livers. In brief, sections on these tissue microarrays were dewaxed in xylene, rinsed in alcohol, and graded in alcohol/water mixtures. Then, hydrogen peroxide at a concentration of 30 mL/L was applied to block endogenous peroxidase activity. After antigen retrieval by two times of 5 min microwaving at high power in a citrate buffer (pH 6.0), the sections were blocked by normal goat serum for 30 min. The specimens were subsequently incubated with the primary antibody at a dilution of 1:50 in PBS overnight at 4°C. After rinsing, staining was performed with the Eli-vision™ plus two-step System (Dako, Carpinteria, CA). The color was developed by reacting with 3,3-diaminobenzidine.

Slides were then counterstained with hematoxylin, dehydrated, cleared, and mounted. Negative controls were performed using sera from nonimmune mice or PBS buffer at the incubation step of the primary antibodies.

3 Results

3.1 Production of mouse mAbs

To generate mAbs in mice, we injected live cells from normal human liver tissues into ten mice. After three doses of immunization, the spleen cells of the mice were isolated and fused with myeloma cells to generate 3000 hybridomas (300/fusion). To select for IHC capable mAbs, seven human cell lines – CCC-HEL-1, QZG, SMMC-7721, ECV304, THP-1, MT4, and Fb – were then used to screen the hybridomas for binding activity. Fifty-four hybridomas were found to secrete mAbs that could recognize proteins in human cells (Fig. 1 and Supporting Information Table 1). Ascites samples of the 54 mAbs were then prepared, and the antibody concentrations were determined to be in the range of 10–20 µg/mL.

3.2 Antigen identification using the human liver protein microarrays

Using a mixture of proteins to immunize mice can rapidly generate many mAbs; however, the following step to identify the corresponding antigens has been time-consuming and costly with low success rate in the past due to a lack of proper tools. To overcome such obstacles, we decided here to construct a human protein chip for antigen identification of the 54 mAbs. On the basis of a literature and database search, a total of 3069 primer pairs were designed to PCR-amplify the full-length cDNAs of the genes known to express in human livers. A commercial “full-length” cDNA library generated from human livers was used as PCR templates and we were able to produce 1144 PCR products with expected sizes from the 3069 PCR amplifications because of the low coverage of the library. We next cloned and verified a total of 1058 full-length human cDNAs in a yeast expression vector that can overproduce the human proteins as N-terminal GST fusions [12]. Based on the known mRNA expression profile found in the databases (*e.g.* <http://www.affimetrix.com>), when *e*-value of 0 were used for sequence matching and cutoff at five times average signal for high expression, among 909 matched, 230 were highly expressed in liver and only 17 were house-keeping genes. We also performed Coomassie staining and Western analysis and estimated that ~85.7% fusion proteins were purified with expected molecular weights (Fig. 2A).

We next fabricated a chip of 1058 unique human liver proteins on poly-L-lysine-coated glass slides with additional control proteins. The amount of immobilized proteins on the protein chips was visualized and quantified using anti-GST antibodies (Fig. 2B). We observed that 98.5% (1042/1058) of

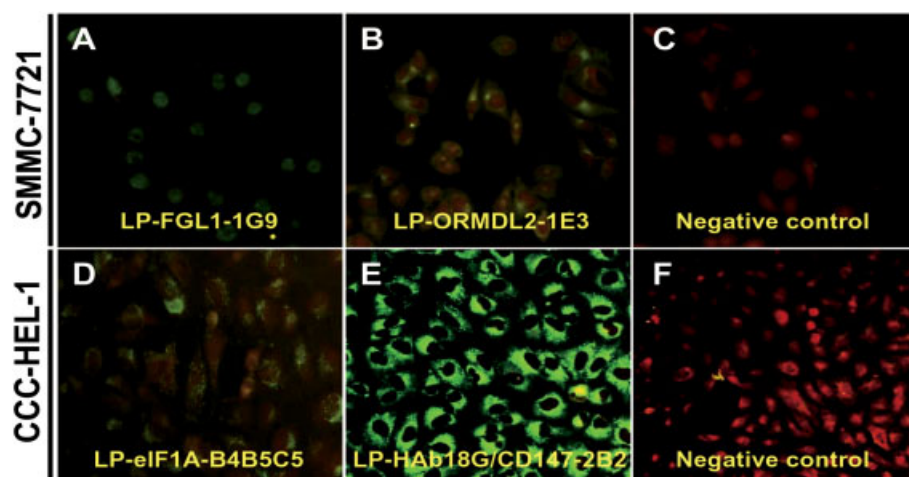


Figure 1. Screening for positive hybridomas using cell immunofluorescent staining. To select IHC capable mAbs, 3000 hybridomas were screened using 7 human cell lines – CCC-HEL-1, QZG, SMMC-7721, ECV304, THP-1, MT4, and Fb – for their binding activity. Fifty-four hybridomas were found to secrete mAbs that could recognize proteins in human cells (Supporting Information Table 1). Positive binding signals are indicated by green fluorescence. The red color is background from Evan's blue buffer. (A, B) SMMC-7721 cells were stained by mAbs LP-FGL1-1G9 and LP-ORMDL2-1E3, respectively. (D, E) CCC-HEL-1 cells were stained by mAbs LP-eIF1A-B4B5C5 and LP-HAb18G/CD147-2B2, respectively. (C, F) SMMC-7721 and CCC-HEL-1 cells were stained by ascites from unimmunized mice as negative controls.

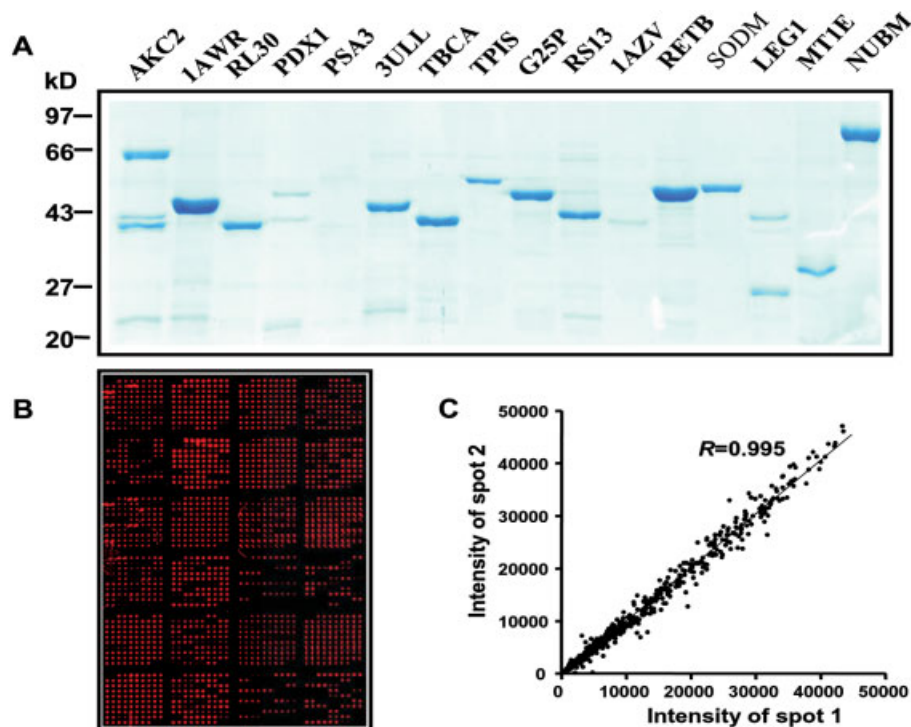


Figure 2. Fabrication of a protein microarray containing 1058 unique human liver proteins. (A) CBB staining of high-level expressed proteins on SDS-PAGE. The 1058 purified proteins were separated with 10% SDS-PAGE and then stained with CBB. Each of the loaded proteins was purified from 1 mL of culture, and 31% of them (329/1058) were visible in gels ($>0.1 \mu\text{g}$). Visible proteins (282 of 329; 85.7%) migrated at their expected molecular weights. (B) Protein microarray probed with anti-GST antibody. All liver proteins were printed on microarray in duplicates (side-by-side) in 24 blocks. Anti-GST antibody was probed to check the printing quality of microarray. The proteins positively detected by the anti-GST antibody are represented in red, and the landmark dots (mouse IgG) at the up-left and down-right corners of each block are invisible. (C) Correlation of spot intensities of duplicates (spot 1 *versus* its duplicate spot 2). The intensities of the duplicate spots of each protein were taken as X and Y positions, respectively, and the correlation coefficient of the resulting diagonal is 0.995, indicating a good reproducibility of the protein spotting.

the proteins showed significant signals above the background and that our protein chips are of high quality (Fig. 2C). Compared with Coomassie staining, which only detected 329 of the 1058 protein (31%) with a detection limit of 100 ng, the detection on the protein chip is much more sensitive, as documented to be as low as 1 ng/mL [13].

To rapidly identify the corresponding antigens of the mAbs, the 54 hybridoma samples were arrayed in a 7 × 8 format to create seven horizontal and eight vertical pools. The resulting 15 pools were separately incubated on the protein chips at a 70 000- or 80 000-fold dilution, and the bound mAbs were detected with Cy3-labeled anti-mouse IgGs. The average signal intensity of the two duplicate spots of each protein was divided by their average GST signal intensity for normalization and then the 1058 proteins were ranked according to their normalized scores. Since the goal was to identify mAbs with high affinity and because we assume that each of the mAbs might cross-react to 2 or 3 proteins on the chips, the top 20 proteins identified by each mAb pool were selected to compare with those by other pools. When vertical and horizontal pools identified one and only one common protein, it indicated that the shared antibody in these two pools specifically recognized that protein without cross-reactivity to other proteins. Thirteen of the 54 mAbs were identified by this means. The normalized scores of 5 of 13 were significantly higher (1.05–2.09) than the rest (0.29–0.66) (see Supporting Information Table 2). Therefore, the top five mAbs with top scores were chosen for further validation. The five mAbs were then individually probed to the human protein microarrays using the above-described protocol. As expected (Fig. 3), they were confirmed to bind to Pirin, fibrinogen-like protein 1 precursor (FGL1), ORM1-like 2 (ORMDL2), eukaryotic translation initiation factor 1A,Y (eIF1AY), and HAB18G/CD147 with high specificity (Table 1). As an example (Figs. 3A and inset, and Fig. 3F), mAb LP-Pirin-3A3b only recognizes a single protein, Pirin, with no obvious cross-reactivity to the other 1057 unique proteins on the array. Comparable specificities were also observed for mAbs LP-FGL1-1G9, LP-ORMDL2-1E3, LP-eIF1A-B4B5C5, and LP-HAB18G/CD147-2B2 against FGL1, ORMDL2, eIF1AY, and HAB18G/CD147, respectively (Figs. 3B–F).

Pirin was first identified as a conserved transcription factor (NFI) binding protein [14]. It is widely expressed in dot-like subnuclear structures in human tissues, particularly

in liver and heart cells [14]. Pirin was later found to bind to an oncoprotein Bcl-3 *in vivo* [15]. Emerging evidence suggests that Pirin may function as a transcription factor regulator to regulate oncogene expression.

FGL1, also known as HFREP-1, and its alternative spliced form LFIRE-1 have been reported to be specifically expressed in normal liver tissues, but not in hepatocellular carcinomas (HCCs) [16]. Although their molecular functions are still unknown, it has been shown that overexpression of wild-type *LFIRE-1/HFREP-1* in human HCC cells suppresses their tumorigenicity in nude mice [16]. On the basis of the facts that LFIRE-1 and HFREP-1 share the most exact amino acid sequences and that they coexpress in many types of tissue, we believe that the IHC staining by LP-FGL1-1G9 reflects the total expression level of both proteins.

ORMDL2 belongs to a newly identified gene family comprising three homologous genes (*ORMDL1*, *ORMDL2*, and *ORMDL3*) in humans and many other eukaryotic organisms, including plants and fungi. Hjelmqvist *et al.* [17] demonstrated that *ORMDL* genes are expressed ubiquitously in human tissues, including liver, lung, and kidney. All three *ORMDL* proteins showed similar ER localization [18]. Studies in yeast also revealed that *Orm2p* might function in protein folding and be involved in the cell-wall-integrity pathway [17, 18].

eIF1AX/Y, the eukaryotic translation initiation factor 1A, stimulates 40S binding of the eukaryotic initiation factor 2 (eIF2)/GTP/Met-tRNA (iMet) ternary complex (TC) and promotes scanning until the complex encounters the initiating AUG codon to form the 40S initiation complex [19]. eIF1A increases the competence of the complex to bind mRNA, and it also stabilizes the TC to the 40S subunit in the absence of mRNA [20]. *eIF1AY* encodes an isoform of *eIF1A* on the human Y chromosome and shares 100% identity with its X-chromosome isoform, eIF1AX, on the amino acid level. As in the case of FGL1, mAb LP-eIF1A-B4B5C5 is not expected to differentiate the expression of the two isoforms in IHC experiments.

The homolog CD147 of HAB18G/CD147 is also known as Basigin or EMMPRIN, a cyclophilin receptor of the immunoglobulin family [21]. Expression of CD147 in different tumor tissues has been reported previously [22], and it can be defined as a therapeutic target and biomarker to predict prognosis in patients [23]. In our previous studies, we

Table 1. Five mAbs and their identified target proteins

mAb	Target protein	Accession no.	MW (kDa)
LP-Pirin-3A3b	Pirin (iron-binding nuclear protein)	NP003653.1	32
LP-FGL1-1G9	Fibrinogen-like protein 1 precursor (FGL1)	Q08830	36
LP-ORMDL2-1E3	ORM1-like 2 (ORMDL2)	NP054901.1	17
LP-eIF1A-B4B5C5	Eukaryotic translation initiation factor 1A, Y (eIF1AY)	NP004672.2	16
LP-HAB18G/CD147-2B2	HAB18G/CD147 fragment	NP001719.2	18/58

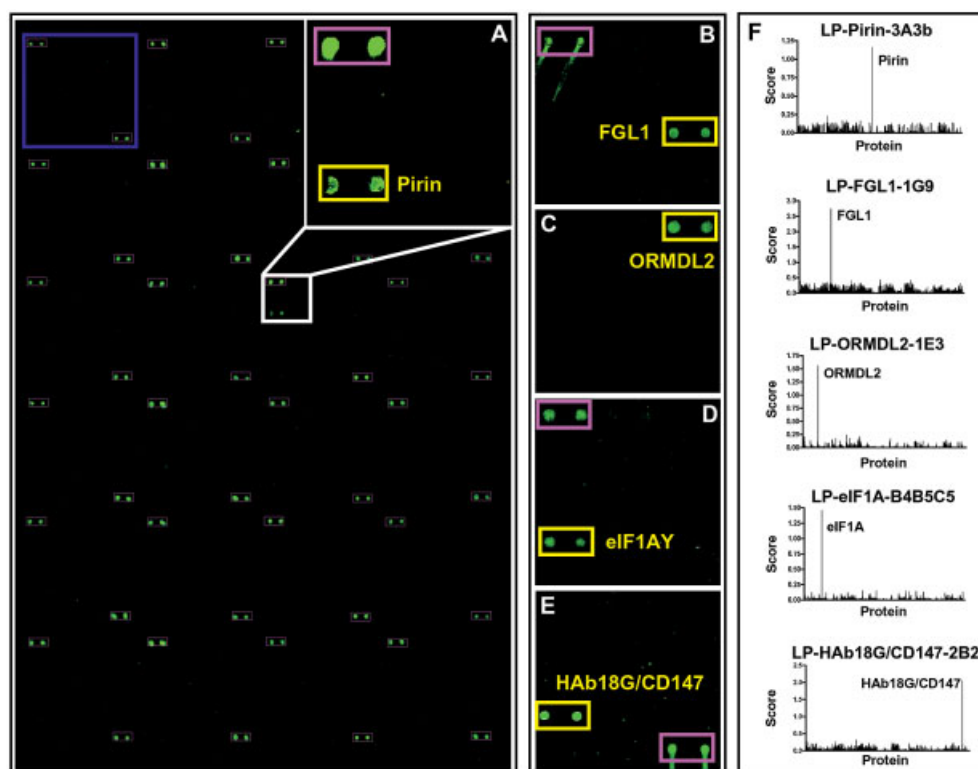


Figure 3. mAb identification on the human liver protein microarrays. Five proteins were specifically recognized by 5 of the 54 unknown mAbs using the human liver protein microarrays. The blue box indicates the first of the 24 blocks; the yellow boxes indicate the target proteins; and the purple boxes indicate the landmarks (mouse IgG). (A) A full image of a human liver protein microarray that was probed with mAb LP-Pirin-3A3b. The inset on the right corner illustrates that a single protein, Pirin, was identified by LP-Pirin-3A3b on the microarray. (B–E) Four more mAbs were confirmed to specifically recognize human proteins, FGL1, ORMDL2, eIF1AY, and HAb18G/CD147 on the human liver protein microarrays. (F) Histograms of the binding signals from protein chips incubated with the five mAbs. The GST-normalized signals of the expected proteins are at least five times higher than those of other proteins.

found that HAb18G/CD147 were expressed highly in HCC cells and correlated to the expression of MMP2, MMP9, and VEGF [24].

3.3 Validation of the mAb–antigen interactions

As a new approach to producing high-quality mAbs and to screening for their corresponding antigens, we sought to confirm the antibody–antigen interactions by using traditional Western blot analysis. We first performed Western blot analysis by using mAbs against the five purified recombinant proteins. As illustrated in Fig. 4A, four GST fusion proteins of Pirin, FGL1, ORMDL2, and eIF1AY, as well as an 18-kDa fragment of HAb18G/CD147 can be recognized by their corresponding mAbs with no cross-reactivity to GST. We next applied Western blot analysis of human liver lysates using the five mAbs. Ascites LP-Pirin-3A3b, LP-eIF1A-B4B5C5, and LP-HAb18G/CD147-2B2 could each recognize a single band at the respective positions of 32, 20, and 50 kDa. The first and the last are in good agreement with the expected molecular weights of Pirin and HAb18G/CD147 (in full-

length), respectively (Fig. 4B). The slight mobility reduction of the eIF1AY band (20 *versus* 16 kDa) might be caused by either low resolution of the gel or protein PTMs such as phosphorylation and/or glycosylation in human liver cells. The other two mAbs, however, did not show any significant signals in Western blot analysis. It is conceivable that these two proteins are in low abundance in human liver tissues.

3.4 Characterization of identified antigens using IHC staining

One of the most useful applications of mAbs is to perform IHC analysis on tissue sections to profile protein expression patterns at various diseased stages, for example, cancer *versus* healthy tissues. To demonstrate that the mAb generated here are useful in IHC studies and that they have the potential to be developed into biomarkers that can specifically differentiate healthy tissues from diseased tissues or *vice versa*, we applied the five mAbs to characterize protein expression patterns of the five identified antigens by using tissue microarrays, each of which contains multiorgan tumor and

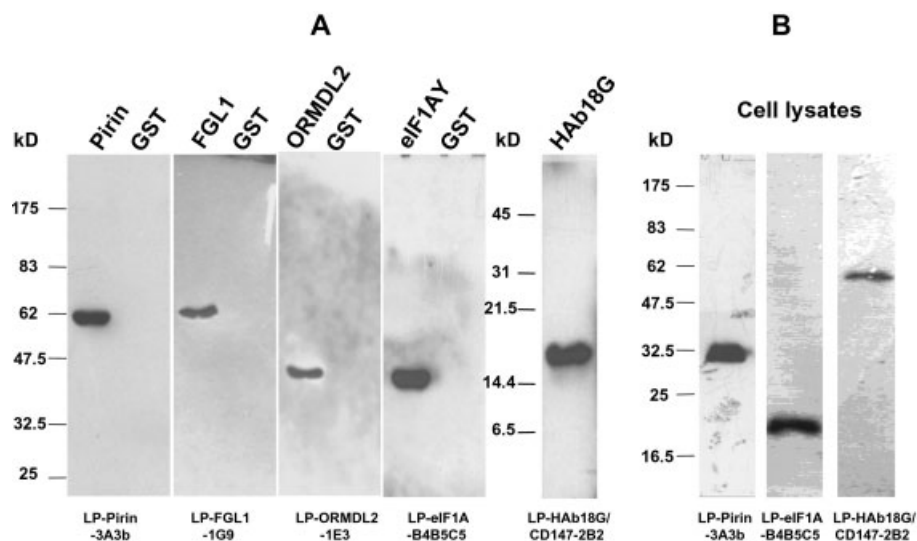


Figure 4. Validation of the microarray data by Western blot analysis. (A) Western blot of the five purified candidate proteins probed with the corresponding mAbs. The control protein, GST, was not detected by any of the mAbs (or by the secondary antibody). (B) Western blot of human liver cell lysates probed with the five mAbs. The mAbs LP-Pirin-3A3b, LP-eIF1A-B4B5C5, and LP-HAb18G/CD147-2B2 specifically recognized their target proteins, *Homo sapiens* Pirin, eIF1A, and HAb18G/CD147, respectively. The other two ascites samples did not give positive results (data not shown).

normal tissues. All but one (LP-Pirin-3A3b) showed distinguishable staining patterns in various tissues, and in some tissues they could unambiguously differentiate normal from carcinoma (Table 2). We also performed multiple negative control experiments using nonimmunized mouse sera or PBS buffer, but we did not observe any significant background staining (data not shown). As summarized in Table 2, FGL1, ORMDL2, HAb18G/CD147, and eIF1A are all expressed in human liver, colon, and esophagus tissues. FGL1 and HAb18G/CD147 are found positive in tissues of all nine organs tested. The expression patterns of ORMDL2 and eIF1A proteins, on the other hand, are more tissue dependent: the ORMDL2 protein is completely absent in stomach, kidney, breast, and ovary tissues; eIF1A protein is completely absent in rectum, lung, and breast tissues.

A close inspection of the results from the IHC staining experiments revealed some interesting expression patterns of the four antigens (Fig. 5). As illustrated in Fig. 5A, the FGL1 proteins are likely localized to the Golgi apparatus in normal liver tissues; however, their expression is much lower and has a more diffuse pattern in liver carcinoma (Fig. 5B and inset). The expression of FGL1 is indistinguishable in normal breast and breast carcinoma tissues (Figs. 5C and D).

We found that ORMDL2 proteins are expressed ubiquitously in many human tissues, including liver, rectum, and lung (Table 2), a finding that agrees with previous studies [17]. Consistent with the results reported by Kemmeren *et al.*, we confirmed that the ORMDL2 proteins are localized to the ER and excluded from the nucleus in normal liver tissues (Fig. 5E and inset). However, in liver carcinoma we observed that in some cells the majority of this protein goes to the nucleus (Fig. 5 and insets). These results suggest that the ER localization of ORMDL2 is less regulated in liver carcinoma, and it might affect its function in protein folding, as proposed in previous studies [18]. As far as we know, we are the first to observe that the ORMDL2 proteins are highly expres-

sed in plasma of goblet and mucous cells in normal, dysplasia, and carcinoma rectum tissues (Figs. 5G and H and insets).

Another interesting result comes from the expression patterns of HAb18G/CD147, a homolog of cyclophilin receptor of the immunoglobulin family (Figs. 5I–L and insets). In normal liver, liver carcinoma, and lung cancer tissues, mAb LP-HAb18G/CD147-2B2 showed strong immunostainings (Figs. 5I, J, and L), whereas in normal lung tissues the signals were barely detectable (Fig. 5K). Its staining patterns in normal and carcinoma liver tissues, however, are strikingly different. In normal liver tissues the HAb18G/CD147 proteins are evenly distributed throughout the entire cytoplasm and excluded from the nuclei; in some cells they are accumulated in dot-like structures, possibly the endosomes (Fig. 5I and inset). However, the HAb18G/CD147 proteins are almost depleted in the cytoplasm and show a dominant localization to the plasma membranes in both liver and lung carcinomas (Figs. 5J and L). Previously, we have demonstrated that transfections of the *Hab18G/CD147* cDNA into HCC cells enhance their potential of metastasis and invasion, probably *via* triggering the release of MMP2 and MMP9 [21, 25, 26]. Given the IHC results of LP-HAb18G/CD147-2B2 shown in this study, it is conceivable that regulations in subcellular localization of HAb18G/CD147 may also play an important role in cancer development and metastasis in liver carcinomas.

Most interesting, perhaps, is the result from the IHC staining with mAb LP-eIF1A-B4B5C5, which recognizes both isoforms of eIF1A (Figs. 5M–P). In normal liver tissues we observed that the eIF1A proteins are present in almost every cell and show a granular staining in the cytoplasm (Fig. 5M and inset). A similar staining pattern was also documented for its yeast ortholog, Tif11 [27]. However, this protein is totally absent in liver carcinomas (Fig. 5N). Since *eIF1A* encodes an essential factor in the translation initiation

Table 2. Detection of four antigens in tissues from nine human organs

Tissue		Number of samples	Number of positive staining			
			FGL1	ORMDL2	HAb18G/CD147	eIF1A
Esophagus	Normal	3	2	1	1	1
	Carcinoma	3	1	3	3	0
Stomach	Normal	3	2	0	3	1
	Carcinoma	3	1	0	1	1
Colon	Normal	3	3	2	1	2
	Carcinoma	3	2	1	1	1
Rectum	Normal	3	3	3	1	0
	Carcinoma	3	3	1	2	0
Liver	Normal	3	2	2	0	2
	Carcinoma	3	0	1	2	0
Lung	Normal	3	3	1	0	0
	Carcinoma	3	1	1	3	0
Kidney	Normal	3	2	0	3	2
	Carcinoma	3	1	0	3	0
Breast	Normal	6	6	0	0	0
	Carcinoma	3	3	0	1	0
Ovary	Normal	3	2	0	0	1
	Carcinoma	3	0	0	3	0

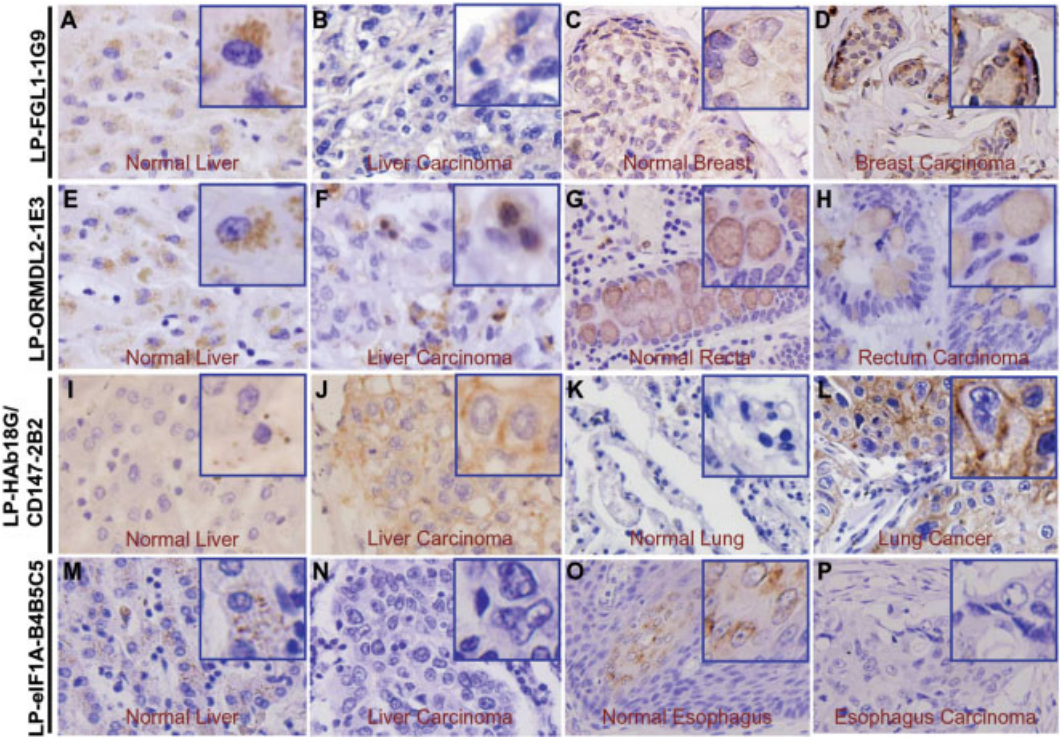


Figure 5. IHC staining of tissue microarray by mAbs LP-FGL1-1G9, LP-ORMDL2-1E3, LP-HAb18G/CD147-2B2, and LP-eIF1A-B4B5C5. (A–D) Normal liver, liver carcinoma, normal rectum, and recta carcinoma tissues stained by mAb LP-FGL1-1G9. (E–H) Normal liver, liver carcinoma, normal stomach, and stomach carcinoma tissues stained by mAb LP-ORMDL2-1E3. (I–L) Normal liver, liver carcinoma, normal lung, and lung carcinoma tissues stained by mAb LP-HAb18G/CD147-2B2. (M–P) Normal liver, liver carcinoma, normal esophagus, and esophagus carcinoma tissues stained by mAb LP-eIF1A-B4B5C5.

machinery, and probably reflects the level of protein translation activity in a cell, it is possible that in liver carcinomas the protein translation machinery is suppressed. Similar phenomena were also observed between normal esophagus and esophagus carcinoma – eIF1A is completely absent in carcinoma esophagus (Figs. 5O and P). Unlike its unanimous staining pattern in normal liver tissues, in normal esophagus tissues high expression of eIF1A is restricted to the polyhedral cells in the middle layer of epithelium, whereas it is not found in the low columnar epithelium or the connective tissues.

Because our long-term goal is to generate as many as possible high-quality mAbs to study human liver proteins, we decided to further characterize the expression patterns of the four antigens in more human liver samples. Each antibody was individually applied to a tissue microarray containing 22 samples of normal, 18 carcinomas, 11 hepatocirrhosis, and 12 hepatitis tissues from human livers. Depending on the signal intensity, we classified the staining signals as *strong*, *moderate*, and *weak* and summarized the results in Table 3. We first noticed that LP-FGL1-1G9 and LP-ORMDL2-1E3 showed moderate to weak staining signals in all four tissues as compared with the other two mAbs. Such differences may reflect variation in affinity or protein expression levels in these tissues (Table 3). In fact, the expression level of ORMDL2 in hepatitis is much reduced – only 2 out of 12 cases were positive. The number of the *weak* class also varies from 10 in the normal to 3 in the carcinoma tissues. The FGL1 proteins show a similar difference in the normal and carcinoma tissues.

Both HAb18G/CD147 and eIF1A show stronger staining signals and a higher percentage of positive staining (Table 3). Although HAb18G/CD147 expressed in most normal and abnormal liver tissues, the signal intensity in carcinomas was significantly stronger than that in the normal tissues ($p = 0.021$). As we demonstrated in liver carcinoma staining (Fig. 5J), the protein also accumulates on cell membranes in hepatitis and hepatocirrhosis tissues (data not shown). As demonstrated in the above multiorgan tissue microarrays, eIF1A is not detectable in liver carcinoma tissues (0/18), but shows *strong* staining in 73% (14/19) in the normal tissues (Table 3). In addition, it can be identified in around 50% of the hepatocirrhosis and hepatitis liver tissues. Its expression level is significantly different between the normal *versus* carcinoma tissues ($p = 0.000$), normal *versus* hepatocirrhosis tissues ($p = 0.026$), hepatocirrhosis *versus* carcinoma tissues ($p = 0.001$), and hepatitis *versus* carcinoma tissues ($p = 0.002$). However, it is not significant between the normal and hepatitis tissues ($p = 0.15$) or the hepatitis and hepatocirrhosis tissues ($p = 1$). These results clearly demonstrated that eIF1A can be developed as a potential biomarker for human liver diseases, and the others are useful in studying diseases and cancer development in livers.

4 Discussion

Using complex samples as antigens to immunize mice demands additional steps to identify the corresponding antigens. In previous studies, screening an expression library

Table 3. IHC results in normal and diseased human liver tissues

Liver tissue		Intensity of staining ^{a)}			
		Strong	Moderate	Weak	None
FGL1	Normal	0	6	6	10
	Carcinoma	0	5	0	13
	Hepatocirrhosis	0	3	1	7
	Hepatitis	0	3	3	6
ORMDL2	Normal	0	4	10	8
	Carcinoma	0	4	3	11
	Hepatocirrhosis	0	2	4	5
	Hepatitis	0	1	1	10
HAb18G/CD147	Normal	4	8	5	5
	Carcinoma	10	5	1	2
	Hepatocirrhosis	3	4	2	2
	Hepatitis	3	6	1	2
eIF1A	Normal	14	4	1	3
	Carcinoma	0	0	0	18
	Hepatocirrhosis	4	2	1	7
	Hepatitis	2	3	0	4

a) The score system is conducted according to Reimers *et al.* [22]: none (no staining at all); weak (one staining regardless of positive cell percentages or two staining of 30% of cells); moderate (two staining of 30% of cells or three staining of 50% of cells); and strong (three staining of 50% of cells).

and immunoprecipitation (IP) coupled with MS have been the most common approaches [28]. The success of the first method is heavily affected by the coverage of the library, and it is tedious and difficult to scale up. The second approach requires expertise in IP and MS techniques, and it is biased against proteins with low abundance in cells because they are more difficult to be immunoprecipitated. It is also known that a fraction of antibodies are not good for performing IP experiments due to the unknown nature of the antibodies.

Here, we present a novel approach that employs a combined force of high-throughput hybridomas production and protein chip technology to produce high-quality mouse mAbs and to identify the corresponding antigens in a high-throughput fashion [12, 29]. To our knowledge, this is the first protein microarray of more than 1000 human proteins that are liver associated.

The protein chip approach to screening for antigens offers several significant advantages. First, the assay consumes minimum amounts of antibodies. Second, such screening is very sensitive and specific. While it allows us to identify an antigen of a particular mAb, a global-binding profile of the antibody is simultaneously generated. In our case, each of the five positive mAbs recognized only a single protein on an array of 1058 unique proteins. Such high specificity was reconfirmed for three mAbs on the basis of the observation that only a single band was detected for each antibody in a Western blot analysis using the whole cell lysates from human livers (Fig. 4). Third, it is suitable for high-throughput screening of antibodies. As we are highly interested in obtaining mAbs with high specificity and affinity, we can apply uniform binding and washing conditions to ensure that both the specificity and affinity of an antibody are above a certain threshold. Such uniformity lends itself well to automation.

Another advantage of our approach is reflected by the usefulness of the mAbs in both Western blot and IHC analyses. Four of the five mAbs could show distinct staining patterns between healthy and carcinoma tissues in IHC assays, resulted in a wealth of information of how their corresponding antigens are differentially regulated in normal *versus* carcinoma tissues. Among the four mAbs tested, the expression pattern of eIF1A detected by LP-eIF1A-B4B5C5 is most interesting. This protein is expressed at a high level in 86% of the normal, 52% of the peri-cancer (hepatocirrhosis and hepatitis tissues), and 0% of the carcinoma tissues. eIF1A plays an important role in the eukaryotic translation initiation [17]. It enhances the ability of eIF1 to dissociate aberrantly assembled complexes from mRNA, and these factors synergistically mediate 48S complex assembly at the initiation codon [30]. Unlike many housekeeping genes, the expression of eIF1A varies in different types of cells, although protein translation is an essential function for cells [31]. It has been documented that the expression of eIF1A is significantly lower in human hepatoma cells (HuH7) than in HCV-infected HuH7 cells [32]. Taken together, it is plausible that protein translation in cancer cells is highly regulated.

The unambiguous and specific expression pattern of eIF1A makes the mAb, LP-eIF1A-B4B5C5, an excellent candidate to serve as a potential biomarker for liver carcinoma prognosis. The coverage of our protein chip at the time when this study was performed was still small, with ~1000 proteins representing around 10% of the human liver proteome. Consistent with its coverage, we identified antigens for ~10% (5/54) of the unknown mAbs. With increased coverage of our human protein chips in the near future, we expect to observe a higher success rate of antigen identification.

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RESEARCH ARTICLE

A protein chip approach for high-throughput antigen identification and characterization

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Proteomics research in humans and other eukaryotes demands a large number of high-quality mAbs. Here, we report a new approach to produce high-quality mAbs against human liver proteins using a combined force of high-throughput mAb production and protein microarrays. After immunizing mice with live cells from human livers, we isolated 54 hybridomas with binding activities to human cells and identified the corresponding antigens for five mAbs *via* screening on a protein microarray of 1058 unique human liver proteins. Finally, we demonstrated that using the five mAbs we could characterize the expression profiles of their corresponding antigens by using tissue microarrays. Among them, we discovered that eIF1A expressed only in normal liver tissues, not in hepatocellular carcinoma in humans.

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1 Introduction

The human liver is the largest organ in the body, and its complexity is probably second only to the brain [1]. It displays various physiological functions involved in metabolism, secretion, biosynthesis, and biotransformation (detoxification). Liver diseases, such as viral hepatitis, liver carcinoma, alcoholic and drug-related liver injuries, and autoimmune

liver disease, pose a great threat to the public health. Understanding of the global molecular networks of liver physiology and pathology will greatly promote the development of diagnostics and therapeutics for these diseases. Thus, the Human Liver Proteome Project (HLPP) was launched in 2002 [2, 3] to address the above issue.

Large-scale proteomic studies require affinity reagents, such as mAbs, to specifically recognize thousands of proteins in a complex sample. Such specific recognition can be used to establish the identity of a given protein. Moreover, mAbs can be used to monitor changes in protein concentration,

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Abbreviations: HCC, hepatocellular carcinoma; HLPP, Human Liver Proteome Project, IHC, immunohistochemical; IP, immunoprecipitation

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localization, and clustering pattern *in situ* in cells and tissues under normal and pathological conditions [4]. The understanding of diseases and protein functions at the proteomic level will be greatly benefited when specific antibodies against each protein are derived and made generally available.

Two major methods have been developed to produce mAbs [5]: the traditional hybridoma fusion [6] and phage antibody display techniques [7]. Although effective and adopted by many companies, the display technologies are not yet user-friendly [8] and still are unable to create high-affinity mAbs without lengthy downstream manipulations [9]. Consequently, most research laboratories use classical hybridoma fusion [10] because, once available, mAbs thus obtained can be produced in relatively large quantity, and they are dimeric, stable, and contain a notably large tag – the Fc domain [8]. Although extremely useful, this technology is not easily adaptable to high-throughput production of the thousands of mAbs required for proteomic studies [4, 5, 8]. One of the major bottlenecks is the need for substantial amounts of highly pure proteins [11].

Here, we report a novel approach to the rapid generation of high-quality mAbs against proteins expressed in human livers and to identification of their corresponding antigens by using protein chips. We further demonstrate that the new mAbs generated in this study are useful in Western blot and immunohistochemical (IHC) analyses and have the potential to be developed into biomarkers for various liver diseases in humans.

2 Materials and methods

2.1 Preparation of immunogens

Human liver tissues from healthy adults were minced and ground into cell suspension in the RPMI-1640 media. The cell suspension was centrifuged at 800 rpm for 10 min, washed three times with 0.01 M PBS, and adjusted to a final concentration of 1×10^8 cells/mL. The remaining tissues were stored in liquid nitrogen for later use. The health human liver tissues were obtained from excised tissues donated by liver injury patients. The study was performed after informed consent being signed and was approved by the Institutional Medical Ethics Committee of FMMU.

2.2 Immunization and generation of hybridomas

Balb/c mice (female, 4–6-wk old) were immunized intraperitoneally with 100 μ L of the liver cell suspension (1×10^8 cells/mL) three times at 2-wk intervals. Three days after the final intraperitoneal immunization, the spleens of the mice were removed, and the spleen cells from the immunized mice were isolated and fused with Sp2/0 myeloma cells at a ratio of 3 Sp2/0 myeloma to 10 spleen cells, using PEG 1500 (Roche). The fused cells were first cultured

in the RPMI-1640 media containing 20% newborn calf serum and 1% hypoxanthine–aminopterin–thymidine (HAT) suspension (Roche).

2.3 Cell immunofluorescent staining

Seven types of cell lines – CCC-HEL-1, QZG, SMMC-7721, ECV304, THP-1, MT4, and Fb – were separately plated at a density of 1×10^5 cells/mL on glass slides and cultured in Petri dishes overnight. Cells were then fixed with cold acetone for 5 min and blocked with 5% nonimmune goat sera for 30 min at 37°C. Appropriate amounts of supernatants of the hybridoma cell culture were added to each slide and incubated for 1 h at 37°C. After several washes with PBS, FITC-conjugated goat anti-mouse IgG diluted 500-fold was added to the cells and incubated for 1 h at 37°C. The hybridomas that showed binding affinity to any of the seven cell lines were scored as positives and selected for further cloning *via* the limiting dilution method.

2.4 Preparation of mAbs

Using the positive hybridoma cells, we prepared mAb using ascites production. 1×10^6 cells *per* mouse were injected intraperitoneally to Balb/c mice. After 8–14 days, ascites were collected and cleaned by centrifuging at 3000 rpm/min for 20 min.

2.5 Cloning of the human full-length ORFs

On the basis of gene annotation and available gene expression data, 3069 pairs of gene-specific primers were designed to PCR-amplify full-length ORFs expressed in human livers. A full-length human liver cDNA library (BD Biosciences, USA) was used as a template in PCR reactions. The products showing the expected sizes were cotransformed with linearized pEGH into yeast cells, and the transformants were selected on SC-URA media [12]. The recombinant plasmids were rescued and sequenced through the upstream junctions between the vector and the ORFs. The resulting sequence traces were batch-blasted against the NCBI database. For those that failed to produce transformants or contained frame-shift mutations, two more attempts were carried out.

2.6 Protein purification and protein chip fabrication

The sequence-verified human ORF constructs were retransformed into yeast, and the yeast transformants were arrayed in a 96-well format and stored as glycerol stocks at –80°C. The recombinant human proteins were purified as described previously [12]. Using a commercial microarrayer, the purified human proteins were spotted onto poly-L-lysine-coated slides at high density.

2.7 mAb identification using protein microarrays

The 54 ascites samples were arrayed in a 7 × 8 format and pooled both vertically and horizontally to form 15 minipools. Each pool of ascites was then diluted 10 000-fold. After blocking, each diluted ascites pool was added to a chip and incubated at room temperature (RT) for 30 min with gentle shaking in humidified chamber. The chips were then washed three times in PBST (1% Tween-20) buffer for 10 min at 42°C with shaking. Anti-mouse IgG antibodies labeled with Cy-3 (Jackson Laboratories, USA, 1:1000 dilution in PBS) were added to the chips and incubated in the dark at RT for 1 h. The chips were then washed three times with prewarmed PBST for 10 min each at 42°C with gentle shaking. After rinsing twice in filter-sterilized, double-deionized water, chips were spun to dryness.

2.8 Data analysis

Each protein microarray was scanned with Scanarray™ Lite scanner (Perkin Elmer, USA). Binding signals were acquired and analyzed using the Genepix Pro 5.1 software (Axon Instruments, USA).

2.9 Western blot

Equal amounts of proteins were separated by SDS-PAGE and transferred onto PVDF membrane by semidry blotting. The membrane was blocked in 5% nonfat milk in PBST for 1 h, incubated with ascites diluted 1:1000 in 5% nonfat milk in PBST for 1 h, and washed three times in PBST. HRP-conjugated anti-mouse IgGs diluted 1:1000 in 5% nonfat milk in PBST was added for a 1-h incubation. After washing three times in PBST, chemiluminescent detection was performed (Pierce, USA).

2.10 Immunohistochemistry

Two kinds of tissue microarrays (Cybrdi, China) were employed to perform IHC staining with mAbs and the Eli-Vision plus method was used for signal visualization. The first type of tissue microarrays contains multiorganic tumor and normal tissues from nine human organs, and the second type contains hepatitis, hepatocirrhosis, hepatoma, and normal tissues from human livers. In brief, sections on these tissue microarrays were dewaxed in xylene, rinsed in alcohol, and graded in alcohol/water mixtures. Then, hydrogen peroxide at a concentration of 30 mL/L was applied to block endogenous peroxidase activity. After antigen retrieval by two times of 5 min microwaving at high power in a citrate buffer (pH 6.0), the sections were blocked by normal goat serum for 30 min. The specimens were subsequently incubated with the primary antibody at a dilution of 1:50 in PBS overnight at 4°C. After rinsing, staining was performed with the Eli-vision™ plus two-step System (Dako, Carpinteria, CA). The color was developed by reacting with 3,3-diaminobenzidine.

Slides were then counterstained with hematoxylin, dehydrated, cleared, and mounted. Negative controls were performed using sera from nonimmune mice or PBS buffer at the incubation step of the primary antibodies.

3 Results

3.1 Production of mouse mAbs

To generate mAbs in mice, we injected live cells from normal human liver tissues into ten mice. After three doses of immunization, the spleen cells of the mice were isolated and fused with myeloma cells to generate 3000 hybridomas (300/fusion). To select for IHC capable mAbs, seven human cell lines – CCC-HEL-1, QZG, SMMC-7721, ECV304, THP-1, MT4, and Fb – were then used to screen the hybridomas for binding activity. Fifty-four hybridomas were found to secrete mAbs that could recognize proteins in human cells (Fig. 1 and Supporting Information Table 1). Ascites samples of the 54 mAbs were then prepared, and the antibody concentrations were determined to be in the range of 10–20 µg/mL.

3.2 Antigen identification using the human liver protein microarrays

Using a mixture of proteins to immunize mice can rapidly generate many mAbs; however, the following step to identify the corresponding antigens has been time-consuming and costly with low success rate in the past due to a lack of proper tools. To overcome such obstacles, we decided here to construct a human protein chip for antigen identification of the 54 mAbs. On the basis of a literature and database search, a total of 3069 primer pairs were designed to PCR-amplify the full-length cDNAs of the genes known to express in human livers. A commercial “full-length” cDNA library generated from human livers was used as PCR templates and we were able to produce 1144 PCR products with expected sizes from the 3069 PCR amplifications because of the low coverage of the library. We next cloned and verified a total of 1058 full-length human cDNAs in a yeast expression vector that can overproduce the human proteins as N-terminal GST fusions [12]. Based on the known mRNA expression profile found in the databases (*e.g.* <http://www.affimetrix.com>), when *e*-value of 0 were used for sequence matching and cutoff at five times average signal for high expression, among 909 matched, 230 were highly expressed in liver and only 17 were house-keeping genes. We also performed Coomassie staining and Western analysis and estimated that ~85.7% fusion proteins were purified with expected molecular weights (Fig. 2A).

We next fabricated a chip of 1058 unique human liver proteins on poly-L-lysine-coated glass slides with additional control proteins. The amount of immobilized proteins on the protein chips was visualized and quantified using anti-GST antibodies (Fig. 2B). We observed that 98.5% (1042/1058) of

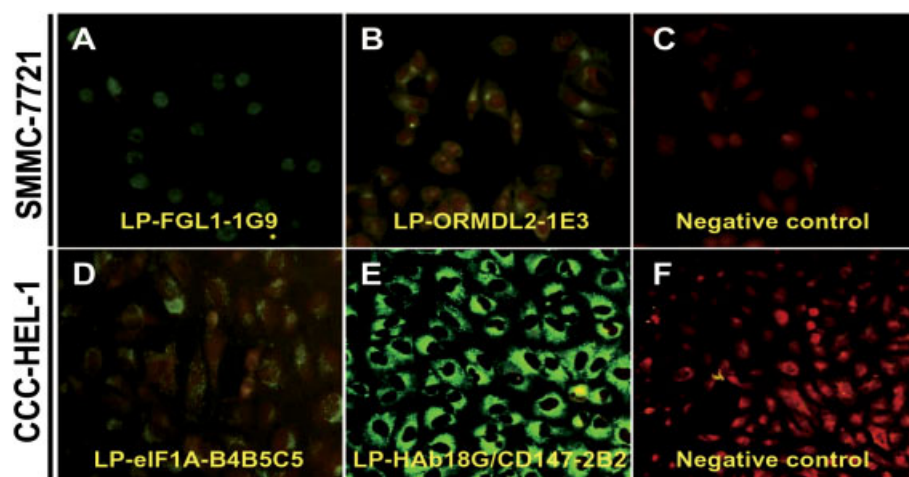


Figure 1. Screening for positive hybridomas using cell immunofluorescent staining. To select IHC capable mAbs, 3000 hybridomas were screened using 7 human cell lines – CCC-HEL-1, QZG, SMMC-7721, ECV304, THP-1, MT4, and Fb – for their binding activity. Fifty-four hybridomas were found to secrete mAbs that could recognize proteins in human cells (Supporting Information Table 1). Positive binding signals are indicated by green fluorescence. The red color is background from Evan's blue buffer. (A, B) SMMC-7721 cells were stained by mAbs LP-FGL1-1G9 and LP-ORMDL2-1E3, respectively. (D, E) CCC-HEL-1 cells were stained by mAbs LP-eIF1A-B4B5C5 and LP-HAb18G/CD147-2B2, respectively. (C, F) SMMC-7721 and CCC-HEL-1 cells were stained by ascites from unimmunized mice as negative controls.

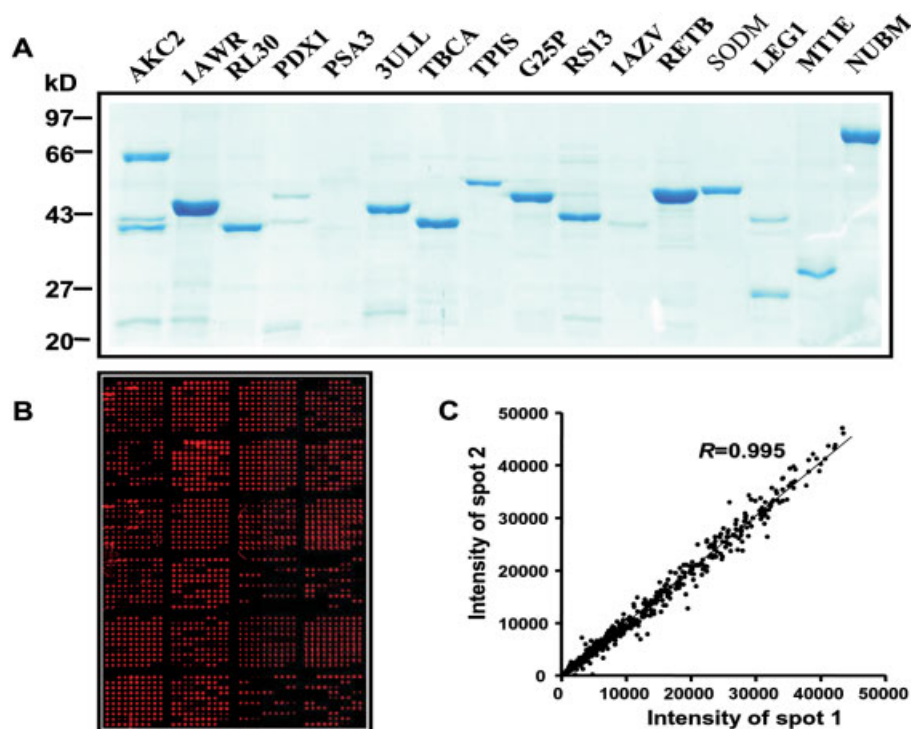


Figure 2. Fabrication of a protein microarray containing 1058 unique human liver proteins. (A) CBB staining of high-level expressed proteins on SDS-PAGE. The 1058 purified proteins were separated with 10% SDS-PAGE and then stained with CBB. Each of the loaded proteins was purified from 1 mL of culture, and 31% of them (329/1058) were visible in gels ($>0.1 \mu\text{g}$). Visible proteins (282 of 329; 85.7%) migrated at their expected molecular weights. (B) Protein microarray probed with anti-GST antibody. All liver proteins were printed on microarray in duplicates (side-by-side) in 24 blocks. Anti-GST antibody was probed to check the printing quality of microarray. The proteins positively detected by the anti-GST antibody are represented in red, and the landmark dots (mouse IgG) at the up-left and down-right corners of each block are invisible. (C) Correlation of spot intensities of duplicates (spot 1 *versus* its duplicate spot 2). The intensities of the duplicate spots of each protein were taken as X and Y positions, respectively, and the correlation coefficient of the resulting diagonal is 0.995, indicating a good reproducibility of the protein spotting.

the proteins showed significant signals above the background and that our protein chips are of high quality (Fig. 2C). Compared with Coomassie staining, which only detected 329 of the 1058 protein (31%) with a detection limit of 100 ng, the detection on the protein chip is much more sensitive, as documented to be as low as 1 ng/mL [13].

To rapidly identify the corresponding antigens of the mAbs, the 54 hybridoma samples were arrayed in a 7 × 8 format to create seven horizontal and eight vertical pools. The resulting 15 pools were separately incubated on the protein chips at a 70 000- or 80 000-fold dilution, and the bound mAbs were detected with Cy3-labeled anti-mouse IgGs. The average signal intensity of the two duplicate spots of each protein was divided by their average GST signal intensity for normalization and then the 1058 proteins were ranked according to their normalized scores. Since the goal was to identify mAbs with high affinity and because we assume that each of the mAbs might cross-react to 2 or 3 proteins on the chips, the top 20 proteins identified by each mAb pool were selected to compare with those by other pools. When vertical and horizontal pools identified one and only one common protein, it indicated that the shared antibody in these two pools specifically recognized that protein without cross-reactivity to other proteins. Thirteen of the 54 mAbs were identified by this means. The normalized scores of 5 of 13 were significantly higher (1.05–2.09) than the rest (0.29–0.66) (see Supporting Information Table 2). Therefore, the top five mAbs with top scores were chosen for further validation. The five mAbs were then individually probed to the human protein microarrays using the above-described protocol. As expected (Fig. 3), they were confirmed to bind to Pirin, fibrinogen-like protein 1 precursor (FGL1), ORM1-like 2 (ORMDL2), eukaryotic translation initiation factor 1A,Y (eIF1AY), and HAB18G/CD147 with high specificity (Table 1). As an example (Figs. 3A and inset, and Fig. 3F), mAb LP-Pirin-3A3b only recognizes a single protein, Pirin, with no obvious cross-reactivity to the other 1057 unique proteins on the array. Comparable specificities were also observed for mAbs LP-FGL1-1G9, LP-ORMDL2-1E3, LP-eIF1A-B4B5C5, and LP-HAB18G/CD147-2B2 against FGL1, ORMDL2, eIF1AY, and HAB18G/CD147, respectively (Figs. 3B–F).

Pirin was first identified as a conserved transcription factor (NFI) binding protein [14]. It is widely expressed in dot-like subnuclear structures in human tissues, particularly

in liver and heart cells [14]. Pirin was later found to bind to an oncoprotein Bcl-3 *in vivo* [15]. Emerging evidence suggests that Pirin may function as a transcription factor regulator to regulate oncogene expression.

FGL1, also known as HFREP-1, and its alternative spliced form LFIRE-1 have been reported to be specifically expressed in normal liver tissues, but not in hepatocellular carcinomas (HCCs) [16]. Although their molecular functions are still unknown, it has been shown that overexpression of wild-type *LFIRE-1/HFREP-1* in human HCC cells suppresses their tumorigenicity in nude mice [16]. On the basis of the facts that LFIRE-1 and HFREP-1 share the most exact amino acid sequences and that they coexpress in many types of tissue, we believe that the IHC staining by LP-FGL1-1G9 reflects the total expression level of both proteins.

ORMDL2 belongs to a newly identified gene family comprising three homologous genes (*ORMDL1*, *ORMDL2*, and *ORMDL3*) in humans and many other eukaryotic organisms, including plants and fungi. Hjelmqvist *et al.* [17] demonstrated that *ORMDL* genes are expressed ubiquitously in human tissues, including liver, lung, and kidney. All three ORMDL proteins showed similar ER localization [18]. Studies in yeast also revealed that Orm2p might function in protein folding and be involved in the cell-wall-integrity pathway [17, 18].

eIF1AX/Y, the eukaryotic translation initiation factor 1A, stimulates 40S binding of the eukaryotic initiation factor 2 (eIF2)/GTP/Met-tRNA (iMet) ternary complex (TC) and promotes scanning until the complex encounters the initiating AUG codon to form the 40S initiation complex [19]. eIF1A increases the competence of the complex to bind mRNA, and it also stabilizes the TC to the 40S subunit in the absence of mRNA [20]. *eIF1AY* encodes an isoform of *eIF1A* on the human Y chromosome and shares 100% identity with its X-chromosome isoform, eIF1AX, on the amino acid level. As in the case of FGL1, mAb LP-eIF1A-B4B5C5 is not expected to differentiate the expression of the two isoforms in IHC experiments.

The homolog CD147 of HAB18G/CD147 is also known as Basigin or EMMPRIN, a cyclophilin receptor of the immunoglobulin family [21]. Expression of CD147 in different tumor tissues has been reported previously [22], and it can be defined as a therapeutic target and biomarker to predict prognosis in patients [23]. In our previous studies, we

Table 1. Five mAbs and their identified target proteins

mAb	Target protein	Accession no.	MW (kDa)
LP-Pirin-3A3b	Pirin (iron-binding nuclear protein)	NP003653.1	32
LP-FGL1-1G9	Fibrinogen-like protein 1 precursor (FGL1)	Q08830	36
LP-ORMDL2-1E3	ORM1-like 2 (ORMDL2)	NP054901.1	17
LP-eIF1A-B4B5C5	Eukaryotic translation initiation factor 1A, Y (eIF1AY)	NP004672.2	16
LP-HAB18G/CD147-2B2	HAB18G/CD147 fragment	NP001719.2	18/58

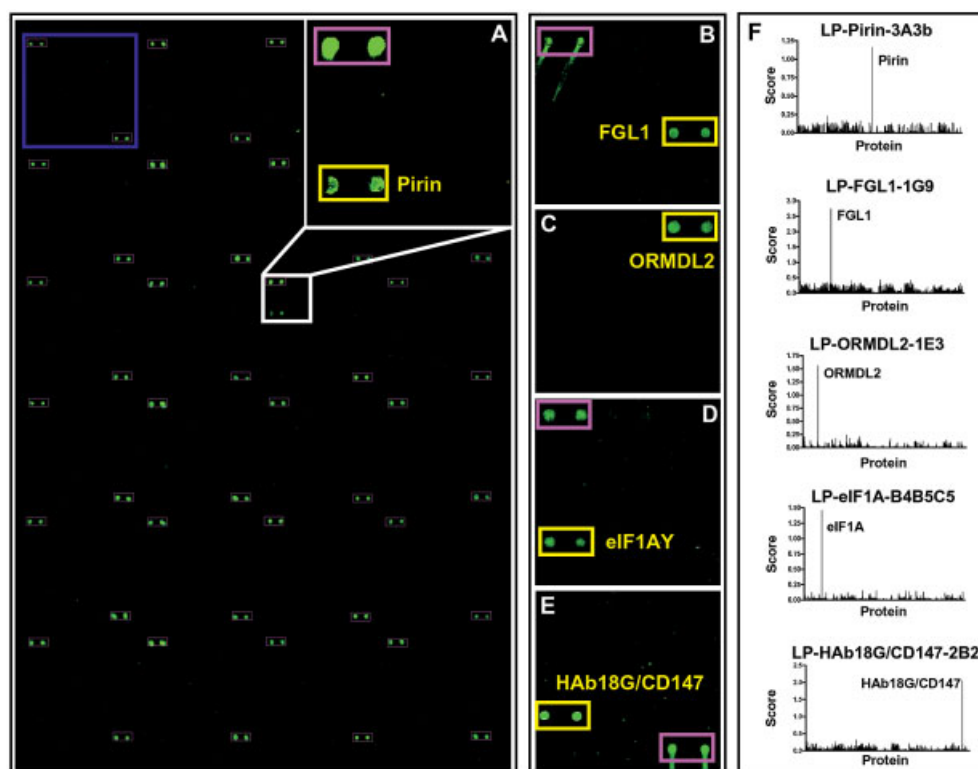


Figure 3. mAb identification on the human liver protein microarrays. Five proteins were specifically recognized by 5 of the 54 unknown mAbs using the human liver protein microarrays. The blue box indicates the first of the 24 blocks; the yellow boxes indicate the target proteins; and the purple boxes indicate the landmarks (mouse IgG). (A) A full image of a human liver protein microarray that was probed with mAb LP-Pirin-3A3b. The inset on the right corner illustrates that a single protein, Pirin, was identified by LP-Pirin-3A3b on the microarray. (B–E) Four more mAbs were confirmed to specifically recognize human proteins, FGL1, ORMDL2, eIF1AY, and HAb18G/CD147 on the human liver protein microarrays. (F) Histograms of the binding signals from protein chips incubated with the five mAbs. The GST-normalized signals of the expected proteins are at least five times higher than those of other proteins.

found that HAb18G/CD147 were expressed highly in HCC cells and correlated to the expression of MMP2, MMP9, and VEGF [24].

3.3 Validation of the mAb–antigen interactions

As a new approach to producing high-quality mAbs and to screening for their corresponding antigens, we sought to confirm the antibody–antigen interactions by using traditional Western blot analysis. We first performed Western blot analysis by using mAbs against the five purified recombinant proteins. As illustrated in Fig. 4A, four GST fusion proteins of Pirin, FGL1, ORMDL2, and eIF1AY, as well as an 18-kDa fragment of HAb18G/CD147 can be recognized by their corresponding mAbs with no cross-reactivity to GST. We next applied Western blot analysis of human liver lysates using the five mAbs. Ascites LP-Pirin-3A3b, LP-eIF1A-B4B5C5, and LP-HAb18G/CD147-2B2 could each recognize a single band at the respective positions of 32, 20, and 50 kDa. The first and the last are in good agreement with the expected molecular weights of Pirin and HAb18G/CD147 (in full-

length), respectively (Fig. 4B). The slight mobility reduction of the eIF1AY band (20 *versus* 16 kDa) might be caused by either low resolution of the gel or protein PTMs such as phosphorylation and/or glycosylation in human liver cells. The other two mAbs, however, did not show any significant signals in Western blot analysis. It is conceivable that these two proteins are in low abundance in human liver tissues.

3.4 Characterization of identified antigens using IHC staining

One of the most useful applications of mAbs is to perform IHC analysis on tissue sections to profile protein expression patterns at various diseased stages, for example, cancer *versus* healthy tissues. To demonstrate that the mAb generated here are useful in IHC studies and that they have the potential to be developed into biomarkers that can specifically differentiate healthy tissues from diseased tissues or *vice versa*, we applied the five mAbs to characterize protein expression patterns of the five identified antigens by using tissue microarrays, each of which contains multiorgan tumor and

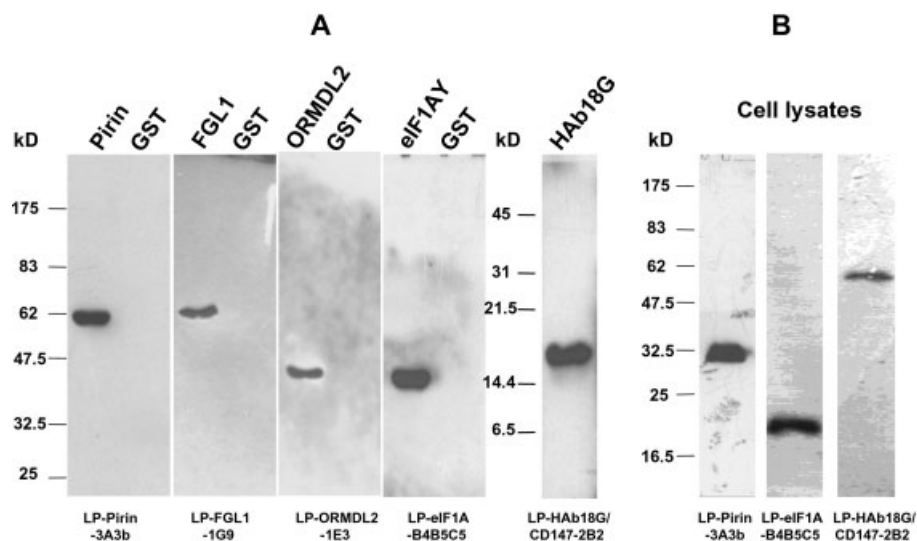


Figure 4. Validation of the microarray data by Western blot analysis. (A) Western blot of the five purified candidate proteins probed with the corresponding mAbs. The control protein, GST, was not detected by any of the mAbs (or by the secondary antibody). (B) Western blot of human liver cell lysates probed with the five mAbs. The mAbs LP-Pirin-3A3b, LP-eIF1A-B4B5C5, and LP-HAb18G/CD147-2B2 specifically recognized their target proteins, *Homo sapiens* Pirin, eIF1A, and HAb18G/CD147, respectively. The other two ascites samples did not give positive results (data not shown).

normal tissues. All but one (LP-Pirin-3A3b) showed distinguishable staining patterns in various tissues, and in some tissues they could unambiguously differentiate normal from carcinoma (Table 2). We also performed multiple negative control experiments using nonimmunized mouse sera or PBS buffer, but we did not observe any significant background staining (data not shown). As summarized in Table 2, FGL1, ORMDL2, HAb18G/CD147, and eIF1A are all expressed in human liver, colon, and esophagus tissues. FGL1 and HAb18G/CD147 are found positive in tissues of all nine organs tested. The expression patterns of ORMDL2 and eIF1A proteins, on the other hand, are more tissue dependent: the ORMDL2 protein is completely absent in stomach, kidney, breast, and ovary tissues; eIF1A protein is completely absent in rectum, lung, and breast tissues.

A close inspection of the results from the IHC staining experiments revealed some interesting expression patterns of the four antigens (Fig. 5). As illustrated in Fig. 5A, the FGL1 proteins are likely localized to the Golgi apparatus in normal liver tissues; however, their expression is much lower and has a more diffuse pattern in liver carcinoma (Fig. 5B and inset). The expression of FGL1 is indistinguishable in normal breast and breast carcinoma tissues (Figs. 5C and D).

We found that ORMDL2 proteins are expressed ubiquitously in many human tissues, including liver, rectum, and lung (Table 2), a finding that agrees with previous studies [17]. Consistent with the results reported by Kemmeren *et al.*, we confirmed that the ORMDL2 proteins are localized to the ER and excluded from the nucleus in normal liver tissues (Fig. 5E and inset). However, in liver carcinoma we observed that in some cells the majority of this protein goes to the nucleus (Fig. 5 and insets). These results suggest that the ER localization of ORMDL2 is less regulated in liver carcinoma, and it might affect its function in protein folding, as proposed in previous studies [18]. As far as we know, we are the first to observe that the ORMDL2 proteins are highly expres-

sed in plasma of goblet and mucous cells in normal, dysplasia, and carcinoma rectum tissues (Figs. 5G and H and insets).

Another interesting result comes from the expression patterns of HAb18G/CD147, a homolog of cyclophilin receptor of the immunoglobulin family (Figs. 5I–L and insets). In normal liver, liver carcinoma, and lung cancer tissues, mAb LP-HAb18G/CD147-2B2 showed strong immunostainings (Figs. 5I, J, and L), whereas in normal lung tissues the signals were barely detectable (Fig. 5K). Its staining patterns in normal and carcinoma liver tissues, however, are strikingly different. In normal liver tissues the HAb18G/CD147 proteins are evenly distributed throughout the entire cytoplasm and excluded from the nuclei; in some cells they are accumulated in dot-like structures, possibly the endosomes (Fig. 5I and inset). However, the HAb18G/CD147 proteins are almost depleted in the cytoplasm and show a dominant localization to the plasma membranes in both liver and lung carcinomas (Figs. 5J and L). Previously, we have demonstrated that transfections of the *Hab18G/CD147* cDNA into HCC cells enhance their potential of metastasis and invasion, probably *via* triggering the release of MMP2 and MMP9 [21, 25, 26]. Given the IHC results of LP-HAb18G/CD147-2B2 shown in this study, it is conceivable that regulations in subcellular localization of HAb18G/CD147 may also play an important role in cancer development and metastasis in liver carcinomas.

Most interesting, perhaps, is the result from the IHC staining with mAb LP-eIF1A-B4B5C5, which recognizes both isoforms of eIF1A (Figs. 5M–P). In normal liver tissues we observed that the eIF1A proteins are present in almost every cell and show a granular staining in the cytoplasm (Fig. 5M and inset). A similar staining pattern was also documented for its yeast ortholog, Tif11 [27]. However, this protein is totally absent in liver carcinomas (Fig. 5N). Since *eIF1A* encodes an essential factor in the translation initiation

Table 2. Detection of four antigens in tissues from nine human organs

Tissue		Number of samples	Number of positive staining			
			FGL1	ORMDL2	HAb18G/CD147	eIF1A
Esophagus	Normal	3	2	1	1	1
	Carcinoma	3	1	3	3	0
Stomach	Normal	3	2	0	3	1
	Carcinoma	3	1	0	1	1
Colon	Normal	3	3	2	1	2
	Carcinoma	3	2	1	1	1
Rectum	Normal	3	3	3	1	0
	Carcinoma	3	3	1	2	0
Liver	Normal	3	2	2	0	2
	Carcinoma	3	0	1	2	0
Lung	Normal	3	3	1	0	0
	Carcinoma	3	1	1	3	0
Kidney	Normal	3	2	0	3	2
	Carcinoma	3	1	0	3	0
Breast	Normal	6	6	0	0	0
	Carcinoma	3	3	0	1	0
Ovary	Normal	3	2	0	0	1
	Carcinoma	3	0	0	3	0

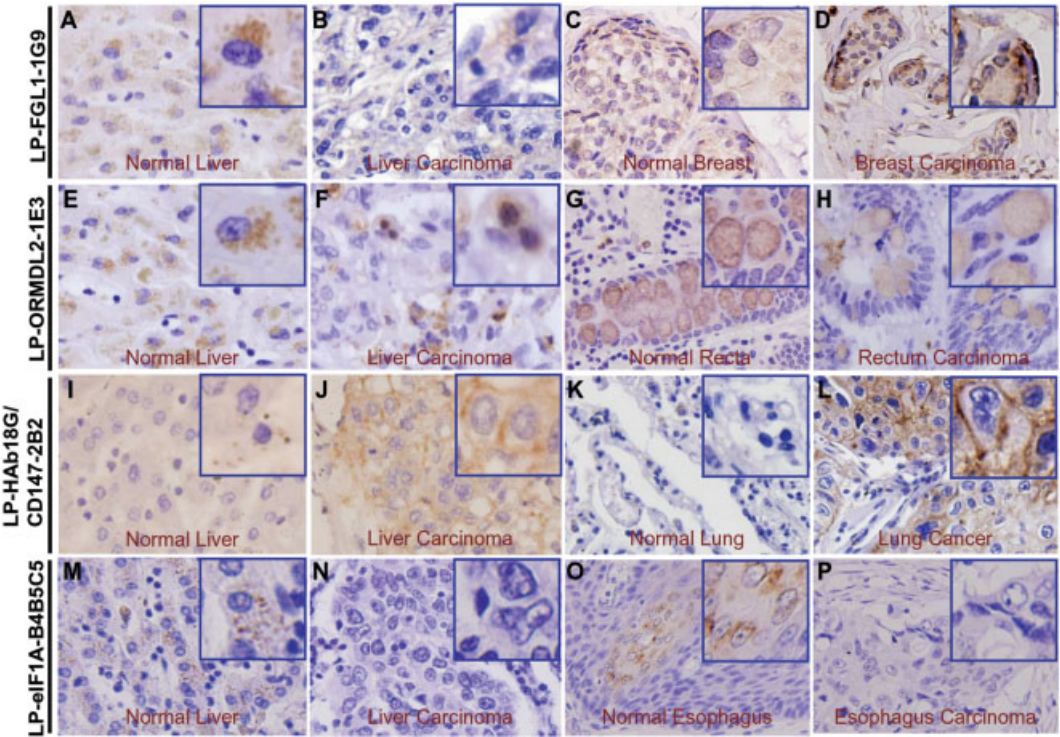


Figure 5. IHC staining of tissue microarray by mAbs LP-FGL1-1G9, LP-ORMDL2-1E3, LP-HAb18G/CD147-2B2, and LP-eIF1A-B4B5C5. (A–D) Normal liver, liver carcinoma, normal rectum, and recta carcinoma tissues stained by mAb LP-FGL1-1G9. (E–H) Normal liver, liver carcinoma, normal stomach, and stomach carcinoma tissues stained by mAb LP-ORMDL2-1E3. (I–L) Normal liver, liver carcinoma, normal lung, and lung carcinoma tissues stained by mAb LP-HAb18G/CD147-2B2. (M–P) Normal liver, liver carcinoma, normal esophagus, and esophagus carcinoma tissues stained by mAb LP-eIF1A-B4B5C5.

machinery, and probably reflects the level of protein translation activity in a cell, it is possible that in liver carcinomas the protein translation machinery is suppressed. Similar phenomena were also observed between normal esophagus and esophagus carcinoma – eIF1A is completely absent in carcinoma esophagus (Figs. 5O and P). Unlike its unanimous staining pattern in normal liver tissues, in normal esophagus tissues high expression of eIF1A is restricted to the polyhedral cells in the middle layer of epithelium, whereas it is not found in the low columnar epithelium or the connective tissues.

Because our long-term goal is to generate as many as possible high-quality mAbs to study human liver proteins, we decided to further characterize the expression patterns of the four antigens in more human liver samples. Each antibody was individually applied to a tissue microarray containing 22 samples of normal, 18 carcinomas, 11 hepatocirrhosis, and 12 hepatitis tissues from human livers. Depending on the signal intensity, we classified the staining signals as *strong*, *moderate*, and *weak* and summarized the results in Table 3. We first noticed that LP-FGL1-1G9 and LP-ORMDL2-1E3 showed moderate to weak staining signals in all four tissues as compared with the other two mAbs. Such differences may reflect variation in affinity or protein expression levels in these tissues (Table 3). In fact, the expression level of ORMDL2 in hepatitis is much reduced – only 2 out of 12 cases were positive. The number of the *weak* class also varies from 10 in the normal to 3 in the carcinoma tissues. The FGL1 proteins show a similar difference in the normal and carcinoma tissues.

Both HAb18G/CD147 and eIF1A show stronger staining signals and a higher percentage of positive staining (Table 3). Although HAb18G/CD147 expressed in most normal and abnormal liver tissues, the signal intensity in carcinomas was significantly stronger than that in the normal tissues ($p = 0.021$). As we demonstrated in liver carcinoma staining (Fig. 5J), the protein also accumulates on cell membranes in hepatitis and hepatocirrhosis tissues (data not shown). As demonstrated in the above multiorgan tissue microarrays, eIF1A is not detectable in liver carcinoma tissues (0/18), but shows *strong* staining in 73% (14/19) in the normal tissues (Table 3). In addition, it can be identified in around 50% of the hepatocirrhosis and hepatitis liver tissues. Its expression level is significantly different between the normal *versus* carcinoma tissues ($p = 0.000$), normal *versus* hepatocirrhosis tissues ($p = 0.026$), hepatocirrhosis *versus* carcinoma tissues ($p = 0.001$), and hepatitis *versus* carcinoma tissues ($p = 0.002$). However, it is not significant between the normal and hepatitis tissues ($p = 0.15$) or the hepatitis and hepatocirrhosis tissues ($p = 1$). These results clearly demonstrated that eIF1A can be developed as a potential biomarker for human liver diseases, and the others are useful in studying diseases and cancer development in livers.

4 Discussion

Using complex samples as antigens to immunize mice demands additional steps to identify the corresponding antigens. In previous studies, screening an expression library

Table 3. IHC results in normal and diseased human liver tissues

Liver tissue		Intensity of staining ^{a)}			
		Strong	Moderate	Weak	None
FGL1	Normal	0	6	6	10
	Carcinoma	0	5	0	13
	Hepatocirrhosis	0	3	1	7
	Hepatitis	0	3	3	6
ORMDL2	Normal	0	4	10	8
	Carcinoma	0	4	3	11
	Hepatocirrhosis	0	2	4	5
	Hepatitis	0	1	1	10
HAb18G/CD147	Normal	4	8	5	5
	Carcinoma	10	5	1	2
	Hepatocirrhosis	3	4	2	2
	Hepatitis	3	6	1	2
eIF1A	Normal	14	4	1	3
	Carcinoma	0	0	0	18
	Hepatocirrhosis	4	2	1	7
	Hepatitis	2	3	0	4

a) The score system is conducted according to Reimers *et al.* [22]: none (no staining at all); weak (one staining regardless of positive cell percentages or two staining of 30% of cells); moderate (two staining of 30% of cells or three staining of 50% of cells); and strong (three staining of 50% of cells).

and immunoprecipitation (IP) coupled with MS have been the most common approaches [28]. The success of the first method is heavily affected by the coverage of the library, and it is tedious and difficult to scale up. The second approach requires expertise in IP and MS techniques, and it is biased against proteins with low abundance in cells because they are more difficult to be immunoprecipitated. It is also known that a fraction of antibodies are not good for performing IP experiments due to the unknown nature of the antibodies.

Here, we present a novel approach that employs a combined force of high-throughput hybridomas production and protein chip technology to produce high-quality mouse mAbs and to identify the corresponding antigens in a high-throughput fashion [12, 29]. To our knowledge, this is the first protein microarray of more than 1000 human proteins that are liver associated.

The protein chip approach to screening for antigens offers several significant advantages. First, the assay consumes minimum amounts of antibodies. Second, such screening is very sensitive and specific. While it allows us to identify an antigen of a particular mAb, a global-binding profile of the antibody is simultaneously generated. In our case, each of the five positive mAbs recognized only a single protein on an array of 1058 unique proteins. Such high specificity was reconfirmed for three mAbs on the basis of the observation that only a single band was detected for each antibody in a Western blot analysis using the whole cell lysates from human livers (Fig. 4). Third, it is suitable for high-throughput screening of antibodies. As we are highly interested in obtaining mAbs with high specificity and affinity, we can apply uniform binding and washing conditions to ensure that both the specificity and affinity of an antibody are above a certain threshold. Such uniformity lends itself well to automation.

Another advantage of our approach is reflected by the usefulness of the mAbs in both Western blot and IHC analyses. Four of the five mAbs could show distinct staining patterns between healthy and carcinoma tissues in IHC assays, resulted in a wealth of information of how their corresponding antigens are differentially regulated in normal *versus* carcinoma tissues. Among the four mAbs tested, the expression pattern of eIF1A detected by LP-eIF1A-B4B5C5 is most interesting. This protein is expressed at a high level in 86% of the normal, 52% of the peri-cancer (hepatocirrhosis and hepatitis tissues), and 0% of the carcinoma tissues. eIF1A plays an important role in the eukaryotic translation initiation [17]. It enhances the ability of eIF1 to dissociate aberrantly assembled complexes from mRNA, and these factors synergistically mediate 48S complex assembly at the initiation codon [30]. Unlike many housekeeping genes, the expression of eIF1A varies in different types of cells, although protein translation is an essential function for cells [31]. It has been documented that the expression of eIF1A is significantly lower in human hepatoma cells (HuH7) than in HCV-infected HuH7 cells [32]. Taken together, it is plausible that protein translation in cancer cells is highly regulated.

The unambiguous and specific expression pattern of eIF1A makes the mAb, LP-eIF1A-B4B5C5, an excellent candidate to serve as a potential biomarker for liver carcinoma prognosis. The coverage of our protein chip at the time when this study was performed was still small, with ~1000 proteins representing around 10% of the human liver proteome. Consistent with its coverage, we identified antigens for ~10% (5/54) of the unknown mAbs. With increased coverage of our human protein chips in the near future, we expect to observe a higher success rate of antigen identification.

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