Identification of the Antigens Predominantly Reacted with Serum from Patients with Hepatocellular Carcinoma

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BACKGROUND. To identify antigens specifically recognized by the immune surveillance system in patients with hepatocellular carcinoma (HCC), the authors examined two complementary DNA (cDNA) libraries of moderately differentiated HCC by serologic analysis of recombinant cDNA expression libraries (SEREX).

METHODS. The libraries were screened with autologous patients’ sera, and sequences of the reacted clones were determined. To study the immunoreactivity of the antigens, sera from 20 patients with HCC, from 20 healthy volunteers, and from 16 patients with chronic viral hepatitis were examined.

RESULTS. Twenty-seven antigens were identified. They included SART1, p57Kip2, ROCK-1, β-catenin, and heat shock proteins, which are classified as tumor-associated genes. Three of 27 antigens—Tat-binding protein-1 (TBP-1), β4 integrin-binding protein (p27BBP), and ribosomal protein L30 (rpL30)—were reacted predominantly with sera from patients with HCC (55% of patients, 45% of patients, and 20% of patients, respectively). Patients in the control group had no antibodies against these three antigens. Seventy percent of patients with HCC had the antibody against at least one of these antigens.

CONCLUSIONS. Disease-specific humoral immune response against TBP-1, p27(BBP), and rpL30 was induced in patients with HCC, and the antibodies against these antigens also may be used as tumor markers. Cancer 2003;97:2474–9. © 2003 American Cancer Society. DOI 10.1002/cncr.11374

KEYWORDS: hepatocellular carcinoma, tumor-associated antigens, tumor markers, Tat binding protein-1, β4 integrin-binding protein, ribosomal protein L30.

Hepatocellular carcinoma (HCC) is a major cause of death in Asian countries, including Japan, and is a terminal complication of chronic inflammatory and fibrotic liver diseases.1,2 Regardless of recent developments in therapeutic modalities, the prognosis for patients with advanced HCC remains poor. Several trials of nonspecific immunotherapy for patients with HCC have been performed to overcome this difficulty.3–5 One of the nonspecific immunotherapies, lymphokine-activated killer cell (LAK) therapy, prevented the recurrence of HCC and demonstrated the usefulness of immunotherapy for the treatment of patients with HCC.3

Recently, vaccine therapies with specific peptides of tumor antigens have been used for the treatment of patients with various malignant tumors, especially melanoma.6–9 In a clinical trial with peptides of a testis carcinoma antigen, NY-ESO-1, the induction of a specific CD8-positive T-cell response was observed in four of seven patients with melanoma; and, in five of those seven patients, the...
treatment resulted in regression or growth suppression of tumors.\textsuperscript{9} We previously reported the expression of tumor antigens MAGE-1, MAGE-3, GAGE1-2, GAGE1-6, and BAGE in HCC; and at least one of those genes was expressed in 88% of patients with HCC.\textsuperscript{10,11} In addition, NY-ESO-1 also was expressed in about 40% of patients with HCC (unpublished data). All of these antigens may be potential targets for antigen specific immunotherapy in patients with HCC. However, most of the antigens were identified originally in patients with melanoma, and it is uncertain which antigens are immunogenic in patients with HCC.

Serologic analysis of recombinant cDNA expression libraries (SEREX) is an immunoscreening method of cDNA libraries prepared from tumor tissues by means of autologous sera.\textsuperscript{12} Because this method does not require cultured tumor cell lines or tumor specific cytotoxic T lymphocytes (CTLs), it is applicable to a wide variety of malignancies. Many tumor antigens, including new cancer/testis antigens, have been discovered by SEREX analyses in different solid tumors.\textsuperscript{13–15} Some antigens were known to induce CTL response.\textsuperscript{16} This means that SEREX can identify antigens that induce a cellular immune response as well as a humoral immune response. In this study, we identified antigens that are immunogenic in patients with HCC by SEREX analysis and discovered that antibodies against the antigens may be used as tumor markers for HCC. Preliminary results were published previously in abstract form.\textsuperscript{17}

**MATERIALS AND METHODS**

**Tissues and Serum Samples**

Two moderately differentiated HCC surgical resection samples, one from a woman age 66 years who had hepatitis C virus (HCV)-related hepatitis (Patient A) and one from a man age 58 years who had alcoholic hepatitis (Patient B), were used for the construction of cDNA libraries. Both patients had no history of other malignancies, autoimmune diseases, or hyper-\(\gamma\)-globulinemia due to chronic liver injury that may have caused a nonspecific immune response. To examine gene expression of the clones obtained by SEREX analysis, five surgically resected HCC specimens and corresponding samples from uninvolved regions of the liver were stored at \(-80\) °C.

Sera from 20 normal volunteers, from 16 patients with hepatitis B virus-related or HCV-related chronic liver injury, and from 20 patients with HCC were used for analysis of the antibodies. The serum \(\alpha\)-fetoprotein (AFP) and des-\(\gamma\)-carboxy prothrombin (DCP) levels in patients with HCC were measured by enzyme-linked immunosorbent assay, and cut-off values were set at 20 ng/mL AFP and 40 mAU/mL DCP. All samples were obtained according to the guidelines of our institution, and informed consent was obtained from all patients.

**RNA Extraction and Construction of cDNA Libraries**

Total RNA and Poly A(+) RNA were extracted using a QuickPrep mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ). The cDNA library was constructed in a ZAP Express vector using a cDNA cloning kit (Stratagene, La Jolla, CA). The cDNA fragments were packaged into a \(\lambda\)-ZAP express vector and then transected into *E. coli*. Approximately 3.5 \(\times\) 10\(^5\) clones and 2.5 \(\times\) 10\(^5\) clones obtained from Patient A and Patient B, respectively, were used for further study.

**Immunoscreening and Sequencing**

All sera (1:10 dilution) were absorbed with lysate from *E. coli* that was coupled to Sepharose 4B (5-Prime 3-Prime Inc., Boulder, CO). The sera were diluted (1: 200) in 0.25% bovine serum albumin/Tris-buffered saline and stored at 4 °C.

The primary recombinant cDNA library was screened with autologous patient serum, as described elsewhere.\textsuperscript{13} Briefly, the phage libraries were treated with isopropyl \(\beta\)-D-thiogalactoside for 6 hours and blotted onto nitrocellulose membranes. The membranes were incubated in the preabsorbed serum overnight at 20 °C. Immunoreactive clones were detected by peroxidase-conjugated goat antihuman immunoglobulin G (Jackson ImmunoResearch, West Grove, PA) and stained with 3-3'-diaminobenzidine (Sigma Chemical Company, St. Louis, MO). Positive clones were subcloned into pBK-CMV phagemid vector (Stratagene), and the nucleotide sequences of cDNA inserts were determined using a BigDye cycle terminator ready reaction kit and ABI Prism automated DNA sequencer (PE Applied Biosystems, Foster City, CA).

**Screening of Patient Sera**

The clones were tested for reactivity against sera from patients with HCC and from normal control participants by using the immunoscreening assay described above. Clones that reacted with > 20% of HCC sera and with 0% of control sera were analyzed further for the presence of the antibody in patients with chronic viral liver disease. A \(\lambda\)-ZAP clone without an insert was co-plated at a ratio of 1:2 and was included in each assay as a negative control.

**Reverse Transcription-Polymerase Chain Reaction Analysis**

mRNA expression of the selected cDNA clones that reacted only with sera from patients with HCC was
Results

Thirty reactive clones were identified by immunoscreening of the two primary cDNA expression libraries with autologous sera (8 from Patient A and 22 from Patient B). Three clones overlapped (clones 19, 23, and 25); thus, 27 different antigens were identified by nucleotide sequences (Table 2). Five of the 27 antigens were registered previously in a SEREX data base (clones 3, 4, 8, 15, and 18; available online from URL: http://www.licr.org/SEREX.html).

Among the 27 antigens identified, 25 antigens were the product of known genes, including SART1, p57Kip2, ROCK-1, γ-catenin, and heat-shock proteins, all of which were classified as tumor-associated genes.18–23 Antibodies to the antigens γ-catenin and heat-shock protein 90 have been reported in sera from patients with thymic tumors and osteosarcoma, respectively.24,25 The remaining two antigens (clones 12 and 22) were hitherto unknown genes that have homology with the draft sequence of the human genome.

Three other antigens—Tat-binding protein-1 (TBP-1; clone 1), β4 integrin-binding protein (p27[BBP]; clone 2), and ribosomal protein L30 (rpL30; clone 3)—reacted only with sera from patients with HCC. The frequency of their response was 55%, 45%, and 20% for TBP-1, p27[BBP], and rpL30, respectively. Down-regulator of transcription 1 (Dr1; clone 4) reacted with sera from patients with HCC and not with control sera; however, Dr1 also reacted with sera from patients with chronic viral liver disease. Seventeen antigens reacted with sera from both normal samples and patient samples (clones 5–21), and 6 antigens reacted only with autologous sera (clones 22–27). The expression of HCC specific antibody against three antigens (clones 1–3) was compared with clinical parameters in 20 HCC samples (Table 3). Although 6 patients were negative for the 3 antibodies, 70% of patients with HCC reacted with at least 1 of the 3 antigens, and serologic responses were induced even in patients with HCC tumors that measured < 2 cm in greatest dimension. The presence of the antibodies did not correlate with patient age, gender, tumor size, the grade of differentiation, tumor markers, or the type of hepatitis virus.

Although the three antigens were immunogenic only in patients with HCC, mRNAs from the three antigens were expressed constitutively in all HCC samples and their corresponding, uninvolved liver samples by RT-PCR analyses. Six antigens that reacted only with autologous serum also were expressed constitutively in all HCC samples and their corresponding, nondiseased liver samples (clones 22–27).

Discussion

In the current study, we applied the SEREX analysis technique to the identification of tumor antigens in human HCC and found that antibodies against TBP-1, p27[BBP], and rpL30 were produced specifically in patients with HCC. The expression of these antigens was not disease specific; however, they seem to be...
good diagnostic markers for HCC, because the frequency of antibody production was high. Among eight patients who were negative for both AFP and DCP, six patients had an antibody against TBP-1. In addition, an antibody against TBP-1 was found even in patients with small HCC tumors, suggesting that anti-TBP-1 antibody may be a useful and complementary marker for the diagnosis of HCC. TBP-1 originally was described as a transcriptional factor of human immunodeficiency virus 1, which interacts with the tat protein and is a component of the 26S proteasome. It is known that TBP-1 binds with the \( \text{erbB} \) receptor family and inhibits cell growth. It also has been reported that TBP-1 binds with hepatitis virus X protein and inhibits the replication of hepatitis B virus.

p27(BBP) was identified originally as a cytoplasmic interactor of integrin \( \beta4 \). Overexpression of p27(BBP) in human colorectal carcinoma has been reported. Overexpression of rpL30 also has been reported in patients with prostate carcinoma. Despite the specific reactivity of HCC with the antigens, mRNAs of TBP-1, p27(BBP), and rpL30 were expressed constitutively in liver tissues. Although noncancerous hepatocytes repeat cell death and regeneration due to hepatitis virus infection, rapid turnover of HCC cells may increase the chance of antigen presentation and result in antibody production against these genes in patients with HCC.

We defined 27 antigens from two different cDNA libraries of HCC; however, there was no overlap between the libraries. Recently, a SEREX analysis of a patient with hepatitis B virus-related HCC was reported that identified 19 distinct antigens; however, there was no overlap between the 19 antigens from that report and our 27 antigens. Five of our defined antigens were identical to the antigens recognized previously by SEREX analysis of other types of malignancies, whereas the remaining 22 antigens have not been registered in the SEREX data base (http://www.lirc.org/SEREX.html). The diversity of antibody repertoire among different SEREX analyses and the exis-

### TABLE 2

Genes Identified by Serologic Analysis of Recombinant cDNA Expression Libraries of Hepatocellular Carcinoma and Reactivity of Allogenic Sera against the Antigens

| Clone no. | Gene Description | GenBank Access no. | HCC (n = 20)* | Control (n = 20) | CH (n = 16) |
|-----------|------------------|--------------------|--------------|----------------|------------|
| 1         | Tat binding protein-1 (TBP-1) | M34079 | 11 (55%) | 0 | 0 |
| 2         | \( \beta4 \) integrin-binding protein (p27(BBP)) | AF084733 | 9 (45%) | 0 | 0 |
| 3         | Ribosomal protein L30 (rpL30) | BC002327 | 4 (20%) | 0 | 0 |
| 4         | Down-regulator of transcription 1 (Dr1) | M97388 | 6 | 0 | 2 |
| 5         | Retinoic acid receptor responder 2 (TGF2) | U77584 | 7 | 1 | — |
| 6         | Junction plakoglobin, isoform 1 (\( \gamma \)-catenin) | Z68228 | 4 | 1 | — |
| 7         | Transgelin 2 (TAGLN2) | D21261 | 13 | 7 | — |
| 8         | Activin A receptor, type IIIB | X77533 | 12 | 8 | — |
| 9         | Hypothetical protein BC008201 (LOC20840) | BC008201 | 11 | 11 | — |
| 10        | Heat-shock 90 kD protein 1 (HSPA) | M27024 | 11 | 9 | — |
| 11        | Solute carrier family 25, member 1 (SLC25A1) | U25147 | 9 | 4 | — |
| 12        | Unknown-1 (genomic DNA, chromosome 9, clone RP11-213G2) | AL333743 | 8 | 6 | — |
| 13        | Cyclin-dependent kinase inhibitor 1C (p57Kip2) | U22398 | 5 | 3 | — |
| 14        | Heat-shock 70 kD protein 9B (mortalin-2) | L15109 | 3 | 1 | — |
| 15        | Rho-associated, coiled-coil containing protein kinase 1 (ROCK-1) | U43195 | 2 | 3 | — |
| 16        | Squamous cell carcinoma antigen recognized by T cells (SART1) | AB006198 | 2 | 1 | — |
| 17        | Hypothetical protein FLJ16346 | AK000371 | 2 | 1 | — |
| 18        | P8 protein (candidate for metastasis 1) | AF069073 | 2 | 1 | — |
| 19        | Hypothetical protein FLJ01456 | AK027362 | 1 | 1 | — |
| 20        | Hairy (Drosophila)-homolog (HRY) | AK000415 | 1 | 1 | — |
| 21        | Solute carrier family 26, member 6 (SLC26A6) | AF279265 | 1 | 1 | — |
| 22        | Unknown-2 (genomic DNA, chromosome 11q, clone RP11-765H23) | AP00810 | 1 | 0 | — |
| 23        | Hypothetical protein FLJ13770 | AK023832 | 1 | 0 | — |
| 24        | Zinc finger protein 281 | AF125158 | 1 | 0 | — |
| 25        | CCG-99 protein | AF151857 | 1 | 0 | — |
| 26        | Hypothetical protein FLJ00447 | AK074076 | 1 | 0 | — |
| 27        | Hypothetical protein MGC2040 | BC006505 | 1 | 0 | — |

HCC: hepatocellular carcinoma; CH: chronic hepatitis.

* Includes autologous patients' sera. Clone 3–5, 8, 13, 14, and 23 were obtained from the cDNA library of Patient A.
tence of antibodies that react only with autologous serum indicate that patients with HCC have many nonspecific immune responses and only a few antigens that can induce a disease specific response.

In this study, we demonstrated that anti-TBP-1, anti-p27(BBP), and anti-rpL30 antibodies were induced in patients with HCC, and these antibodies could be used as tumor markers for HCC. Elucidation of the mechanism of this cancer specific antigen production may make it possible to use these antigens in the future for immune therapy in patients with HCC.

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