Definition of Tumor-associated Antigens in Hepatocellular Carcinoma

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Abstract

With an estimated annual incidence of about one million cases, hepatocellular carcinoma (HCC) is one of the most common neoplasms worldwide. Of all malignant diseases, it is the major cause of death in some regions of Africa and Asia. The pathogenic mechanisms responsible for HCC are not well defined, and therapeutic means, especially in inoperable HCCs, are still unsatisfactory and await improvement. In the quest for tumor antigens exploitable for gene therapy, we studied immune responses in the context of HCC. A cDNA library derived from a human HCC sample was screened using the SEREX approach. Nineteen distinct antigens reactive with autologous IgG were identified. Sequence analysis revealed three of the cDNA clones to code for hitherto unknown proteins and 16 known genes products. Proteins as diverse in function as LDH, albumin, and kinectin were found. Furthermore, proteins involved in the transcription/translation machinery had elicited an immune response in the autologous host. A panel of allogenic sera including sera from patients with hepatitis, liver cirrhosis, HCC, and other tumor entities, as well as sera from normal individuals, was used for frequency analysis of antibody responses. Whereas allogenic sera of HCC patients detected most antigens at a high percentage, control sera were rarely antibody-positive. The nature of the major fraction of antigens described here are linked to liver. Thus, our findings demonstrate not only the complexity of the humoral immune response against HCC, but may also offer new insight into mechanisms underlying transformation of the liver cell.

Introduction

A major goal of tumor immunology is the definition of tumor-associated structures that could serve as targets for diagnostic or therapeutic approaches.

Different approaches for this aim have been exploited, each with its own inherent strength and weakness. For the most part, tumor-associated antigens have been defined either from a cellular or a humoral basis. The list of both is rapidly growing, and today there is little doubt that the immune system recognizes malignancy and responds to it. The extent of efficiency of these immune defenses against tumors is the subject of ongoing discussion and presently cannot be answered.

SEREX is a method used to analyze tumor-associated antigenic structures by examining antibody responses in the tumor bearing host. In the past, some tumor associated antigens had been identified by conventional serology, however, with the introduction of SEREX, the amount of antibody detectable antigens has risen rapidly (1). The broad applicability with respect to different tumor entities and the fast access to molecular characterization are the major advantages of this method.

There is growing evidence that antitumor immune response is a process integrating different effectors of the immune system (1, 2). Humoral responses, especially in the case of high-titered antibodies, as in SEREX, are T-helper-cell-dependent. Previous studies have demonstrated the capability of SEREX to retrieve tumor-associated antigens that were initially defined using T-lymphocyte cell approaches (2–4). Recent work has shown conversely that cytotoxic T-cell populations against SEREX antigens exist (5).

To identify genes that contribute to the etiology of tumor in general, and specifically in HCC, we applied the SEREX technology to this tumor entity. The present study examines the diversity of antibody responses in the context of HCC.

Materials and Methods

Sera, Tissues, and Cell Lines. The study was approved by the local ethical review board (“Ethikkommission der Ärztekammer des Saarlandes”). Recombinant DNA work was done with the official permission and according to the rules of the state government of Saarland. Sera and tumor tissues were obtained during routine diagnostic or therapeutic procedures and were stored at −80°C until use. Normal tissues were collected from autopsies of tumor-free patients. For sera controls, routinely drawn blood from patients with confirmed HBV+, HCV- or alcohol-related cirrhosis as well as from 20 healthy donors was studied. The tissue sample for the construction of a cDNA library was obtained from a 30-year-old patient from Guanxi, China. Intraoperational, the excised tumor measured 6 × 10 × 9 cm. There was no metastasis and no ascites at the time of surgery.

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4 The abbreviations used are: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; HBS-Ag, hepatitis B surface antigen; EGF, epidermal growth factor; PSP, perichloric acid-soluble rat protein; HSP, heat-shock protein.
initial treatment. Histopathologically the tumor was found to be moderately differentiated and liver cirrhosis was confirmed. There were no risk factors besides a chronic HBV infection. Moreover, there was no history of alcohol, hemochromatosis, or other rare diseases leading to cirrhosis. According to the scheme of Okuda et al. (6), the clinical stage was I.

**Hepatitis Serology.** The infectious status of each patient and reference sera was determined by routine diagnostic tests; ELISAs for the presence of antibodies to HCV (anti-HCV) and HBS-Ag. The patient’s status was found to be anti-HCV-negative and HBS-Ag-positive.

**p53 Status.** Keeping in mind the high incidence of p53 alterations in HCC patients in Guanxi, we assessed the status of p53 using a site-specific PCR to amplify the known hotspot at codon 249. [Primers used were p53ex75s (TTGGCTCTGACT-GTACCACC) and p53ex83s (ATTCTCCATCCAGTG-GTTTC).] The primary cDNA expression library served as template (see below). The PCR products were cloned into pCR2.1 TOPO-vector (Invitrogen, Carlsbad, CA) and were subsequently sequenced.

**Construction of cDNA Library.** Total RNA was isolated from the patient’s tumor biopsy. Poly(A) RNA was prepared with a mRNA isolation kit (Stratagene, La Jolla, CA). A cDNA expression library was constructed by starting with 10 μg of poly(A) RNA. First-strand synthesis was performed using an oligo(dT) primer with an internal Xhol site and 5-methyl-CTP. cDNA was ligated to EcoRI adapters and digested with Xhol. cDNA fragments were cloned in sense direction with respect to the lacZ promoter into the bacteriophage expression vector λ-ZAPII (Stratagene); they were packaged into phage particles and were used to transfet Escherichia coli, which resulted in 1.65 × 10⁶ primary recombinants.

**Autologous and Allogeneic Immunoscreeening of Transfecteds.** Immunoscreeening for the detection of reactive clonies was performed with autologous serum. E. coli transfected with recombinant λ-ZAPII phages were plated onto LB-agar plates. Expression of recombinant proteins was induced with isopropyl β-D-thiogalactoside (IPTG). Plates were incubated at 37°C until plaques were visible and then blotted onto nitrocellulose membranes. The membranes were blocked with 5% (wt/vol) low-fat milk in Tris-buffered saline and were preincubated with an alkaline phosphatase-conjugated antibody specific for human IgG for 1 h. Reactive clones representing expressed IgG heavy chains were visualized by staining (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium; BCIP/NBT) and marked by alkaline phosphatase-conjugated antibody specific for human IgG. Reactive clones were purified and then mixed with nonrecombinant phages as internal controls in a 1:10 fashion. This mixture was tested against preabsorbed sera of interest. In this study, we tested the sera of 20 healthy controls, 20 patients bearing different tumors, and 20 patients chronically infected with HBV or HCV as well as from 4 patients suffering from alcohol-induced liver cirrhosis.

**Western Blot and in Vitro Translation.** To confirm the actual translation of the clone, we translated the pBK-CMV plasmids in vitro (Boehringer, Mannheim, Germany) and confirmed the predicted protein sizes by SDS-electrophoresis and Western blotting.

**Northern Blot Analysis.** Northern blots were performed with RNA extracted from tumors and normal tissues (9). The integrity of the RNA was checked by electrophoresis in formalin-4-morpholinepropanesulfonic acid gels. Gels containing 20 μg RNA per lane were blotted onto nylon membranes. After prehybridization, the membranes were incubated with the specific 32P-labeled cDNA probes overnight at 65°C in hybridization solution (6× SSC, 5× Denhardt’s solution, and 0.2% SDS). The membranes were then washed at progressively higher stringency; the final wash was in 1× SSC and 0.1% SDS at 65°C. Autoradiography was conducted at ~70°C for up to 7 days using Kodak X-OMAT-AR film and intensifying screen. After exposure, the filters were stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase probe to prove RNA integrity.

**Results**

Immunoscreeening of a HCC cDNA expression library with autologous sera led to the isolation of 99 immunoreactive clones. These clones represent 19 distinct antigens, 16 complete or partial cDNAs coded for known or closely related proteins (Table 1). Three were hitherto unknown. At least five of the former genes can be linked to liver, either by function or by predominant expression in this organ (albumin, SMP-30, p14.5, LDH-A, and metallopanstinin). There were no hepatitis-associated proteins among the isolated antigens.

**p53 and Aflatoxin.** A major role of aflatoxin B1 in the pathogenesis of this case could not be demonstrated. p53 was found to be wild type on the mRNA expression level. The frequent aflatoxin B1-associated mutation at codon 249 could be ruled out by PCR and sequencing as described above. Other mutations were not detected within the examined regions of p53 (exon 7 and exon 8).

**LDH-A.** Two different clones (HOM-HCC 2–3.2b, HOM-HCC 6–8a), both encompassing the full length coding region of LDH-A, were detected by autologous antibodies. Three of 20 healthy control had detectable antibodies against LDH-A.

**Albumin.** In comparison, albumin antibodies could be detected solely in infectious [3 of 5 HCC’s (HBV-positive)], 4 of 10 HBV, 2 of 10 HCV) but neither in toxic liver disease (0 of 4 primary cirrhosis) nor in any other examined tumor (0 of 20) or control sera (0 of 20). In a series of studies Sakata and Atassi (11) have described six major antigenic sites of antibody recognition in serum albumin. These sites seem to be species-
independent and serve as a good model for structural and conformational singularity. Whereas the clone 3.12 possesses all of these sites, clone 29.5 coding for a COOH-terminal fragment of albumin contains only two of these, epitope 3 and 6. Because epitope 6 is a discontinuous antigenic site, it is unlikely that clone 29.5, a fragmented fusion protein, displayed its correct superstructure. Thus, it can be assumed that epitope 3, the farthest continuous antigenic site, elicited the immune response. For this peptide, cross-reactivity with T cells has been successfully demonstrated (12).

Clone 14.1: A HSP. HSP89αDN, the homologue of clone 14.1, has been isolated from a subtractive cDNA library enriched for clones elevated in pancreatic cancer (13). HSP89αDN is a classical HSP.

SEC63 (21.7.2) Is a DNAJ-like Protein. 21.7.2 codes for a COOH-terminal part of SEC63, a DNAJ-like protein that is involved in activation processes of HSPs/chaperones.

Clone 6.8.2 Represents p14.5. This Mr 14,500 protein was first isolated by Schmiedeknecht et al. in 1996 (14) from mononuclear phagocytes. p14.5 displays low mRNA and protein expression in a variety of undifferentiated proliferating liver and kidney tumor cells. This led to the assumption that p14.5 prevents hepatocytes from entering the cell cycle (15). In accordance with this, we found p14.5 to be down-regulated in four of five HCCs studied by Northern blotting. However, one HCC patient (clinical stage I) exhibited a more than 3-fold overexpression compared with normal liver tissue (Northern blot). Serologically, this patient had antibodies against p14.5, but no alteration of p14.5 was detected. Antibodies were found in four of five HCC cases but not in any tumor-bearing host, hepatitis case, or healthy control.

GCF2 (2.5.1). Clone 2.5.1 codes for a fragment of the GCF2 protein, a family member of the GC-binding factors. It includes three of four potential phosphorylation sites and the N-glycosylation site but lacks COOH-terminal part of the DNA binding site and the putative nuclear localization signal. In Northern blot studies by Reed et al. (16) RAJI cells (Burkitt’s lymphoma), T98G cells (glioblastoma), and HUT-102-cells (T-cell lymphoma) expressed the highest levels of GCF2. GCF2 had elicited the broadest allogenic immune response. Remarkably, antibody responses against clone 2.5.1 were found not only in other tumors (such as melanoma, lymphoma, renal cell carcinoma) but in chronic hepatitis B patients (3 of 10) in a high frequency. All of the hepatitis-C

### Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>HOM-HCC designation</th>
<th>Size (kb)</th>
<th>Identity (Gen Bank accessions no.)</th>
<th>Functional significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HOM-HCC 2.3.2</td>
<td>1.5</td>
<td>LDH-A</td>
<td>Metabolic enzyme, tumor marker, transcriptional modulator</td>
</tr>
<tr>
<td>2</td>
<td>HOM-HCC 3.12.3</td>
<td>1.9</td>
<td>Albumin</td>
<td>HSP in fetal liver</td>
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<tr>
<td>3</td>
<td>HOM-HCC 29.5.1</td>
<td>0.84</td>
<td>Hsp89αDN (AF028832)</td>
<td>HSP</td>
</tr>
<tr>
<td>4</td>
<td>HOM-HCC 29.5.1</td>
<td>1.8</td>
<td>SEC63</td>
<td>ER membrane protein, contains DNAJ-like domain; interacts with HSPs</td>
</tr>
<tr>
<td>5</td>
<td>HOM-HCC 6.8.2</td>
<td>0.95</td>
<td>14.5 kDa protein (X93884)</td>
<td>Translation inhibitor</td>
</tr>
<tr>
<td>6</td>
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<td>1.65</td>
<td>GCF2 (U69609)</td>
<td>Transcriptional repressor</td>
</tr>
<tr>
<td>7</td>
<td>HOM-HCC 7.14.1</td>
<td>3.0</td>
<td>Metallopanstimulin 1</td>
<td>Growth factor-inducible gene with a zinc-finger structure and DNA-binding ability</td>
</tr>
<tr>
<td>8</td>
<td>HOM-HCC 22.5</td>
<td>1.1</td>
<td>SMP-30 (D31815)</td>
<td>Ca$^{2+}$ binding protein, proteinsynthesis regulator</td>
</tr>
<tr>
<td>9</td>
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<td>2.5</td>
<td>Cg1 protein,</td>
<td>Vesicle motility, transmembrane trafficking</td>
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<tr>
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<td>Kinecin (L25616)</td>
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<tr>
<td>11</td>
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<td>2.3</td>
<td>3JS protein (X99145)</td>
<td>Up regulated in thyroid on TSH stimulation</td>
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<tr>
<td>12</td>
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<td>1.6</td>
<td>31-ATPase, β subunit (X03559)</td>
<td>Catalytic site of 31-ATPase the major enzyme for ATP synthesis</td>
</tr>
<tr>
<td>13</td>
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<td>0.6</td>
<td>Pre-apolipoprotein CIII (X01388)</td>
<td>Ribosomal protein</td>
</tr>
<tr>
<td>14</td>
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<td>2.2</td>
<td>Galactose-1-phosphate-uridyltransferase (GALT) (M96264)</td>
<td>Metabolic enzyme</td>
</tr>
<tr>
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<td>1.0</td>
<td>DNA polymerase Δ, small subunit (U21090)</td>
<td>DNA processing</td>
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<tr>
<td>16</td>
<td>HOM-HCC 26.3</td>
<td>0.5</td>
<td>Mitochondrial DNA (J01415)</td>
<td>Mitochondrial DNA</td>
</tr>
</tbody>
</table>

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patients were negative, but two of ten healthy controls were positive.

**Metallopanstimulin** (7.14.1). 7.14.1 represents unaltered MPS-1, a zinc finger protein that is located in the nucleus of the cell. MPS-1 was originally cloned from a mammary carcinoma cell line that had been stimulated with transforming growth factor β1 in the presence of cyclohexamide (17).

**SMP-30 Protein** (22.5). Clone 22.5 is a nonmutated COOH-terminal fragment of SMP-30, a protein previously described to be involved in Ca$$^{2+}$$ channel regulation and aging (18, 19). The clone lacks the first 38 amino acids of the original clone. This results in a loss of the putative first protein kinase I phosphorylation site. Besides being detected in HCC patients, antibodies against SMP-30 were detected in 1 of 20 healthy controls and 1 of 10 hepatitis-B patients.

**Kinectin, Human CG1 Protein** (1.8.1–27.12). The most abundant antibody response, five different clones found in this screening, was directed against CG1-kinectin. Apart from the 5′ untranslated region and the first 55 amino acids, the whole CG1 protein was covered by different clones. Allogenic screening consistently caught antibodies versus different clones in four of five examined HCC patients but not in hepatitis or cirrhosis patients nor in healthy controls. Kinectin is an evolutionary conserved integral membrane protein anchored in the endoplasmic reticulum via a transmembrane domain (20). It is critically involved in kinesin-driven vesicle motility (21). The detected and sequenced fragments in this study did not reveal any mutations of CG1 but an NH$_2$-terminal mutation could not be ruled out.

**Discussion**

The young age of the patient and the early stage of disease made the patient a good candidate for this study because a strong cellular and humoral immune answer was expected. The diversity of antibodies that have developed in this setting, ranging from antibodies against ubiquitous antigens to very tissue-restricted antigens, demonstrates the intensity of the humoral immune response against malignant and damaged tissue associated with tumor growth. Antigens that have long been known to be involved in tumor formation and development, such as LDH, have been detected by SEREX in the past and were retrieved in this study again. The fact that no virus-related proteins, namely hepatitis, were among the isolated antigens may be related to the fact that chronic hepatitis, as found in this patient, leads to immunogenic indifference or that viral proteins are not major players in the tumorigenicity of HCC.

The antigens can be grouped according to their established or putative function in normal cell function and malignancy.

**Distorted Transcription/Translation.** GCF2, a relative of the GC-binding factor, is a transcriptional repressor of the EGF gene (16). Besides GCF and GCF2 proteins, Sp1, Ap2, and p53 bind to the EGFR promoter in an analogous region underlining the regulatory importance of transcription repression (22). EGFR is involved in cell growth and development (23, 24) and is known for its capacity for transformation on overexpression (25). Several types of cancer overproduce EGFR because of gene amplification or as a result of transcriptional or posttranscriptional deregulation (26). Several agents have been described that alter EGFR expression level (27, 28). In colorectal carcinoma, another member of the transcriptional regulator protein family, namely, TRIP4 protein, has been described as a tumor-associated antigen (29). HOM-MEL-40, a KRAB domain-containing protein that is overexpressed in melanomas is another example of a transcriptional regulator detected by autologous antibodies.

p14.5 is a strong translational repressor. Like a homologous PSP, it is most abundant in the cytosolic fraction of hepatocytes and renal distal tubular cells. Like PSP, the $M_s$ 14,500 protein strongly inhibits protein synthesis in vitro. Furthermore, the recently published mouse homologous translational inhibitor (Hrp12) has been shown to be a heat-shock responsive protein that is down-regulated in highly proliferating hepatocytes. Our Northern blotting study (Figs. 1 and 2) confirms that p14.5 is usually down-regulated in tumor cells, but there are exceptions. The patient had almost undetectable levels of mRNA at the time of surgery, but, nevertheless, a strong immune response had been established. Either the p14.5 mRNA levels at this stage were already down-regulated, or they may not have corresponded to the cytosolic protein concentration. Another patient had more than a 3-fold elevated expression of p14.5, as seen by Northern blotting (Figs. 1 and 2) and
SMP-30 rescues cell death by enhancing plasma membrane expression. This suggestion is in accordance with a finding that in liver and kidney in a very restricted pattern. Aging tissues energetic supply while sustaining transformation. Beside its al. (32) reported that c-Myc transactivates LDH-A to ensure an been unexplained on a molecular basis. Only recently, Shim Warburg effect), despite being intensely investigated, has long result of increased glycolysis. This phenomenon (named the production of lactic acid from glucose by malignant cells as a course and levels of S-LDH rather than S-AFP has been re-
elevated serum LDH (S-LDH) levels. In Burkitt’s lymphoma, tumors. For example, lymphomas and testicular, ovarian, pros-
tate, and small-cell lung cancers are often accompanied by elevated serum LDH (S-LDH) levels. In Burkitt’s lymphoma, S-LDH is an established tumor marker with prognostic value (30). In the case of HCC, a closer correlation between clinical course and levels of S-LDH rather than S-AFP has been reported (31).

Elevation of S-LDH is most likely due to the excessive production of lactic acid from glucose by malignant cells as a result of increased glycolysis. This phenomenon (named the Warburg effect), despite being intensely investigated, has long been unexplained on a molecular basis. Only recently, Shim et al. (32) reported that c-Myc transactivates LDH-A to ensure an energetic supply while sustaining transformation. Beside its function in intermediary metabolism, the LDH-A isoenzyme has also been linked to transcriptional modulation of gene expression and DNA replication (33–35).

**Altered Cell Death.** SMP-30 has been shown to be expressed in liver and kidney in a very restricted pattern. Aging tissues have decreasing amounts of SMP-30 protein, so that our results may point to a proliferate stimulus in malignancy when over-
expressed. This suggestion is in accordance with a finding that SMP-30 rescues cell death by enhancing plasma membrane Ca2+-pumping activity in Hep G2 cells (36).

**Antigen Presentation and Chaperoning.** HSP90αΔN, SEC63 (DNAJ-like protein), albumin, and CG1 are the representatives of this group.

HSPs have been seen as chaperones in oncogenic transformation, in cell cycle control, and in antigen presentation (13, 37, 38). From our experience with SEREX, various HSPs have been found in different cancers. One likely hypothesis is that HSPs are innocent bystanders detected while they act as chaperones for antigen-presenting molecules. Instead of reacting with the antigen presented, antibodies are then directed against the HSP. On the other hand, a possible role in transforming cells remains to be excluded.

**Others.** HOM-HCC-26–3: The interpretation of this finding is somehow conflicting. This clone partially encodes a sequence that was initially published by Katoh et al. (39) as the 5′UTR of Wnt-13, a member of the Wnt gene family. Wnt proteins are secreted glycoproteins, which are involved in normal development, differentiation, and carcinogenesis. A subsequent report by another group (40) indicated that this part of Wnt-13 belongs to mitochondrial DNA, and the authors concluded that the initial nucleotide sequence was erroneous and derived from a chimeric clone. It is known that Wnt-13 maps to chromosome 1p13, a region often affected by loss of heterozygosity in germ cell tumors (41). Hence, the chimeric sequence may not be an artifact that is accidentally discovered but a product of genetic instability, e.g., translocation of malignant cells. The detection of this DNA by SEREX, together with the specific antibodies, could then indicate that either the mitochondrial DNA has its own role in a tumor or it is in a favorable location to translocate to chromosome 1p13.

In addition to the clones discussed above, other proteins involved in cell homeostasis and metabolism have evoked an antibody response (see antigens 10–16, Table1). These proteins can be seen as recruits of a tumor that needs to sustain its energy supply and perform rapid growth. They represent the diverse means of the cell apparatus exploited to secure the malignant cell’s advantage over adjacent normal tissues.

In conclusion, the data generated by this study provide an insight into processes involved in HCC, and, when compared with previous studies (29, 42), in tumor formation and progression in general. At first glance, the antigenic molecules represent a panel of very diverse proteins. But taken together, like pieces of a puzzle fitted together, they present a picture of a cell machinery that has gone out of control and an immune system reacting to it.

The known antigens found in the context of HCC reflect a spectrum of genes with a diversity of functions in the cellular apparatus. Most of them share features of being involved in the regulation of transcription, translation, chaperoning, and presenting of proteins. Their immunogenicity is most likely a result of overexpression. It is tempting to speculate that they are overexpressed as a result of a futile attempt of cells to suppress the protein machinery going out of control. The zinc finger proteins deserve special attention because this protein family is, suspiciously, often seen in antitumor immune responses. Taken together, alteration of a variety of regulators of transcription have been seen to be affected in the tumor development of HCC and can be demonstrated by SEREX.

Some of the described antigens have been clearly linked to malignancy in the past (LDH-A, GFC2, MPS-1). As for the others, having no known association with cancer or autoimmu-
nity, they should be revisited in that context.

The role and function of the yet undetermined genes that were found here is the obvious next task, and experiments to elucidate their role are underway.

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**References**


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