Proteomic-based approach for the identification of tumor markers associated with hepatocellular carcinoma

Philip Shalhoub, Sarah Kern, Sophie Girard and Laura Beretta*

Department of Microbiology and Immunology and Cancer Center, University of Michigan Medical School, Ann Arbor, MI 48109, USA

There is increasing evidence for an immune response to cancer in humans, demonstrated in part by the identification of autoantibodies to tumor antigens. The identification of panels of tumor antigens that elicit a humoral response may have utility in cancer screening, diagnosis or in establishing prognosis. Several approaches are currently available for the identification of tumor antigens. We have used a proteomic-based approach for the identification of tumor antigens that induce an antibody response which we have applied to hepatocellular carcinoma, a major type of cancer worldwide. Two-dimensional gel electrophoresis allows simultaneous separation of several thousand individual proteins from tumor tissue or tumor cell lines. Proteins eliciting a humoral response in HCC were identified by 2-D Western blotting using sera from patients with hepatocellular carcinoma, followed by mass spectrometry analysis and database search. The common occurrence of autoantibodies to specific proteins may have utility for HCC screening and diagnosis.

1. Introduction

Primary liver cancer is a prevalent tumor worldwide. The incidence of hepatocellular carcinoma (HCC), the major histological form of primary liver cancer, has substantially increased in Japan [1], Western Europe [2, 3], and the United States [4] over the past two decades. The age-specific incidence of this cancer has also shown a progressive shift towards younger people (40

to 60 years old). Human hepatocarcinogenesis is a multistage process with the involvement of a multifactorial aetiology and many gene-environment interactions. It is important to emphasize the heterogeneity of the histological background on which the tumor develops. The majority of HCCs are associated with cirrhosis (at least 90% in America and Europe). These tumors often have a poor prognosis, with five-year survival rates of less than 5%. Some tumors will occur in livers with minimal histological changes and "benign" adenomas can even develop in normal livers. This heterogeneity likely reflects different environmental, as well as possibly genetic factors. A large number of epidemiological and molecular studies have clearly indicated the major importance of environmental factors in the development of primary liver cancers in humans. The role of genetic factors has also been raised but is difficult to properly address, due to confounding variables such as intrafamilial transmission of HBV [5].

2. Risk factors for the development of HCC

Some factors in the pathogenesis of HCC have been defined and almost all tumors occur in the context of chronic liver-cell injury, inflammation, and increased turnover of hepatocytes. Cirrhosis is a very important risk factor for HCC [6], with the risk increasing by a factor of approximately 200 after the onset of cirrhosis, and up to 30–50% of patients with cirrhosis developing an HCC upon 10 years follow up.

Chronic infection by hepatitis B (HBV) or C (HCV) virus is also a major risk factor for HCC, and development of a chronic carrier state is a most frequent event following acute viral infection [7,8]. The global distribution of HCC correlates well with the geographic prevalence of chronic carriers of HBV, who number 400 million worldwide. With persistent HBV infection, the risk of HCC increases by a factor of 100 [9].

* Address for correspondence: Laura Beretta, Dept. of Microbiology and Immunology, University of Michigan, 1510 MSRB-I, 1150 W. Medical Ctr. Dr., Ann Arbor, MI 48109-0666, USA. Tel.: +1 734 615 5964; Fax: +1 734 615 6150; E-mail: berettal@umich.edu.

Among those who become infected with HBV at birth, men have an estimated lifetime risk of HCC of 50%, while women have a risk of 20% [10]. Persistent infection with HCV is also an important risk factor for HCC development. As is true for HBV, the relative risk of HCC among persons with chronic HCV infection is approximately 100 times the risk in uninfected persons. Persistent HCV infection is the cause of 70% of the cases of HCC in Japan [11], and the most likely reason for the rising incidence of HCC in the United States is the spread of HCV infection in the population. Four million people in the United States have chronic HCV infection and persistent HCV infection is the cause of approximately 30–50% of the HCC cases in the United States.

The mechanisms involved in virally related liver carcinogenesis still remain largely undefined. There is evidence for direct effects of HBV in this process; in contrast, there is presently only very preliminary information regarding the carcinogenic role of HCV. Persistent viral infection causes inflammation, increased cell turnover and cirrhosis. Furthermore, the HBV genome may be integrated into the chromosomes of hepatocytes, contributing to genomic instability. As in persistent HBV infection, persistent HCV infection also initiates inflammation, cellular injury, regeneration, and cirrhosis, all of which may contribute to the oncogenic process. Prevention of chronic infection with these viruses by immunization is a high priority, and childhood vaccination against HBV carries the greatest potential for reducing the liver cancer burden. Safe and effective HBV vaccines are available, both plasma-derived and recombinant DNA-derived, although they are not yet used universally because of cost. An HCV vaccine is not yet available and may be difficult to develop because of the high mutation rate of the viral genome and variability of genotypes.

Epidemiological studies have demonstrated a strong association between exposure to aflatoxin and an increased incidence of HCC [12]. Aflatoxins have been found as contaminants in food, particularly in corn, peanut oil, soya sauce and fermented soya beans. Excessive alcohol consumption and tobacco smoking may also be associated with increased risk of HCC.

3. Prevention of HCC

A goal of cancer prevention is the detection of latent premalignant or malignant clones before they expand to a clinically detectable tumor. Resection of small HCC

remains an important approach in achieving long-term HCC survival and to improving 5-year survival rates. It is more effective than treatment of large HCC, surgical cure being rarely possible. Therefore, it is accepted that early detection, diagnosis and treatment of HCC remains an important target to be achieved before a breakthrough appears on the primary prevention of HCC. As high risk factors for developing HCC have been established, mass health screening for early detection of HCC is available. Initial screening should be done as early as possible on patients with chronic HBV and HCV infection, cirrhosis, and persons who had blood transfusions or a family history of HCC. The screening test currently used and widely practiced for HCC includes serum alpha-fetoprotein (AFP) levels and abdominal ultrasound examinations. Annual screening with ultrasound and AFP fails to identify potentially curable tumors because the diagnosis is often only made at a late stage of the disease [13]. Therefore a follow-up every 3 to 6 months is necessary. Although the screening can identify small tumors, survival may not be improved because the presence of cirrhosis may limit the number of patients who can undergo resections. In addition, recurrences or second primary tumors are common. Screening all liver cirrhosis patients is a questionable approach because it is very expensive and its benefit in terms of patient survival is poor. Conflicting data on the utility and efficacy of screening patients with cirrhosis for early detection of HCC and failures in the screening of chronic hepatitis B or C virus-infected patients with ultrasound and AFP have been reported [14]. Additional targeted screening programs with definite risk factors are dramatically needed.

4. Tumor markers

Several tumor markers of HCC have been identified. However, no evidence has been obtained indicating that the detection of these markers precedes clinical diagnosis of HCC. Aberrant expression of the AFP gene in serum is characteristic of a majority of HCC cases and is widely used as tumor marker in the evaluation of prognosis and management of patients with HCC [15]. Long considered the fundamental marker for diagnosis of HCC, the usefulness of AFP as a marker has slowly become overshadowed by its inability to efficiently diagnose early-stage tumors. A high false-negative diagnosis rate, sometimes reaching 40% [16], is also a problem associated with this marker. A fucosylated

subtype of AFP, termed Lens culinaris agglutinin A reactive AFP, has been shown to be significantly better in identifying early HCC than general AFP levels [17–19].

The search for new tumor markers has long been sought. Many searches are not aimed at replacing this rather valuable marker, but instead hope to couple AFP serum levels with another marker to produce a more definitive diagnosis. An example of such a marker is des-gamma-carboxy prothrombin (DCP), an abnormal prothrombin which has long been considered a reasonable indicator of HCC, particularly when used as a complementary marker with AFP [20–28]. It has been suggested that elevation of DCP levels in HCC patients are due to a vitamin K deficiency in the cancerous tissue [29]. DCP serum level was found to be one of the most significant indicators of recurrence of HCC in patients [30]. DCP levels have also been found to be a good indicator of portal venous invasion (PVI) in HCC patients, which heralds the progression of the disease [31]. One issue concerning DCP is the difficulty in detecting a small level in the serum, which is often a diagnostic indicator in early stage HCC patients.

New generations of sensitive immunoassays for detection of minute serum DCP levels have recently been reported to be effective in patients with small-sized HCC [26,27,32].

The diagnostic value of serum gamma-glutamyl transferase (GGT) has also been under evaluation [33], particularly that of an isoenzyme specific to HCC. Recently, hepatoma-specific bands of this enzyme (HS-GGT) were identified and found to be significantly increased in patients with HCC as compared to acute hepatitis, chronic hepatitis, cirrhosis, and extrahepatic tumors, and thus were found to be a useful tumor marker for AFP-negative HCC patients. Its use in combination with AFP serum levels has been proposed for monitoring chronic liver disease patients and diagnosing HCC [16]. The methylation status of the GGT gene has also been given consideration as hypomethylation of the CCGG sites of the GGT gene has been implicated in the abnormal expression of GGT in HCC patients [16].

Platelet-derived endothelial cell growth factor (PD-ECGF) has been demonstrated to be highly expressed in advanced stage HCC patients and has been suggested to induce rich neovascularization of tissue in HCC [34]. Although there is little usefulness for PD-ECGF measurement as an early diagnostic marker for HCC, its value lies in the prospect of using PD-ECGF as a prognostic marker of tumor development in advanced stage patients. Plasma vascular endothelial growth factor (VEGF) levels were also shown to be significantly in-

creased in patients with HCC. More specifically, the largest increase was seen after the disease has metastasized, which suggests some utility as a cancer stage specific marker [35]. It has been found to be a useful marker in detecting metastasis of HCC, and may have valuable prognostic value in patient care. A recent study has confirmed both VEGF and PD-ECGF as useful markers for identifying HCC, and has also reported a strong correlation between portal vein tumor thrombus (PVTT) development in HCC patients and serum levels of these markers [36].

A few additional tumor markers have been proposed, such as: plasma nitrate/nitrite [37,38], MXR7 [39] fibroblast growth factor [40], the CD24 gene [41], alpha-L-fucosidase [42], serum C-reactive protein [43], and activin-A [44]. Further research will determine whether or not these markers will prove valuable in diagnosing HCC.

5. Identification of new HCC tumor-associated antigens using proteomics

The common occurrence of autoantibody formation to certain cancer-related proteins may also have value in cancer screening and diagnosis. For example, mutations in the p53 tumor suppressor gene are present in up to 37% of patients with HCC. Conformational change and cellular accumulation can initiate an immune response with generation of circulating autoantibodies to p53 protein. However, the presence of p53 autoantibodies in patients with chronic liver disease is not completely specific for HCC and no direct evidence has been obtained that indicates p53 autoantibody formation precedes the clinical diagnosis of HCC [45,46]. Antigens that have been shown to induce a humoral response in HCC include diverse other nuclear proteins [47–51], cyclin B1 [52] and a novel cytoplasmic protein with RNA-binding motifs [53].

Methods have been developed to identify tumor associated antigens such as molecular cloning in expression systems [54,55] or using a biochemical strategy, based on the extraction of antigenic peptides bound to major histocompatibility complex class I molecules from tumor cells [56,57]. These methods have allowed the recognition of several human tumor antigens [58–62]. A method called SEREX, ‘serological identification of antigens by recombinant expression cloning’, has been recently used for the identification of tumor antigens [63]. The SEREX analysis is based on screening of autoantibodies in sera from cancer patients against an

Fig. 1. 2-D PAGE protein profile of hepatoma cell lines and tumor tissues. Total proteins from the hepatoma cell lines Huh7 and HepG2 (A) and from tumor tissues obtained from 2 patients with HCC (B) were separated by 2-D electrophoresis and subsequently silver-stained.

Fig. 2. Detection of autoantibodies in sera from patients with HCC. The proteins from the hepatoma cell line Huh7 were separated by 2-D electrophoresis and subsequently transferred on PVDF membranes for Western-blotting experiments using sera from a healthy individual (left panel) or from a patient with HCC (right panel) as a first antibody.

expression library made with the RNA from the autologous tumor. Through application of this strategy, an unexpected frequency of tumor antigens that elicit specific immune responses in the autologous host has been observed [63–66]. The SEREX approach is limited by the necessity to construct expression libraries and the analysis is usually restricted to one or a few patients. In addition, the approach does not allow the identification of antibodies directed against post-translational modifications. A SEREX study of hepatocellular carcinoma

has uncovered reactivity to diverse proteins involved in the transcription/translational machinery as well as to chaperone proteins [67].

In order to identify proteins eliciting humoral responses in HCC patients, we used a proteome-based approach that has been recently implemented in other studies [68–70]. Several thousand cellular proteins from hepatoma cell lines or from liver tumor tissues, were separated by 2-D PAGE. Figure 1 shows the protein profile of 2 commonly used hepatoma cell lines,

Huh7 and HepG2 (Fig. 1(A)) and of tumor tissues isolated from 2 patients with HCC (Fig. 1(B)). Proteins from hepatoma cell lines or from liver tumor tissues were then transferred onto membranes. Sera from HCC cancer patients were screened individually for antibodies that react against separated proteins. The autoantigens were detected using a secondary antibody directed against human IgM or IgG, followed by autoradiography. As shown in Fig. 2, a greater number of reactive proteins were detected in general with sera from patients with HCC than with sera from healthy individuals. Proteins that specifically reacted with sera from HCC patients were located on silver-stained 2-D gels after superimposition with the blots, extracted from the gel, digested and identified by mass spectrometric analysis and/or amino acid sequencing. We identified 8 proteins for which autoantibodies were detected in sera of more than 10% of 37 patients with HCC tested, but not in sera from healthy individuals (manuscript submitted). Autoantibodies against four of these proteins (beta-tubulin, hsp60, cytokeratin 18 and creatine kinase-B) were detected at a comparable frequency in sera from patients with chronic hepatitis. The other four proteins, which consisted of calreticulin isoforms, cytokeratin 8, nucleoside diphosphate kinase A and F1-ATP synthase beta subunit all induced autoantibodies among patients with HCC independently of their HBV/HCV status. The protein F1-ATP synthase beta subunit was previously reported to be antigenic in patients with HCC, by SEREX [67]. Calreticulin and a protein spot with an estimated MW of 32 kDa most frequently elicited autoantibodies among patients with HCC (27%). We previously identified this 32 kD protein as a new truncated form of calreticulin corresponding to the C-terminal end of the protein, and designated this novel form Crt32 [71]. The protein calreticulin has been identified as an autoantigen in various rheumatic diseases [72]. However, whereas the epitopes eliciting a humoral response in patients with autoimmune diseases have been reported to be located in the N-terminal part of the molecule, the epitopes eliciting a humoral response in patients with HCC in our study, are located in the C-terminal portion. In addition, autoantibodies against Crt32 were largely restricted to liver cancer patients among the different cancer sera we have analyzed.

6. Conclusion

A proteome-based approach allows individual screening of a large number of patient sera as well as detection

of autoantibodies directed against post-translational modifications of specific targets. We observed a distinct repertoire of autoantibodies associated with HCC, reflecting the heterogeneity of the tumor. These autoantibodies may have utility in early diagnosis of HCC among high-risk subjects with chronic hepatitis and/or cirrhosis and may be used in combination assay with serum AFP or other marker levels. This global analysis also emphasizes the need for specific markers used in targeted screening programs with defined risk factors.

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