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Mini-Array of Multiple Tumor-Associated Antigens to Enhance Autoantibody Detection for Immunodiagnosis of Hepatocellular Carcinoma

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Abstract

Liver cancer, especially hepatocellular carcinoma (HCC), is particularly prevalent in Africa and Asia. HCC affects the Hispanic population of the United States at a rate double that of the white population. The majority of people with HCC will die within 1 year of its detection. This high case-fatality rate can in part be attributed to lack of diagnostic methods that allow early detection. How to establish a methodology to identify the high-risk individuals for HCC remains to be investigated. The multi-factorial and multi-step nature in the molecular pathogenesis of human cancers must be taken into account in both the design and interpretation of studies to identify markers which will be useful for early detection of cancer. Our recent studies demonstrated that a mini-array of multiple tumor-associated antigens (TAAs) might enhance autoantibody detection for diagnosis of HCC, especially for the alpha fetoprotein (AFP)-negative cases. It also suggested that different types of cancer might require different panels of TAAs to achieve the sensitivity and specificity required to make immunodiagnosis a feasible adjunct to tumor diagnosis.

Keywords

Tumor-associated antigen; Autoantibody; Immunodiagnosis; Hepatocellular carcinoma

1. Introduction

Studies in autoimmune diseases have provided abundant evidence suggesting that the autoantibodies are antigen-driven responses and that autoantibodies can be viewed as reporters from the immune system revealing the identity of antigens which might be playing roles in the pathophysiology of the disease process [1]. The highly specific autoantibody response in systemic autoimmune disease generally predicts the biologic phenotype of the disease, making autoantibodies clinically valuable and diagnostically useful. Whether a similar mechanism is operating in the humoral immune response in cancer remains to be established, but appears to be a possibility. Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide, particularly prevalent in Africa and Asia. The majority of people with HCC will die within 1 year of its detection. This high case-fatality rate can in part be attributed to lack

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of diagnostic methods that allow early detection. How to establish a methodology to identify the high-risk individuals for HCC remains to be investigated. Although serum alpha fetoprotein (AFP) is the most effective marker available to detect HCC, the sensitivity and specificity is not optimal, especially in patients with small tumor or in well- to moderately-differentiated HCC cases. Therefore, there is a need for the development of more sensitive and specific methods that supplement AFP in the early detection of this cancer. The purpose of this review was not mainly directed at addressing the methodology of identification of TAAs in HCC. The review will focus on the recent advances in our studies primarily associated with the idea and possibility that a mini-array of multiple TAAs can be used for immunodiagnosis of HCC.

2. Tumor-associated antigens (TAAs)

Many studies demonstrated that cancer sera contain antibodies which react with a unique group of autologous cellular antigens called tumor-associated antigens (TAAs) [1–3]. One of the most extensively studied TAAs is p53, the tumor suppressor protein. Autoantibodies to p53 in cancer were first reported in 1982 [4] and since then there have been numerous reports confirming and extending this finding [reviewed in 5]. The types of cellular proteins which induce autoantibody responses are quite varied and include oncogene products such as HER-2/neu [6], cellular proteins which shield mRNAs from natural physiological degradation such as p62 [7] and CRD-BP [8], onconeural antigens in the paraneoplastic disorder syndromes [9], differentiation-antigens such as tyrosinase and the cancer/testis antigens [10]. Factors leading to the production of such autoantibodies are not completely understood but the available data show that many of the target antigens are cellular proteins whose aberrant regulation or overexpression could lead to tumorigenesis, such as p53 [4,5], HER-2/neu [6] and CENP-F [11], or are proteins whose dysregulation could have tumorigenic potential including mRNA binding proteins such as p62 [7] and cell-cycle control proteins such as cyclin B1 [12]. A highly informative study showed that lung tumors contained several types of p53 gene mutations including missense, stop codon and frameshift mutations, but it was the missense mutations with overexpression of protein which altered function and increased stability that correlated with antibody production [13]. In the case of p62 which is a fetal protein absent in adult tissues, immunogenicity appears to be related to abnormal expression of p62 in tumor cells [14] and with the onconeural antigens in paraneoplastic neurological disorders, antibody responses are thought to be related to ectopic expression of neuron-restricted cellular proteins in tumor cells [9]. The immune system in certain cancer patients appears to have the capability of sensing these abnormalities and it was proposed that autoantibodies might be regarded as reporters identifying aberrant cellular mechanisms in tumorigenesis [1]. In recent years there have been a steadily increasing number of studies describing and characterizing autoantibodies in cancer. Research on antibody immunity to cancer-associated proteins has received a great attention. As the detection of antibody immunity to tumor antigens becomes more routinary, investigators have evolved to begin to address specific clinical questions such as the role of antibody immunity as a marker for patients exposed to cancer, as a tool to monitor therapy, or as an indicator of disease prognosis.

3. Identification of TAAs

The approach which we have used in the identification of putative TAAs has involved initially examining the sera from cancer patients using extracts of tissue culture cells as source of antigens in Western blotting and by indirect immunofluorescence on whole cells. With these two techniques, we identify sera which have high-titer fluorescent staining or strong signals to cell extracts on Western blotting and subsequently use the antibodies in these sera to isolate cDNA clones from cDNA expression libraries. In this manner, several novel TAAs including HCC1 [15], SG2NA [16], CENP-F [17], p62 [7] and p90 [18] have been identified. Several novel as well as previously defined tumor antigens have been recently identified with

autoantibodies from patients with different types of cancer [3] using a methodology called SEREX (serological analysis of recombination cDNA expression libraries) [19]. The rationale is that intracellular proteins which are involved in carcinogenesis are provoking autoantibody responses and therefore autoantibodies can be used to immunoscreen cDNA expression libraries to isolate, identify and characterize proteins which might potentially be involved in malignant transformation. Using this approach, we have successfully isolated several novel TAAs such as p62 [7] and p90 [18]. A proteome-based approach has been recently implemented for identifying tumor-associated antigens in cancer patients [20]. Compared to SEREX, the proteome-based technology allows individual screening of a large number of sera, as well as determination of a large number of autoantigens. Proteome-based approach can also distinguish isoforms and the detection of autoantibodies directed against post-translational modifications of specific targets. The practical utility of this approach remains to be established with the proviso that efforts should be made to identify tumor-associated from tumor-irrelevant antigens.

4. Using a mini-array of multiple TAAs to enhance antibody detection in HCC

A feature of HCC is that antecedent liver cirrhosis and chronic hepatitis are common precursor conditions and during transition to malignancy some patients develop autoantibodies which were not present during the preceding chronic liver disease phase [21–23]. A hypothesis which has been proposed is that these antibody responses may be stimulated by cellular proteins which are involved in carcinogenesis. Many investigators have been interested in the use of autoantibodies as serological markers for cancer diagnosis, especially because of the general absence of these autoantibodies in normal individuals and in non-cancer conditions. Enthusiasm for this approach has been tempered by low sensitivity. We have observed that this drawback can be overcome by using a panel of carefully selected TAAs and that different types of cancer may require different panels of TAAs to achieve the sensitivity and specificity required to make immunodiagnosis a feasible adjunct to tumor diagnosis. This feature is one of the innovative notions we have proposed in our studies. One of our previous studies showed that detection of autoantibodies in cancer can be enhanced by using mini-array of seven TAAs as target antigens [24,25]. The mini-array of TAAs comprised full-length recombinant proteins expressed from cDNAs encoding c-myc [26], p53 [5], cyclin B1 [12], p62 [7,27,28], Koc [27,29], IMP1 [30] and survivin [31,32]. Antibody frequency to any individual TAA was variable, and ranges from 10.8 to 24.6 percent in HCC. With the successive addition of TAAs to a final total of seven antigens, there was stepwise increase of positive antibody reactions up to 56.9 percent in HCC. In our recent study, p16, a cyclin-dependent kinase inhibitor, which has been implicated as an important tumor suppressor [33], was evaluated as a TAA and added into our previously constituted mini-array of seven TAAs [24]. As shown in Table 1, the frequency of antibodies to eight TAAs in sera from patients with chronic hepatitis, liver cirrhosis and HCC using Enzyme-linked immunosorbent assay (ELISA). Positive results were also confirmed by slot blot, Western blot and immunoprecipitation. Antibody frequency to any individual TAA in HCC was variable from 9.9%–21.8% but rarely exceeded 20%. With the successive addition of TAAs to a final total of eight antigens, there was a stepwise increase of positive antibody reactions up to 59.9%, which was significantly higher than the frequency of antibodies in chronic hepatitis (20%), liver cirrhosis (30%) and normal individuals (12.2%). The sensitivity in diagnosing HCC was 59.9%, and the specificity was 87.8%, 80.0% and 70.0%, respectively, compared to normal individuals, chronic hepatitis and liver cirrhosis cases (34). This study further demonstrates that malignant transition in HCC is associated with autoantibody responses to certain cellular proteins which might have a role in tumorigenesis, and suggests that a mini-array of eight TAAs may enhance antibody detection for diagnosis of HCC. The fact is that antibodies to any individual antigen such as anti-p53, anti-p62 or anti-c-Myc do not reach levels of sensitivity which could become routinely useful in diagnosis [24]. The data from our studies in one way indicates that the combination of antibodies might

acquire higher sensitivity for diagnosis of cancer. On the other hand, the data also suggest that in the selection of different antigen-antibody systems, some of the antigens may turn out to be more specific for a certain type of cancer while others may be not. It is conceivable that autoantibody profiles involving different panels or arrays of TAAs might be developed and the results could be useful for diagnosis of certain types of cancer.

5. Alpha fetoprotein and HCC diagnosis

Serum alpha fetoprotein (AFP) is a fetal glycoprotein produced by yolk sac and fetal liver and has been commonly used as a serum marker for diagnosis of HCC. However, the sensitivity of AFP has been reported to be about 60% in HCC [35]. The elevation of AFP is also associated with benign hepatic diseases such as acute and chronic viral hepatitis as well as toxic liver injury, suggesting that AFP specificity in HCC diagnosis is still not reliable. Serum AFP levels remain within normal limits in most patients who have small HCC (<20 mm) or well-differentiated HCC [36]. Moreover, elevated serum AFP levels reflect hepatic regeneration after destruction of hepatocytes in patients with chronic hepatitis and liver cirrhosis [37]. In a recent study [38], we have observed a group of HCC patients from Japan, and found that 7 of 8 HCC patients with autoantibodies against multiple TAAs had a normal range of serum AFP levels. We have also demonstrated that 6 of 8 HCC patients with anti-TAAs had comparatively small nodules of HCC (<30 mm). In addition, all 5 HCC patients who had anti-TAAs and were confirmed histologically had well- to moderately-differentiated HCC. This finding may indicate that anti-TAAs seem to be supplementary serological markers for the diagnosis of HCC, especially for the AFP-negative cases.

6. What are the differences between our approach and others

Recently, Wang et al. developed a phage-display library derived from prostate-cancer tissue, and a phage protein microarray, to analyze autoantibodies in serum samples from patients with prostate cancer and controls [39]. In this study, a 22-phage-peptide detector was constructed for prostate cancer screening, with 81.6% sensitivity and 88.2% specificity. Compared to our approach, we constructed a decision tree for classifying prostate cancer, using five TAAs, with 79% sensitivity and 86% specificity [25]. With a smaller panel, these rates are equivalent to those of Wang et al. and might be improved by the selection of other TAAs. Approaches from Wang et al. as well as our panel of TAAs do not justify adoption in a screening program, and could be improved by the inclusion of additional tumor antigens. In a recent study, we added three selected TAAs (p90, cyclin D1 and cyclin A) into our previously used mini-array system for detection of antibodies in prostate cancer. The results showed that the cumulative positive reactions in prostate cancer sera reached 92.5%, significantly higher than that in benign prostatic hyperplasia (BPH) and other controls [40]. All these studies strongly support the hypothesis and rationale which we have proposed in our study. In searching the literature, we found that the notion of using panels of TAAs for detecting cancer autoantibodies is rarely discussed and is fairly new approach. We stress the notion that panels of “customized” TAAs should be used for different types of tumor and that these customized panels should be rigorously tested for sensitivity and specificity not only against other tumors but also against other disease conditions. In the case of HCC, the natural conditions would be chronic hepatitis and liver cirrhosis. The basis for the notion of the necessity of customized panels of TAAs is based not only on empirical observations but also on retrospective analysis of our own data learned from previously published work [22–24]. The main focus of our studies is to identify a specific panel of TAAs for HCC and compare and contrast this with antigen panels associated with chronic hepatitis and liver cirrhosis.

7. Conclusions

Based on our preliminary study of a mini-array of eight TAAs in detection of HCC, the sensitivity was 59.9%, and the specificity was 87.8%, 80.0% and 70.0%, respectively, in contrast to normal individuals, chronic hepatitis and liver cirrhosis cases. As described in this review, our efforts will be aimed at increasing both the sensitivity and specificity of antibodies as markers in HCC by expending TAA array to include antigens which might be more selectively associated with HCC and not with others. We expect that our mini-array of multiple TAAs can be used as a novel non-invasive approach to identify HCC in normal population and also in high-risk individuals such as in patients with chronic hepatitis and liver cirrhosis. Our concern is that the approach may be not suitable to distinguish HCC (liver cancer) with other types of cancer such as lung, breast and prostate cancer. The reason is that certain TAAs which we have used in our mini-array approach (for example p62, p53 and so on) may be also associated with other types of cancer such as lung, breast and prostate cancer. In the future study, we propose that some of selected antibody-antigen systems may be unique to HCC, and some may not. A comprehensive analysis and evaluation of various combinations of selected antibody-antigen systems will be useful for the development of autoantibody profiles involving different panels or arrays of TAAs in the future, and the results could be useful for diagnosis of certain types of other cancer. Important additional studies would be to determine whether mini-arrays of antibody markers might be useful in identifying in early stage of certain types of other cancer such as lung and breast cancer in high-risk individuals.

Take-home messages

1. The high case-fatality rate in HCC can in part be attributed to lack of diagnostic methods that allow early detection. How to establish a methodology to identify the high-risk individuals for HCC remains to be investigated.
2. Although serum AFP is the most effective marker available to detect HCC, the sensitivity and specificity is not optimal, especially in patients with small tumor or in well- to moderately-differentiated HCC cases. Therefore, there is a need for the development of more sensitive and specific methods that supplement AFP in the early detection of this cancer.
3. The multi-factorial and multi-step nature in the molecular pathogenesis of human cancers must be taken into account in both the design and interpretation of studies to identify markers which will be useful for early detection of cancer.
4. Cancer sera contain antibodies which react with a unique group of autologous cellular antigens called tumor-associated antigens (TAAs). A mini-array of multiple TAAs may enhance autoantibody detection for immunodiagnosis of HCC, especially for the AFP-negative cases.

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Table 1
Frequency of antibodies to eight tumor-associated antigens^a

Cancer	No.	No. and (percentage) of autoantibodies to:								
		IMP1	p62	koc	p53	c-myc	Cyclin B1	survivin	p16	eight TAAs
HCC ^b	142	24(16.9)**	20(14.1)**	14(9.9)*	20(14.1)**	29(20.4)**	19(13.4)*	16(11.3)*	31(21.8)**	85(59.9)**
LC	30	0(0)	1(3.33)	4(13.3)	2(6.7)	1(3.33)	0(0)	0(0)	4(13.3)	9(30)*
CH	30	3(10)	0(0)	2(6.67)	1(3.3)	0(0)	0(0)	0(0)	2(6.7)	6(20)
NHS	82	2(2.4)	1(1.2)	1(1.2)	2(2.4)	0(0)	2(2.4)	2(2.4)	1(1.2)	10(12.2)

^a Cutoff value: mean + 3 SD of 82 NHS; *P* Values between cancer and NHS were calculated to be <0.05 (*) or 0.01 (**), (from reference 34).

^b Abbreviations: HCC, hepatocellular carcinoma; LC, liver cirrhosis; CH, chronic hepatitis; NHS, normal human sera