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# Immunoprotemics views: Many cancers one goal

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## ABSTRACT

There is an important need to find relevant biomarkers that show high sensitivity and specificity for early diagnosis and prognosis of many cancers. To day, immunoproteomics is a relatively new concept in the field of proteomics and an increasingly powerful technology in the hunt for new and novel diagnostic and prognostic biomarkers. For cancer diagnostic, it is important that circulating autoantibodies reflect a molecular imprint of those antigens (low abundance) that are specifically related to tumor. Identification of tumor-associated antigens and their cognate autoantibodies is a promising strategy for the discovery of relevant biomarkers. Importantly, sera from tumor patients contain tumor specific antibodies directly against antigenic proteins. Also, autoantibodies are highly stable compared to many other serum proteins, they seem ideal to be implemented in clinical diagnostic assays for the detection of antigen-associated diseases. To facilitate autoantibody discovery, approaches that allow the simultaneous identification of multiple autoantibodies are preferred. During the past few years, proteomic approaches, including SEREX and SERPA, have been the dominant strategies used to identify tumor-associated antigens and their cognate autoantibodies. In this review, we will outline the advances applications of immunoproteomics for the discovery and analysis of clinically relevant protein targets and discuss their merits in clinical applications of the most common cancers. 

## KEYWORDS

Autoantibodies;
Biomarkers;
Cancer;
Serum;
Tumor-associated antigens.

## INTRODUCTION

Cancer is the second leading cause of death worldwide. In 2002, there were reportedly 11 million new cases of cancer and 7 million cancer-related deaths, leaving approximately 25 million people alive with cancer[1]. Despite the availability of protein products that could serve as cancer biomarkers, it is widely recognized that their combined use with the available clinical information is still insufficient for early cancer diagnosis and for guiding individualized therapeutic interventions and predicting outcomes. There remains a need for the development of innovative diagnostic and prognostic tools that effectively exploit biomarkers for the management of human cancers. Autoantibodies against autologous cellular proteins called tumor-associated antigens (TAAs) have generated increasing interest as biomarker[2]. These autoantibodies may be employed

as potential prognostic and diagnostic markers as well as therapeutic targets for the treatment of patients and can offer a novel technology for cancer detection[2].

Actually, autoantibodies have opened the door to the possibility that these proteins could be exploited as serological tools for the early diagnosis and management of cancer[3,4]. Proteomics approaches open new horizons in many research areas of life sciences. This is particularly true for work in the field of medicine, in which clinical proteomics research may accelerate the discovery of protein disease markers useful for clinical diagnoses. Improvements in the technology of two-dimensional electrophoresis (2-DE) analysis and mass spectrometry (MS) have made proteomics a suitable, valuable and powerful tool for studying human diseases in terms of the characterization of proteins involved in the initiation, maintenance and progression of tumor disease[5-7].

Presently there is a growing enthusiasm for applying proteomic approaches to the identification of serum biomarkers for the early diagnosis of cancer and for monitoring tumor progression. These approaches include direct profiling of human sera, using 2-DE/ MS to identify distinctive protein signatures characteristic of different tumor types, and the exploitation of the serum autoantibody repertoire from cancer patients for the identification of the TAA panels[3,4]. Identification of tumor antigens eliciting immune response may have utility in cancer screening, diagnosis and immunotherapy[4]. Nowadays, immunoproteomics, which defines the proteins involved in the immune response, especially in humoral response, holds considerable promise for the discovery of serological markers in tumor. This approach could not only identify tumor-associated antigens eliciting immune response, but also detect isoforms and post-translational modifications of these antigens/proteins[2]. Several terms have been proposed for this approach, such as SERPA (serological proteome analysis) and SEREX (serological analysis of tumor antigens by recombinant cDNA expression cloning)[3,4,8]. All of these approaches are implicated to the identification of the cognate antigens that elicit humoral immune response in patients by proteomics technology. In this review, we given an overview will outline the advances applications of immunoproteomics approaches for the discovery of clinically relevant protein biomarkers. Furthermore, the definition and the clinical utility of the immunoproteomics based assays of different approach (SEREX and SERPA), which allow simultaneous identification of multiple autoantibodies. Herein, we reviewed the autoantibodies found in the most common cancers and lastly, we discussed their merits in clinical applications of many cancers.

## IMMUNE SYSTEM AND AUTOANTIBODIES PRODUCTION

In the 1960s, Robert W. Baldwin, who was a pioneer in the immune system and demonstrate that this system could react to a developing tumor[9]. The immune response is composed of simultaneous cellular and humoral responses. Although little is known about the origin of this immune response, it is now largely established that cancer patients produce autoantibodies to mutated tumor proteins, truncated, misfolded, or over-expressed proteins and proteins that are ectopically expressed[3,4]. Thus, identification of antigenic proteins and their associated antibodies appears to be a good strategy for the discovery of new tumor biomarkers. In reality, immune system protects organisms from infection with three subsequent layers of defence: (i) physical barriers that prevent pathogens from entering the body, (ii) the innate immune system that provides an immediate, but nonspecific response and (iii) the adaptive immune system that can adapt its response during an infection to improve its recognition of the pathogen. The latter response is retained in the form of an immunological memory, and allows the adaptive immune system to react faster and stronger when a pathogen enters the organism for a second time. The adaptive immune system can be divided in a T cell mediated cellular component, and a B cell-mediated humoral component. T cells will recognise antigens only when these are presented in a processed form by the MHC of infected (or cancerous) cells. Killer T cells can trace and directly kill infected host cells by the secretion of cytotoxins that form pores in the membrane of the affected cells. Helper T cells control the mobilisation of other cells, such as macrophages, to confine the source of infected or malignant cells[10]. In contrast to T cells, B cells can recognise 'protrudes' directly with their surface-associated antibodies, which can bind to a specific antigen without the need of antigen processing. Importantly, each B cell is programmed

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to make one specific antibody[10]. Thus, the complete set of B cell antigen receptors represents the full humoral potential of a given organism. After binding, the antigen/antibody complex is internalised by the B cell and processed into peptides. These antigenic peptides will be displayed on MHC molecules to mobilise matching helper T cells, which in return release cytokines that activate this specific B cell[11]. Consequently, the activated B cell begins to divide and its progeny secretes millions of copies of the antibody that recognises this particular antigen. Next, these antibodies can bind to pathogens expressing this antigen and mark them for destruction. Antibodies have long been used for the diagnosis and classification of autoimmune diseases. Autoantigens represent the fraction of the tissue proteome that is targeted by aberrant immune responses in autoimmunity, and multiplex analysis of autoantibody responses against spectra of candidate antigens represents a powerful screening tool to delineate biomarker signatures in autoimmunity and in tumor pathology. In fact, autoimmune diseases comprise a wide variety of systemic or organ-specific inflammatory diseases, characterized by aberrant activation of immune cells to target self tissues. Autoimmunity arises from complex interactions of genetic and environmental factors, and there is substantial heterogeneity in the clinical manifestations, disease courses and outcomes among patients. The etiology and pathogenesis of autoimmunity remain poorly understood. Autoimmune diseases are manifested by a immunological attack against self molecules (autoantigens) that aremistaken by the immune systemas nonself. Alterations in genes that control pathways regulating self tolerance are critical in the pathogenesis of these diseases.

## THE MAJOR GOAL: EARLY CANCER DETECTION

The ultimate utility of autoantibodies lies in early cancer detection. Many of the well-known available tumor-associated biomarkers, such as carcinoembryonic antigen (CEA) for colon cancer, CA-15-3 for breast cancer, cancer antigen CA19-9 for gastrointestinal cancer and CA-125 for ovarian cancer, lack sufficient specificity and sensitivity for use in early cancer diagnosis. The immune response to TAAs occurs at an early stage during tumorigenesis, as illustrated by the detection of high titers of autoantibodies in patients with early stage cancer. The immune response to TAAs has also been shown to correlate with the progression of malignant transformation[3,4,12]. Thus, the production of autoantibodies can be detected before any other biomarkers, rendering such autoantibodies indispensable as biomarkers for early cancer detection[13]. In addition, autoantibodies possess various characteristics that enable them to be valuable early cancer biomarkers. First, autoantibodies can be detected in the asymptomatic stage of cancer, and in some cases, may be detectable as early as 5 years before the onset of disease[13]. Second, autoantibodies against TAAs are found in the sera of cancer patients where they are easily accessible to screening. Third, autoantibodies are inherently stable and persist in the serum for a relatively long period of time because they are generally not subjected to the types of proteolysis observed in other polypeptides. The persistence and stability of the autoantibodies give them an advantage over other biomarkers. Moreover, the autoantibodies are present in considerably higher concentrations and they are amplified by the immune system in response to a single autoantigen. Identification of such autoantibodies is of great interest as early detection of cancers may enhance treatment options, thereby increasing survival rates and providing for better disease management. Although serum biomarkers have great potential as effective screening tools, most of those currently available are far from ideal, and more research is needed in this area to develop and validate biomarkers for early detection or to develop panels of similar biomarkers. Moreover, the variety of reagents and techniques available for antibody detection, in proteomics approaches, facilitates the development of assays for these autoantibodies.

## PROTEOMICS APPROACHES

The proteome project, initiated in 1995, was made possible by 2-DE combined with MS. The project main objective was and remains the identification of all proteins expressed by serum/plasma, cell, tissue or organism in a given time and condition (Figure 1)[14-16]. Following this objective, the global profiling of proteins in health versus pathological state by the 2-DE/MS-based proteomic approach has contributed to the elucidation of the basic mechanisms of disease by discovering can-

didate disease biomarkers and disease targets for new drug development. Nowadays, 2-DE and mass spectrometry analysis is the basic strategy in proteomics technology (Figure 1). 2-DE is a key technology which is used to separate very complex protein mixtures with extremely different physicochemical properties and abundances according to charge and molecular weight. Although the main advantage of 2-DE technology is its capacity to provide a global view of a sample proteome at a given time by resolving hundreds to thousands of proteins simultaneously on a single gel[3,4,16]. Also, 2-DE has led to great improvements in terms of its reproducibility and sample loading capacity attributable to the development of immobilized pH gradients strips in recent years. 2-DE is widely applied in proteomics study due to its powerful ability to separate more than one thousand proteins at the same time and to show protein post-translational modifications directly. In earlier period, 2-DE/ Western blotting is a usual analysis approach in proteomics combined with antibody-based antigen identification. This approach employs 2-DE to separate cellular proteins from tissue or cell lines. The separated proteins are then transferred onto membranes which were subsequent immunodetection of relevant antigens among the several thousand individual proteins separated by 2-DE. Usually, sera from patients are used as the primary antibody for Western blotting analysis and are screened individually for antibodies that react against separated proteins. Enzyme-conjugated anti-human IgG antibody is used as secondary antibody[4]. In reality, the visual image of the blots (protein pattern on a 2-DE gel) must be captured in a digital format before computer-based image analysis. The objective of computerized 2-DE image analysis is to identify protein spots that have increased or decreased in size and intensity, by comparison with control gels. The critical parameter in computer-based image analysis is the quality of the images. Good quality requires high quality and reproducible 2-DE gels and also high quality acquisition of the image with image capturing devices.

After comparing blots with sera from the patients to that with sera from normal individuals, positive protein spots are cut out, from the gels, digested, usually with trypsin, and the resulting mixture of peptides is introduced into a mass spectrometer for protein identification. The most common systems for doing this are MALDITOF/ MS and ESI-MS to identify the proteins, which may be used as potential markers of a particular disease (Figure 1). The compatibility of 2-DE with Western blotting detection is advantageous and represents the basis for the combination of proteome analysis and antibody-based antigen identification. However, denaturing conditions can cause a potential loss of conformational epitopes as recently demonstrated. It shows that several antibodies failed in recognizing their respective antigens on 2-DE gel-derived blots, although these antibodies recognized their specific antigens on blots generated from conventional SDS-PAGE gels[4].

## IMMUNOPROTEOMICS METHODS FOR IDENTIFYING TUMOR AUTOANTIBODIES

Serological screening methods have been used extensively to identify autoantigens in cancer[3,4,8]. With advances in the development of technologies for autoantibody identification, several high-throughput methods available for uncovering autoantibodies have become increasingly well defined. Immunoproteomics have been implemented to define antigens eliciting humoral responses in cancer patients. This method allows the individual screening of a large number of patient's sera, the determination of the occurrence of relevant autoantigens eliciting a humoral response, the distinction of isoforms and the detection of autoantibodies directly against post-translational modifications of specific targets. This suggests that immunoproteomics are valuable alternatives for the identification of tumor-associated antigens. So far, this experimental approach has been applied to various different types of cancers[3,4,9,17-19]. For more than two decades, numerous groups made use of the SEREX (serological profiling of tumor antigens or Immunomics approach) technology where recombinant expression libraries are screened with cancer patient sera. In order to represent more closely the natural sources of immune responses in cancer patients, including protein modifications, the proteome of cells or tumors has been used as the antigen source for autoantibody profiling. This approach called SERPA (serological proteome analysis or PROTEOMEX), involves performing 2-DE-Western blots on cells or tumor lysates using untreated human cancer sera as the source of antibody.

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## SEREX: SEROLOGICAL ANALYSIS OF TUMOR ANTIGENS BY RECOMBINANT CDNA EXPRESSION CLONING

Serological analysis of tumor antigens by recombinant cDNA expression cloning (SEREX) was first developed in 1995 and led to the identification of a large group of autoantigens in cancer patients' sera[17]. This emerging area of research, termed "cancer immunomics," allows a global analysis of the autoantibodies produced by neoplasms against their antigens. Many autoantigens have been cloned with recognition of antibodies in patient's sera; however, efforts to predict malignant disease based on autoimmunity to individual antigens have thus far been largely unsuccessful. Although in aggregate these studies strongly suggest that autoantibodies have potential as biomarkers, thus far they have not resulted in serological markers with definitive predictive ability for cancer in the clinical arena, and none have been selected for cancer diagnosis. SEREX involves the identification of TAAs by screening patient sera against a cDNA expression library obtained from the autologous tumor tissues[9]. In the SEREX approach, a cDNA library constructed from fresh tumor specimen is expressed recombinantly, and the recombinant proteins transferred onto membranes are identified as tumor-associated antigens by their reactivity with IgG antibodies present in the patient's serum. This methodology allowed identification of several antigens. By using SEREX, Sahin et al.[17] showed that CTAs elicited a humoral response in cancer patients. Subsequently, a large number of TAAs associated with numerous cancer types have been identified using this method[17]. More than 2000 of these autoantigens are documented in a public access online database known as the Cancer Immunome Database (CID) http://ludwig-sun5.unil.ch/CancerImmunomeDB[18,19]. The application of SEREX has facilitated the identification of TAAs as potential cancer biomarkers in various types of cancer[20-23]. The panel of SEREX-defined immunogenic tumor antigens include CTAs (NY-ESO-1, MAGE), mutational antigens (like p53), differentiation antigens (like tyrosinase, SOX2, ZIC2) and embryonic proteins. Although many of these TAAs are potential serological biomarkers.

## SERPA: SEROLOGICAL PROTEOME ANALYSIS

Another commonly used technique is the proteomics based approach termed SERPA[4]. It involves the discovery of TAAs using a combination of 2-D electrophoresis, western blotting and MS (Figure 2). Proteins from tumor tissues or cell lines are extracted and separated by 2-D electrophoresis, transferred onto membranes by electroblotting and subsequently probed with sera from healthy individuals or patients with cancer[4]. The respective immunoreactive profiles are compared and the cancer-associated antigenic spots are identified by MS. 2-D electrophoresis is indisputably the classical technique for proteome analysis. Proteins are first separated according to their isoelectric points and then according to their molecular weights[4]. Despite some limitations, 2D electrophoresis is still the best method for the high-resolution separation of a complex mixture of proteins, and its efficacy in distinguishing post-translationally modified proteins and protein isoforms is unparalleled. Consequently, when coupled with western blotting for serological screening, autoantibodies can be used to detect TAAs that have undergone post-translational modifications. Most of these antigens can be subsequently identified with the aid of MS.

## CANCER-ASSOCIATED AUTOANTIBODIES

The hunt for relevant autoantibodies has intensified in recent years, as evidenced by a search for 'autoantibodies, proteomics approaches and cancer' on PubMed. Autoantibodies and TAAs have been found many cancers such as HCC, and in lung, colorectal, breast, stomach, prostate and pancreatic cancers. The growing list of TAAs identified in cancers include oncoproteins, tumor suppressor proteins, survival proteins, cell cycle regulatory proteins, mitosis-associated proteins, mRNA-binding proteins, and differentiation and CTAs[3,4,8]. The following section shall discuss the identification of autoantibodies in the most major cancers.

## BREAST CANCER

Breast cancer is a major health problem and one of the leading causes of death among women worldwide.

Its incidence is steadily rising in developing countries. In Tunisia, the incidence of breast cancer is approximately 19 new cases per 100,000women per year[24]. Invasive carcinomas represent 70-80% of all breast cancer and among these, infiltrating ductal carcinomas (IDCA) are the most aggressive forms and have a poor prognosis. Up to now, poor diagnosis of breast cancer is due in great part to a lack of specific biomarkers of this disease. A novel oncogenic protein that regulates RNA-protein interaction (designated RS/DJ-1) has been identified in breast cancer by immunoproteomics approaches[3]. A large number of studies have suggested an important association between cancer and autoimmunity, clearly indicating that various types of human cancer can trigger an immune reaction to tumor-associated antigens. By SEREX which represent a major advancement in immunoscreening that resulted in the identification of a wide array tumor-associated antigens eliciting B-cell responses in breast and other cancer patients against a host of tumor antigens, among others, NY-ESO-1 and SSX2, two cancer-testis antigens, ING1, a tumor suppressor, fibulin-1, a breast cancer-related glycoprotein, MAGE-3 and MAGE-6, as well as the novel gene products NY-BR-62 and NY-BR 85, the latter two found to be overexpressed in breast cancer[25-28]. A novel breast cancer antigen, NY-BR-1 was found to be expressed only in testis and breast[26,27]. Minenkova et al.[29] also attempted to improve the potency of the SEREX approach by combining it with phage display technology using lambda phage as a display vector[29]. They identified several breast cancer-associated antigens including topoisomerase-II-beta and topoisomerase I-binding protein[29]. Fernández Madrid et al.[30] have identified a panel of breast cancer-associated autoantigens[30]. Several autoantigens including annexin XI-A, the p80 subunit of the Ku antigen, the ribosomal protein S6, and other known and unknown autoantigens could significantly discriminate between breast cancer and non-cancer control sera[26,27]. Several autoantigens identified by serum antibodies have been found to be expressed in breast tumor tissue and their therapeutic potential is presently being explored. Also, we should mention that the availability of serologically defined tumor antigens in breast and other cancers using SEREX has facilitated the identification of proteins recognized by tumor-specific T lymphocytes and has stimulated the interest in vaccine strategies. The use of the immunoproteomic approach SEREX led to the identification of a number of breast cancer-associated autoantigens recognized by cancer patient sera, expressed in tumor tissue and recognized by cellular effectors of the immune system which are potentially relevant for immunotherapy approaches.

Using SERPA methodology, Bechr et al.[4] have detected twenty six immunoreactive proteins against which sera from newly diagnosed patients with infiltrating ductal carcinomas exhibited reactivity. These proteins spots were targeted by mass spectrometry. Based on their functions, the identified proteins can be categorized into different categories, including anti-oxidative proteins (peroxiredoxin-2, protein disulfide isomerase (PDI) and Mn-SOD), chaperoning proteins (HSP60) whose rate of synthesis increases many fold in response to environmental stress and during malignant transformation and alpha Bcrystallin, tumor suppressor proteins (prohibitin), proteins related to cell structure (cytokeratins 8, 18, F-actin and β-tubulin), metabolic enzymes (ornithine aminotransferase, enoyl-CoA hydratase), as well as, proteins involved in cell signalling (heterogeneous nuclear ribonucleoproteins H, A2B1 and K)[4].

Among these antigens, peroxiredoxin-2 (Prx-2) belongs to a family of thiol-specific antioxidant proteins and may have an important role and protect the breast tumor cells against oxidative injury and modulate cell proliferation and apoptosis of malignant cells[31]. HSP60 and alpha B-crystallin are two other immunoréactive proteins most commonly observed in the breast cancer cells (MCF-7). The molecular chaperone HSP60 is involved in protein folding, as well as, in activation of integrin which is a major contributing factor in breast cancer progression and metastasis[32]. Recently, it has been reported that increased expression of HSP60 in breast tumors may have a prognostic value since it correlates with the presence of lymph node metastasis[33]. Prohibitin is another antigen that was recognized in breast cancer cell. This protein is involved in cell cycle control, differentiation and in suppression of tumor progression. In addition, studies have shown that prohibitin interacts with cell cycle regulatory proteins and modulates Rb/E2F, as well as, p53 regulatory pathways[34].

## ESOPHAGEAL CANCER

Esophageal squamous cell carcinoma (SCC) rep-

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resents one of the most malignant tumors. The poor prognosis of this tumor is attributed largely to a delay in diagnosis. Several tumor markers have been used for the diagnosis of esophageal SCC. A previous application of the SEREX method to esophageal cancer resulted in the identification of NY-ESO-1, a cancer-testis antigen expressed in various cancer cells but not in normal tissues[35]. SEREX analysis has also led to the isolation of several antigens with known cancer relatedness, including a mutated version of the p53 tumor suppressor protein, while the presence of antibodies to p53 in serum was associated with poor prognosis in esophageal cancer[35-37]. The tumor suppressor p53, the protein product of the p53 gene, is the most extensively studied cancer-associated B-cell antigen. It was discovered in 1979 by two independent groups of investigators[38]. Mutations in the p53 gene and amino acid substitutions induced by it are found in >=50% of patients with malignant tumors. These mutations change the conformational structure of p53 in such a way that it loses the ability to transactivate p53-dependent genes concomitantly with inhibition of DNA repair and formation of genetically unstable cells with a "switched-off" p53-induced apoptotic mechanism[39]. For the majority of malignant neoplasms, the detection frequency of class IgG anti-p53 antibodies in appropriate cohorts of patients varies from 15 to 20%, being maximal in patients with many cancers. Therefore, p53 represents a highly specific cancer-associated antigen. Two others groups report autoantibodies against heat shock protein (HSP) 70 and peroxiredoxin (Prx) VI in esophageal squamous cell carcinoma (ESCC)[41,42]. Concentrations of serum HSP70 autoantibody are significantly higher for patients with ESCC than for patients with gastric or colon cancer or healthy individuals[40]. About 50% patients (15/30) with ESCC show autoantibody reactivity against PrxVI, only 6.6% (2/30) healthy individuals did[41].

## COLON CANCER

Colorectal cancer (CRC) is the second most prevalent cancer in the western world. The development of this disease takes decades and involves multiple genetic events. CRC remains a major cause of mortality in developed countries because most of the patients are diagnosed at advanced stages. By immunoproteomics studies, autoantibodies against HSP60 and inosine monophosphate dehydrogenase-2 (IMPDH-2) have been identified in colorectal carcinoma[42]. In addition, diffuse and moderate-strong cytoplasmic and membraneous immunoreactivity for HSP60 has been observed in the colorectal carcinoma tissue. Furthermore, antibody titers to HSP60 are significantly higher in the serum from patients with colorectal carcinoma than that in the healthy cases.

## LUNG CANCER

Lung cancer is the most common cancer and the leading cause of cancer-related death worldwide. Sera from 60% of patients with lung carcinoma have exhibited autoantibodies reactivity against glycosylated annexins I and/or II. Annexin II autoantibodies are found only in lung cancer patients, whereas annexin I autoantibodies also are observed in a few patients with other cancers[43]. In the same way, other study demonstrate that the protein gene product 9.5(PGP9.5), also termed ubiquitin COOHterminal esterase L1, or UCHL1 is another tumor antigen identified in lung cancer[44].

## PANCREATIC CANCER

Pancreatic cancer has worst prognosis of all cancers, with a 5-year survival rate of <3%. The poor prognosis for pancreatic cancer is due, in part to lack of effective biomarker useful for the early detection. The autoantibodies directed against calreticulin isoforms have been found to occur in 58.3% of pancreatic cancer patients[45]. DEAD-box protein 48 (DDX48) antibody reactivity in sera occurred in 33.3% pancreatic cancer patients[46]. In addition, some metabolic enzymes and cytoskeletal proteins are specifically reactivity with pancreatic ductal adenocarcinoma sera[47].

## HEPATOCELLULAR CARCINOMA (HCC)

HCC has a poor prognosis, with 5-year survival rates of less than 5%. Chronic infections with hepatitis B (HBV) and C (HCV) viruses are major risk factors for HCC. The proteomic approach to the identification of tumor proteins that induce a humoral response in patients with HCC we have utilized has identified a diverse set of antigens, with substantial heterogeneity be-

tween patients. Antigens that have been shown to induce a humoral response in HCC include p53 and diverse other nuclear proteins[48]. Autoantibodies to cyclin B1 and to a novel cytoplasmic protein with RNA binding motifs have also been reported[49]. A SEREX study of hepatocellular carcinoma has uncovered reactivity to diverse proteins involved in the transcription/translational machinery, as well as to chaperone proteins[50]. In other group's study report autoantibodies against β-tubulin, creatine kinase-B, hsp60, and cytokeratin 18, calreticulin, cytokeratin 8, $F_1$-ATP synthase β-subunit, and NDPKA were largely restricted to HCC patients. Interestingly, the protein $F_1$-ATP synthase β-subunit was reported previously to be antigenic in patients with HCC[50]. With immoproteomic approach we have utilized has allowed identification of several forms of calreticulin including Crt32, a novel truncated form, all of which were recognized by autoantibodies in sera of patients with HCC. 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 this study are located in the C-terminal portion. This suggests a specific mechanism of calreticulin processing during hepatocarcinogenesis. Calreticulin is a component of major histocompatibility complex class I peptide loading complex, and it has been reported recently that this protein elicits tumor- and peptide-specific immunity[51,52]. Calreticulin was reported to be abundant in the nuclear matrix fraction of hepatocellular carcinoma but not in nonmalignant liver tissue[53]. We have shown that Crt32 was up-regulated in HCC tumor tissue as compared with the non-tumor counterpart. This overexpression of Crt32 may contribute to the humoral response observed against calreticulin and Crt32 in liver cancer patients.

## RENAL CELL CARCINOMA

Renal cell carcinoma (RCC) represents the most prevalent malignancy of the kidney and accounts for approximately 3% of all adult cancers in Western countries[54]. Histopathologically, RCCs are classified into four subtypes: the most frequent clear cell, the chromophobic, the chromophilic and the oncocytomic subtype. At present, most RCC patients have developed metastatic disease exhibiting a poor 5-year survival rate of less than 5%. RCC is considered an immunogenic tumor and first used to identify tumor antigens by serological proteome analysis. A combination of 2-DE expression profiling of tumor cell lines or tissues and immunoblotting with patient's and control sera (SERPA approach) might serve as a powerful tool for the identification of TAA. So far, this experimental approach has been successfully implemented in RCC. Utilizing SERPA approach, members of the cytoskeleton and of the heat shock protein family have been identified to elicit a humoral response in RCC[55]. By comparing 2-DE patterns of whole normal kidney epithelium and RCC tissues, a number of polypeptides differentially expressed in RCC have been detected[56]. These include metabolic enzymes which are expressed in normal kidney tissues, but absent in RCC lesions, such as the ubiquinolcytochrome c reductase and the mitochondrial NADH–ubiquinone oxidoreductase complex I, enzymes overexpressed in RCC, such as the glutathione peroxidase and the manganese superoxide dismutase (Mn-SOD) or enzymes with diminished expression in RCC like aldehyde dehydrogenase 1, aminoacylase-I, enoyl-CoA hydratase (ECHM) and α-glycerol-3-phosphatase-dehydrogenase[56,57]. Furthermore, two others antigens have been identified, smooth muscle protein 22-a (SM22-a) and carbonic anhydrase I[55]. Some immunogens have been identified in patients with high-grade disease. One of them, thymidine phosphorylase, is marked up-regulated in RCC and almost absence from most normal tissues, indicating a potential use as a therapeutic target[58]. Superoxide dismutases encoded by two apparently independently evolved genes, SOD1 and SOD2, with shared cellular functions are potent scavengers of superoxide radicals produced by oxidative phosphorylation into $O_2$ and $H_2O_2$ and appear to play a central role in the defense against oxidative stress[59]. Its biological activity is dependent on copper and zinc ($Cu^{2+}$, $Zn^{2+}$) ions and on homodimerformation. In contrast, the gene SOD2 coding for the mitochondrial variant SODM, depends on manganese ($Mn^{2+}$) ions and acts as a tetramer[59]. Overexpressed in RCC leading to the formation of multimeric forms not detectable in normal renal epithelium. Thus, superoxide dismutase overexpression might be associated with resistance to radiotherapy and chemotherapy and therefore may be a potential target for immunotherapies.

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## NEUROBLASTOMA

Neuroblastoma is a common childhood tumor. IgG and IgM autoantibodies against neuroblastoma proteins have been screened in patients sera by means of 2-DE blotting[60]. Neuroblastoma patients sera contain IgG and IgM antibodies against b-tubulin isoforms. Moreover, neuroblastoma patients sera that reacted with b-tubulin and III isoforms in neuroblastoma tissues do not react with b-tubulin and III isoforms found in normal brain tissue.

## HEAD AND NECK CANCER

Immunoprecipitation followed by 2-DE is used in identification of novel targets in head and neck carcinomas. Primary tumor cells or permanent cell lines are used as an antigen-pool. About thirty potential carcinoma antigens are identified[61]. Cytokeratin 8, one of the identified antigens, revealed its de novo expression in hyperlastic tissue, gradual overexpression with increasing malignancy, and ectopic localization on the cell surface. Moreover, a strong prevalence of CK8-specific antibodies occurs in the sera of cancer patients already at early disease stages. Another tumor antigens, KIAA1273/TOB3, e-FABP, hnRNP H, and Grb2 are strongly overexpressed in head and neck carcinomas, as compared to healthy epithelium.

## LEUKEMIA

In chronic myeloid leukemia, we have identified six proteins that elicited a humoral response. Alpha enolase, aldolase A, b-tubulin, HSP70 protein8, tropomyosin isoforms in chronic myeloid leukemia[62]. In acute leukemia patients, autoantibodies against Rho GDP dissociation inhibitor 2 (Rho GDI2) and other five proteins are also found with a high frequency[63].

## ABBREVIATIONS

| | |
|---|---|
| CRC | colorectal cancer |
| DCIS | ductal carcinoma in situ |
| HBV | hepatitis B virus |
| HCC | hepatocellular carcinoma |
| HCV | hepatitis C virus |
| HSP | heat shock protein |
| PGP9.5 | protein gene product 9.5 |
| PTMs | post-translational modifications |
| SEREX | serological analysis of tumor antigens by recombinant cDNA expression cloning |
| SERPA | serological proteome analysis |
| TAAs | tumor-associated antigens |

## CONCLUSIONS AND PERSPECTIVES

With the introduction of proteomics approaches, 2- DE, SERPA, SEREX approaches and MS have been successfully used in a large number of studies in many biological fields. Immunoproteomics has allowed defining tumor-associated antigens that elicit humoral response in tumor patients. The antigenic proteins recognized by autoantibodies in cancer sera have a potential clinical utility and may serve as novel cancer markers in screening, diagnosis or in prognosis. These tumor autoantibodies identified by immunoproteomics approach in different cancers could be employed as potential prognostic and diagnostic biomarkers. So, larger scale validation studies need to be carried out to confirm the reliability of the results. This process is more challenging than the discovery process for several reasons. The most relied-on approach for validation remains the sandwich enzyme-linked immunosorbent assay (ELISA), immunohistochemistry and Western blots are often used. Moreover, statistical analysis is also desirable to estimate and to compare the predictive sensitivity and specificity of the candidate biomarkers.

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