Review

Proteomics in the Diagnosis of Hepatocellular Carcinoma: Focus on High Risk Hepatitis B and C Patients

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Abstract. *Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide. The high morbidity rate associated with this cancer is mainly linked to the late diagnosis, when therapy is no longer effective and this is particularly true for high risk patients, such as hepatitis B and C infected individuals. A biomarker can be defined as a substance, found in an increased amount in the body fluids, such as blood, which can indicate the presence of liver cancer. Current screening methodologies for liver cancer in at-risk patients rely on measuring the serum level of α-fetoprotein (AFP), a biomarker, as well as ultrasound imaging. AFP's sensitivity is very limited since many other liver diseases can result in a very high blood level of AFP similar to that observed in HCC. In addition, AFP is not always elevated in the early stages of cancer development, when therapy is mostly effective. Imaging, on the other hand, depends to a large extent on the operator. Therefore, better diagnostic methods are needed to increase the survival rate in liver cancer patients. Proteomics can be simply defined as the protein expression of the genome; and protein expression can vary depending on the biological state. Antibody microarrays can scan for multiple targets (antigens) within the tissue or in the circulation. This technology is still in its infancy and has great potential as a diagnostic tool for hepatitis liver cancer patients. Another proteomic approach is mass spectrometry, which can detect proteins and present them as charged species (ions). The mass spectrometric technique termed SELDI (surface enhanced laser desorption ionization), releases proteins in a sample from a capturing surface that can specifically bind groups of proteins which share common features (hydrophobic, negatively charged, etc.) and the expression of thousands of proteins can be monitored simultaneously. Proteomic profiles of hepatitis patients, liver cancer patients and healthy individuals can be established and evaluated for diagnosis. Elevated proteins can further be isolated and identified using the well-established mass spectrometric protein identification methods. In this article, the technological SELDI mass spectrometry and antibody microarrays are presented at the basic level. In addition, the current state of the novel liver cancer diagnostic methods (and biomarkers) that have been evaluated with focus on high risk hepatitis B and C patients using proteomic approaches are reviewed and highlighted.*

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Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide (1) Statistically, it is the fifth most prevalent cancer in the world with a continuous increase in the total number of its victims. For example, in the 2001 Annual Report to the Nation on the Status of Cancer in the United States, HCC was the second tumor with the largest increase in the last three decades of the 20th century (2). HCC is globally ranked as the third most fatal cancer after the lung and the stomach carcinomas (1, 3). The high morbidity with HCC tumors can be directly linked to the late diagnosis when traditional therapies are no longer effective. These therapies include surgery, local tumor destruction (*i.e.,* percutaneous ablation) and liver transplantation (4). As with all forms of cancer, early diagnosis is a crucial factor for successful therapy. In fact, the five-year survival rate within liver cancer patients can exceed 70% if diagnosed in the very early stages, when the tumor is less than 2 cm^3 in size (5). Currently, HCC is diagnosed *via* blood tests, imaging and/or biopsy (6); however, these methodologies are not very efficient in detecting small cancerous lesions within the liver. Blood tests, which are a very attractive option because of their simplicity, include measuring the serum level of α-fetoprotein (AFP) (6), a biomarker which is not elevated above its diagnostic cutoff (400 ng/ml) in most patients with small liver cancer lesions (7). AFP can also be highly elevated

within cirrhotic-liver patients leading to diagnostic difficulties (8). Therefore, better biomarkers with higher selectivity and sensitivity are needed for early diagnosis of HCC within high risk patients.

Many factors can increase the risk of developing HCC; these factors can be categorized into two major groups, namely toxins and viral infections. Both categories can induce liver cirrhosis and dysfunction. Aflatoxin is a common toxin that contaminates many food products especially in Asian and African countries (9). Similarly, both hepatitis B (HBV) and C (HCV) viral infections can significantly contribute to the development of HCC (9). From an etiological point of view, more than 75% of HCC patients are also infected with HBV or HCV (3). While HBV is more common in Asia and Africa, HCV infection is substantially higher in Western countries and Japan (10). It is estimated, for example, that more than four million individuals are infected with HCV in the United States alone (11) and accordingly, HCC incidence in the US is projected to rise over the next two decades (12). Similarly in Canada, a 38% increase in HCC incidents was reported between the years 1984-2000 (13). The development of efficient and reliable screening methodologies can assist in early diagnosis of HCC, especially for at-risk hepatitis patients.

Liver cells are very active and continuously secrete various metabolites within the circulation. Therefore, screening human serum for potential biomarkers is an attractive option for early diagnosis of liver cancer. It should be noted that biomarkers can also be substances secreted by other organs in response to the growth of the tumor. Many publications have evaluated potential serum biomarkers that can be more sensitive than AFP, such as des-γ-carboxyprothrombin (14), which consists of variant forms depending on the severity of liver damage (15). More recently, Golgi Protein-73 (GP73) was shown to be up-regulated within diseased livers (16) and was evaluated as a biomarker candidate. It was suggested that GP73 is a better marker than AFP within early stage HCC (17). To validate this finding, more large-scale investigations are required before clinical application.

The fast-growth experienced in cancer biomarker discovery in general, including liver cancer, was boosted through the remarkable advances in genomic and proteomic approaches (18-20). It is now possible to compare diseased *versus* normal state conditions and to identify many biomarker candidates which can also serve as diagnostic or prognostic indicators. In fact, a novel liver cancer protein biomarker namely, TSPY (testis specific Y-encoded protein), first identified at the genome level (21), was proven to be elevated within HCC tissues and may stand as a possible diagnostic marker (22). Furthermore, current technologies have enabled researchers to monitor many biomarkers simultaneously and generate or proteomic-based "signatures" (20). Such "signatures" will probably increase the chances of early diagnosis, resulting in better therapeutic outcomes.

In this article, proteomic-approaches, namely protein microarrays and SELDI mass spectrometry, will be presented at the basic technical level as well as at the research level with relevance to HCC and hepatitis B and C infections.

Mass Spectrometry

Mass spectrometry (MS) has been the center piece of proteomic research and has rapidly evolved during the past 20 years with its various applications extending into every discipline within the life and health sciences. It relys on the formation of gas phase ions that can be isolated based on their mass-to-charge ratio (m/z). In an MS spectrum, the x-coordinate represents m/z values while the y-axis indicates the intensity "quantity". Mass spectrometric analysis can provide important information about the analytes including their structure, purity and composition. The introduction of soft ionization techniques, namely Electrospray Ionization (ESI) and Matrix Assisted Laser Desorption Ionization (MALDI), has enabled researchers to study biological compounds, such as proteins and DNA. Koichi Tanaka of the Shimadzu Corp in Kyoto, Japan and John Fenn of the Virginia Commonwealth University, Richmond, USA shared the 2002 Nobel Prize in chemistry for the invention of MALDI and ESI, respectively. Ions can be extracted from solution using ESI technology and it is usually preceded by separation methodologies, such as liquid chromatography (LC). MALDI, on the other hand, generates gas phase ions from the solid state as the analytes are mixed with a matrix, which causes the whole mixture to crystallize as it dries. The proteins in the mixture are brought into the gas phase *via* a laser beam that hits the sample-matrix crystal, leading to absorption of the laser energy by the matrix and the subsequent desorption and ionization of the proteins in the sample. Similar to ESI, LC separation can precede MALDI; however, two-dimensional gel electrophoresis (2DE) is more commonly associated with MALDI analysis. Studying the serum of HBV-infected HCC patients using 2DE revealed the presence of two down-regulated proteins which were identified using MALDI-MS as the carboxyl terminal fragment of complement C3 and the isoform of apolipoprotein A1 (23). In a similar analysis, 14 proteins were identified as differentially expressed among HBV- and HCV-infected patients compared to patients who developed HCC (24). Both LC and 2DE coupled with MS are tedious, time-consuming and often lack reproducibility. Subsequently, a new technology known as SELDI (surface enhanced laser

desorption ionization) was introduced in 1993 (25) and combines the power of MALDI with the selectivity of a protein chip technique.

The major advantage of SELDI is that the need for purification and separation of proteins before MS analysis is eliminated. The protein chip consists of metal base, chemically active sites and a hydrophobic coating. Samples containing the protein mixture are applied to the active sites, which can capture proteins and peptides in a very specific way. The active sites on the chip are usually based on hydrophobic, metal affinity or ion exchange chromatography (chemical surfaces), or the less common biochemical surfaces that immobilize antibodies, DNA, or receptors (26). The protein chip can therefore act as "chromatography" by isolating a specific group of proteins. In SELDI, the sample-presenting surface plays an active role in the extraction/purification of the analytes before being mixed with the matrix for MS analysis, while MALDI surfaces are merely used for the presentation of the analytes to the laser beam. In addition, modifications of the sample can be achieved on the chip directly. The serum of rat models for liver cirrhosis was purified on the chip by specific washing such that the target remained on the chip while the rest of the proteins were removed. This was followed by on-chip tryptic digestion and identification of the target protein by peptide mass fingerprint and tandem mass spectrometry (MS/MS) (27). SELDI is connected to a ToF analyzer (SELDI-ToF) which relies on the free flight of the ionized molecules in the free field region. ToF has a high resolving power; if two ions (M_1, M_2) are formed at the same time with the same charge but $M_1 < M_2$, M_1 will reach the detector before M_2. Since SELDI is intended to detect proteins in the sample (biomarkers), its combination with ToF will be beneficial. The goal of using SELDI-ToF in cancer-related applications is to detect a specific biomarker associated with cancer for further identification or to generate mass spectrometric fingerprints. The following section will focus on SELDI-MS and its relation to hepatitis HCC diagnosis.

In the case of liver cancer, SELDI-ToF with a metal affinity protein chip revealed the presence of 38 differentially expressed proteins which enabled the discrimination between chronic HCV and HCV-HCC patients with 61% sensitivity and 76% specificity, respectively (28). These values were increased up to 75% and 92%, respectively, when the values for AFP, des-γ-carboxyprothrombin and GP73 serum markers were combined with SELDI peak detection methodology (28). A more comprehensive study was carried by fractioning the serum of HCC patients using anion exchange fractionation followed by SELDI-MS analysis (two dimensions for separation) (29). SELDI-ToF analysis suggested the presence of 250 potential serologic markers of HCC. Using these 250 features, hierarchical clustering showed separation between HCC patients and patients with chronic liver diseases such as HBV and HCV infections (29). The term feature refers to the observed intensities at a particular *m*/*z* value, while the term peak is a local maximum of specific spectra. A new approach for HCC diagnosis using SELDI-MS data was recently reported and it uses *m*/*z* windows rather than precise *m*/*z* values; the system showed 91% sensitivity and 92% specificity (30). SELDI-ToF analysis is also applicable at the tissue level. Melle *et al.* (31) generated proteomic profiles of microdissected HCC central and peripheral tumor cells as well as non-tumorous tissue. The technology was capable of identifying one peak which distinguished central and peripheral cancerous tissue, using two different chip surfaces. The number of potential biomarkers increased to the 20th range when normal tissue was compared against cancer (31).

SELDI-MS is not merely used for peak classifications and building prediction algorithms, but also for identifying diagnostic peaks. It can be coupled with tandem mass spectrometry such that the sequencing information of peptides and proteins can be obtained. MS/MS is based on the isolation of a specific ion (precursor) in the first analyzer of the instrument; this ion can be collided with neutral gas (*i.e.,* nitrogen or argon) in the collision cell (*i.e.,* collision-induced dissociation; CID). The result is a series of fragment ions (daughters) that provide vital information about the structure of the precursor. When used for identification, SELDI-MS/MS is preceded with purification steps using various chromatographic methods and gel electrophoresis. Fractionation from IMAC spin column followed by SELDI-MS/MS resulted, for example, in the identification of the C-terminal fragment of vitronectin, a multifunctional glycoprotein, as a diagnostic peptide within the serum of HCC patients with chronic liver diseases (32). This glycoprotein was first identified during SELDI-MS profiling. Similarly, a rat serum marker, that distinguishes cirrhotic animals from normal, was deduced to be a fragment of histidine-rich glycoprotein (27). In a very recent investigation (33), SELDI-MS fingerprint of 182 hepatitis C-induced cirrhotic patients (77 with HCC) were established and showed that 17 peaks were significantly different between the sera of HCC and non-HCC patients. Two of those peaks were elevated 50% in the serum of HCC and were identified as immunoglobulin light chains κ and λ (33).

The approaches by which SELDI can be used for both fingerprint-based diagnosis and biomarker identification are summarized in Figure 1. As it can be seen, SELDI spectra of two sets of samples from healthy individuals and cancer patients are collected and compared with the aid of bioinformatics tools such that signature pattern can be established. This pattern can then be tested on a testing set

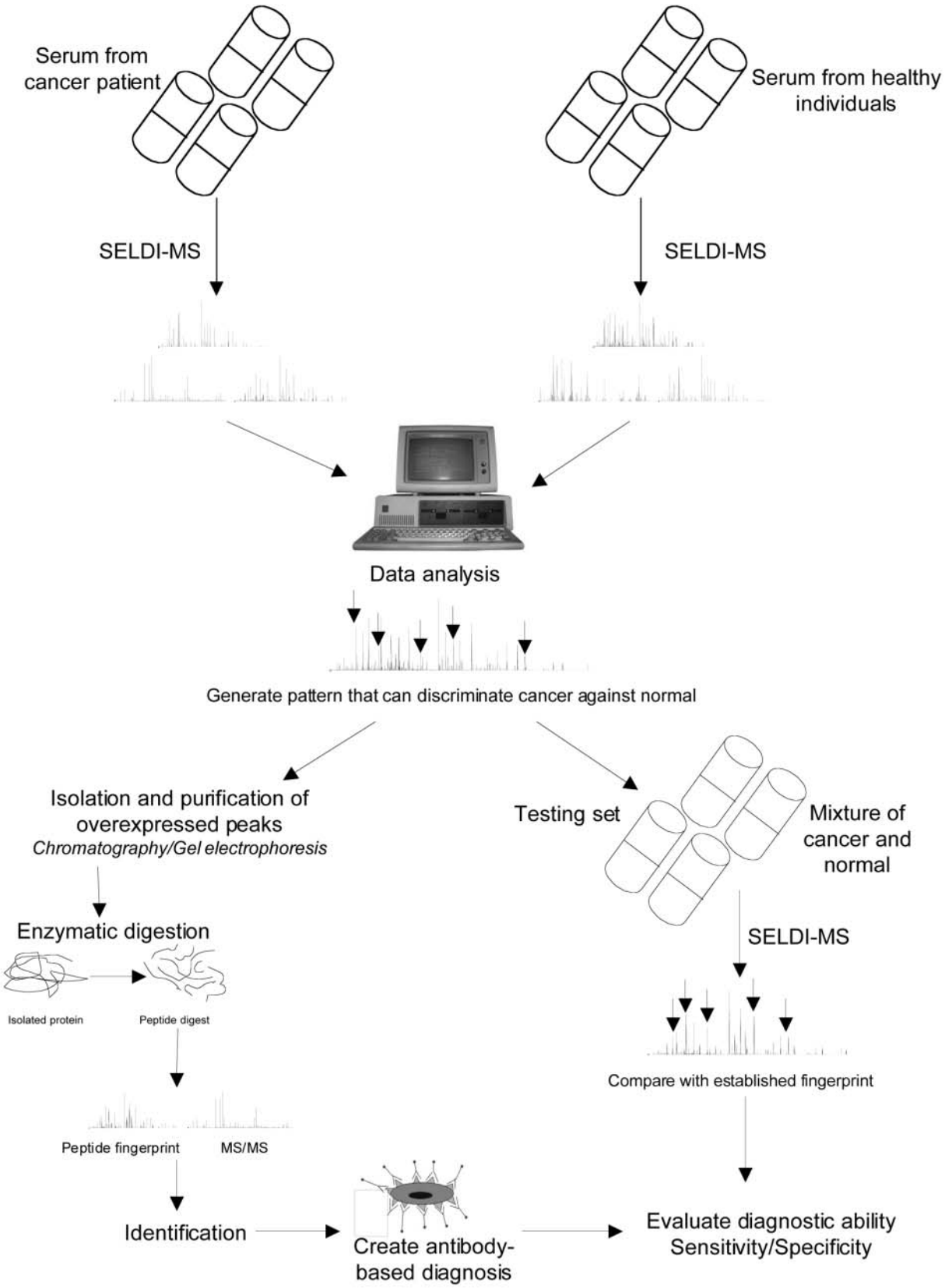

Figure 1. *The general procedures in which SELDI-MS can be used for diagnosis and biomarker discovery.*

to evaluate its detection ability such that sensitivity and specificity can be obtained. On the other hand, overexpressed peaks can be isolated and identified either by peptide mass fingerprint or MS/MS approaches (34); both of which are beyond the scope of this article. Once a potential biomarker is identified, the possibility of creating specific antibodies can be explored and used for diagnosis purposes. From this perspective, SELDI-MS can indeed be a very useful tool for the diagnosis and for the detection of new serum markers within high risk hepatitis patients.

Antibody Microarrays

Protein microarrays were first described in the late 1990s and since then, this technology has flourished and hundreds of proteins can now be detected simultaneously on the same array. Proteins can be arrayed on glass or polymeric surfaces such as hydrogels and nitrocellulose. Protein microarrays can be used to study protein-protein/ protein-DNA interactions (functional arrays); the probe on such an array can be either a protein or capturing agent, known as aptamer (*e.g.* oligonucleotides) (35). The most common arrays however, utilize antibody-antigen interactions.

In antibody arrays, antibodies are arrayed on the surface to capture the antigens in the sample (35). Reverse phase arrays work in the opposite way; samples, whether sera or cell lysate, are immobilized such that they can be detected by suitable antibodies. Antibody microarrays offer the opportunity to monitor multiple targets at the same time. This will most likely increase the sensitivity of the diagnostic procedure, with the hope of fewer false-positive and false-negative outcomes. Since this technology monitors protein expression, it is applicable at the tissue level and for body fluids, including blood. Due to the limited capacity of these arrays (especially when compared to DNA microarrays that are used for genomics studies), the targeted proteins should be chosen based on the available information with a well-identified hypothesis. For example, a protein microarray that specifically immobilized variant forms of *p53* (45 different mutations) was constructed and its interaction with other proteins was evaluated (36). It should be mentioned that protein microarrays are different from tissue microarray technology, a method of relocating multiple tissues from conventional histological paraffin blocks so that tissues from multiple patients/normal individuals can be tested on the same slide. A specific antibody can then be incubated with these tissues such that its reaction pattern can be evaluated across hundreds of samples. It was successfully used within HCC tissues and, for example, that the c-myc oncogene was found to be overexpressed in chronic hepatitis, promoting hepatocarcinogenesis (37).

In an antibody microarray, the targeted antigens can be detected through chemiluminescence or fluorescence labeling. Detection can be achieved *via* direct or indirect labeling (Figure 2). As shown in Figure 2, amplifying the signal in case of low concentrations of the target, can be achieved through the rolling-circle amplification (RCA), which can bring the detection limits as low as 0.5 pg/ml (38, 39). RCA works by attaching an oligonucleotide primer to the secondary antibody. After attaching to its epitope, the oligonucleotide is enzymatically extended using circular DNA as a template, incorporating labeled nucleotides which amplify the signal. This approach can also be used for expression, comparative studies where two sample populations are mixed. It has been shown, using plasma samples from healthy, cirrhotic and liver cancer subjects, that the use of RCA resulted in detecting a greater number of proteins in more samples in comparison to other antibody microarray-based detection methods (38).

Antibody microarrays can be used for dual- or single-color experiments, as shown in Figure 2. Dual-color arrays are aimed at comparing the cancer state *versus* normal and generate "signature" patterns. This approach uses microarrays that host hundreds of antibodies on the surface and usually use the direct labeling approach for antigen detection. Subsequent analysis such as clustering can be performed. Tannapfel *et al.*, for example, showed that 32 proteins were associated with HCC when 83 different capturing antibodies were evaluated simultaneously (40). The choice of these 83 antigen targets was based on previous knowledge of their involvement in oncogenesis, apoptosis and cell-cycle progression. Among the major up-regulated proteins was insulin growth factor (IGF), signal transducers and activators of transcription (STAT) and cyclin D1 (40). The outcomes of this study were confirmed using Western blotting and tissue microarray technology, proving the sensitivity and suitability of antibody microarray for the identification of new proteins associated with carcinogenesis and prognosis of HCC.

Unlike dual-color microarrays that utilize two dyes and usually are used for comparative studies, one-color antibody microarrays use one dye aiming at detecting multiple proteins at the same time and establishing the relationship among those tested factors. They are also used for absolute quantification measurements. Twelve clinically-used tumor markers, including AFP for the liver, were evaluated in the sera of 1147 cancer patients showing 68.18% sensitivity, with the highest detection being observed within HCC patients (92.05%); this percentage dropped to 60% when AFP was used alone (41). The presence of the 12 markers and their simultaneous detection was the major reason for such high sensitivity. For example, CA125 (cancer antigen 125) was elevated in almost 50% of liver cancer patients. On the negative side, the combined 12 markers had false-positive outcomes in 4% of normal sera and 36% in hepatitis patients; the values were 2% and 32%, respectively, with

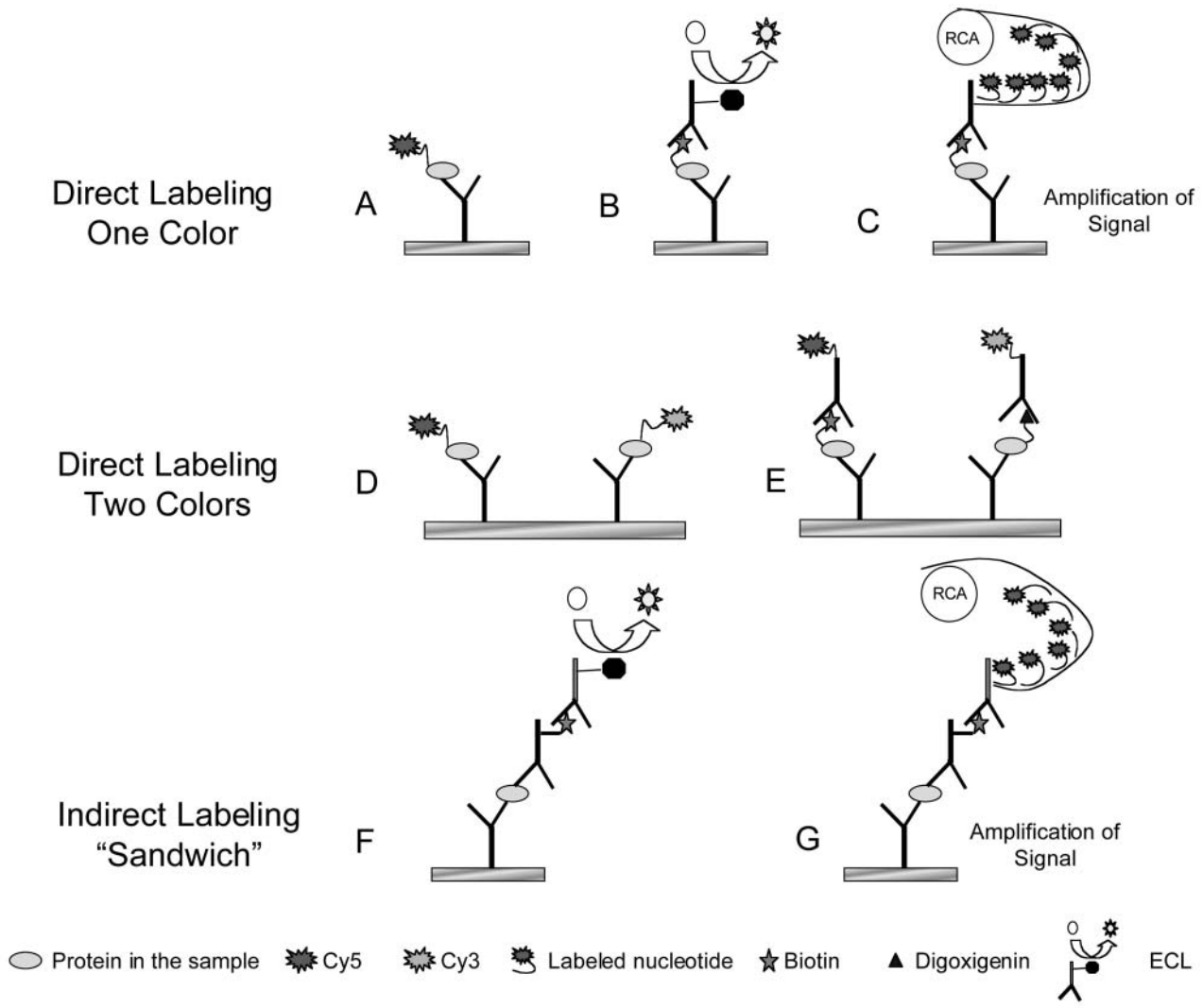

Figure 2. *Different platforms of antibody microarrays. Direct labeling involves fluorescent dyes such as Cy3 and Cy5, being attached to the antigens in the samples (A and D). This form of direct labeling can be used for comparative analysis, similar to the gene expression arrays (D and E). Direct labeling can be disruptive to the antigens and interferes with antigen-antibody interactions. Another type of direct labeling includes using a hapten tag (e.g., biotin and digoxigenin) which can be then detected by a secondary antibody and visualized, usually through fluorescence (E) or chemiluminescence detection (B). On the other hand, indirect labeling (also called the sandwich approach), does not modify the sample as the antigen captured by the antibody on the array is detected through another antibody which carries the detection system, RCA, that amplifies the signal possibly with direct (C) and indirect labeling (G).*

AFP alone. Utilizing an advanced support vector machine algorithm, however, seemed to optimize the outcomes with 0 % false-positive in normal sera and 16% in the hepatitis patients (41).

Protein microarrays, including antibody microarrays, are in their infancy and more efforts are needed to optimize this technology. Yuk *et al.*, for example, constructed a protein microarray for HCV diagnosis. The array hosts four HCV antigens and one control protein. This allowed multiple sample screening, as the membrane can fit in a well of standard 96-microwell plate (*i.e.,* 96 samples can be evaluated simultaneously) (42). Protein microarray technology, once optimized, is expected to add a powerful tool for the diagnosis and biomarker discovery of high risk HCC patients.

General Concerns

The major concern when using high-scale proteomic approaches arises from the fact that multiple targets are monitored simultaneously. Both SELDI-MS and antibody microarrays can monitor a large number of proteins at one time. Due to the complexity of such an endeavor, reproducibility seems to be the main concern within the

proteomic research that focuses on diagnosis. Intra-individual changes can also affect the outcomes such as age, gender, smoking, nutrition states, *etc.* In addition, there is current controversy with regards to the suitability of SELDI profiles under the current experimental design, as well as the robustness of the bioinformatics tools used for diagnosis (43). It was a major drawback when Baggerly *et al.* (44) was not able to reproduce the work of Petricoin *et al.* (45), who suggested that monitoring ovarian cancer with 100% accuracy was achieved using SELDI-MS. Similarly, the sensitivity/specificity of SELDI-MS, empowered with complicated algorithms, to discriminate renal cell carcinoma was 98-100%, however, when the same procedure was repeated after almost a year with a different data set, the sensitivity dropped to 41% (46). Another criticism of SELDI-MS, however, is related to the fact that diagnostic peaks are not usually identified. It is much more desirable to determine the nature of the signature peaks so that they can be related to the disease.

Concerns surrounding the usage of high-throughout SELDI-MS as a clinical diagnostic tool resulted in establishing a collaborative project sponsored by the National Cancer Institute/Early Detection Network to evaluate SELDI-MS protein profiling for prostate cancer. The recently reported work, which involved 6 research sites and 27 individuals, showed very promising outcomes in terms of platform reproducibility (47). With regard to antibody microarray, the risk of cross-reactivity is the major limitation when using antibodies (48), an important issue which needs to be taken into consideration when utilizing this technology.

Conclusion

High-throughput proteomic approaches have revolutionized cancer research and moved it into a stage where many proteins can be studied simultaneously. Valuable information regarding cancer development, therapy and diagnosis can now be obtained with microliter sample volumes. These approaches also open the door wide for comparing many cancerous lesions simultaneously without the need for labor-consuming efforts.

Combined proteomic approaches can increase the positive outcomes of diagnosis and biomarker discovery. For example, the study of interstitial fluids, perfusing the breast tumor microenvironment, using three proteomic approaches, namely mass spectrometry, protein microarray and 2D gel electrophoresis, provided a comprehensive list of proteins that can be evaluated as diagnostic or prognostic indicators (49).

So far, biomarker discovery using proteomic approaches is in its technology-optimization stage. Any findings associated with the SELDI or antibody microarray with relevance to HCC, once passed to the clinical level, will be eventually combined with other diagnosis approaches to hopefully reach the 100% detection level for high-risk patients.

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