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Improvement of protein immobilization for the elaboration of tumor-associated antigen microarrays: Application to the sensitive and specific detection of tumor markers from breast cancer sera

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ABSTRACT

There is an urgent need to identify relevant tumor markers showing high sensitivity and specificity for early diagnosis and prognosis of breast cancer. Protein microarrays have demonstrated to be cost-effective, high through-put and powerful tools for screening and identifying tumor markers with only minute samples. Autoantibodies directed against tumor-associated antigens (TAAs) were shown to be relevant tumor markers. However, due to the variability of immune response from one individual to another and depending on the type of cancer, detection of only one type of anti-TAA autoantibody is not sufficient to give a reliable and precise diagnosis. It is necessary to use a set of several TAAs for determining specific autoimmune profiles. Therefore, combining various TAAs on different surfaces could improve sensitivity and specificity for anti-TAA autoantibody detection. Herein a panel of 10 proteins, including well-known tumor-associated antigens (TAAs) and potential new biomarkers of breast cancer, were immobilized onto microstructured microarray under optimized conditions (spotting pH buffer, surface chemistry, blocking procedure), in order to determine an autoimmune signature of breast cancer. Sera from 29 breast cancer patients and 28 healthy donors were screened in sandwich immunoassays on the miniaturized system to detect the eventual presence of anti-TAAs autoantibodies. Results indicated that the detection level of each anti-TAA autoantibody in a given serum sample was strongly dependant on the surface chemistry. Combining five TAAs (p53, Hsp60, Hsp70, Her2-Fc, NY-ESO-1) on two different surface chemistries (NHS and APDMES) allowed the significant detection of more than 82% breast cancer sera.

1. Introduction

The global burden of cancer has more than doubled in the past 30 years and breast cancer is the most common cancer among women worldwide. It was reported by World Health Organization (WHO) that 636,000 and 514,000 incident cases occurred in developed and developing countries during 2008, respectively. Conventional techniques for breast cancer diagnosis include: mammography, ultrasonography, magnetic resonance imaging (MRI), and positron emission tomography (PET) (Lord et al., 2007). These techniques are often costly and actually not immediately available for many people. With the recent development of proteomic technologies, varieties of tumor markers were identified and employed for cancer detection with immunoassay methods (Ludwig and Weinstein, 2005; Sidransky, 2002). However, the lack of sensitivity and specificity of these biomarkers tested individually and their low frequency and heterogeneity in patient sera are a challenge to testing techniques for cancer diagnosis.

Among the tremendous number of tumor markers relative to breast cancer, oncofoetal protein (CEA), oncoproteins (Her2, c-myc, p53) and carbohydrate antigens (CA15-3, CA27-29) are the most proposed. Besides, mammaglobin, survivin, livin, NY-ESO-1, Annexin XI-A, Endostatin, Hsp60 and p62 are well documented tumor markers (Duffy, 2006; Molina et al., 2005). Since breast cancer is a heterogeneous disease, these tumors express many aberrant proteins and diagnose based on single tumor marker detection of either antigen or antibody usually lack sensitivity and specificity as noticed in most studies. A research group reported that tumor marker guided PET scan in the follow-up of breast cancer patients has a sensitivity of 92%, specificity of 75% and a positive predictive value of 89% in the detection of

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occult tumor recurrence (Suarez et al., 2002). However, PET could not identify low-grade lesions or tumors less than 5 mm in size. Cheung et al. (2000) summarized tumor marker measurements in the diagnosis and monitoring of breast cancer. CA15-3 has a higher sensitivity (73%) than CEA (50%) but with a similar specificity. However, combining several tumor markers demonstrates to be better than any single marker in the diagnosis and monitoring of breast cancer. The sensitivity could reach beyond 90% when the two markers (CEA and CA15-3) are used together.

As emphasized above, multiplex detection of a set of TAAs was shown to be more sensitive and specific than the detection of a single tumor marker (Cheung et al., 2000; Desmetz et al., 2009; Madoz-Gurpide et al., 2008). Thus, protein microarray technology has begun to play a significant role in the detection and quantification of proteins in complex biological samples. In particular such approach is high throughput and multiparametric assay for screening and identifying tumor markers from minute samples (Madoz-Gurpide et al., 2008; Robinson et al., 2002). Unlike traditional tumor markers, autoantobodies against tumor-associated antigens (TAAs) are found in serum from patients with different cancers, and may represent early indicators of tumor development (Desmetz et al., 2009; Liu et al., 2011). In a recent report (Desmet et al., 2011), a panel of 12 proteins involved in cancer pathology was immobilized within the nitrocellulose/cellulose acetate membrane of a 96-well filtering microtiter plate bottom via non-covalent interactions. The captured autoantibodies in cancer sera were detected using a staining approach based on alkaline phosphatase labeling. The results showed that five of these proteins (p53, NY-ESO-1, IMP1, cyclin B1 and c-myc) were able to detect 72.2% of the cancer patients tested (within a panel of 18 patients and 16 healthy donors). The authors demonstrated the advantage to use a multiple antigen panel for increasing the sensitivity of auto-antibody detection in cancer sera. To gain more diagnose efficiency of the immunoassay, it becomes essential to implement the TAA panel with complementary antigens. Moreover, testing a larger population of healthy sera would enable the determination of more accurate cut-off values for each probe in view to avoid false-positive results.

Although protein microarray can perform high throughput detection of tumor markers in single assay, efficient multiplex analysis remains challenge due to biomarkers variability and lack of sensitivity. One of the key parameters in protein microarray elaboration is to improve sensitivity is the surface chemistry. Indeed, immobilization of probe proteins on a surface leads to partial loss of their biological activity. Many efforts have been done to develop surfaces which retain biological activity of immobilized probes and limit non-specific adsorption of proteins (Olle et al., 2005; Lee and Shin, 2005; Monchaux and Vermette, 2007; Nijdam et al., 2009). However, because of the variability of protein structure and function, no universal surface chemistry meets this schedule of conditions. In a previous study, we demonstrated that the immobilization yield was dependent on the type of protein (bovine serum albumin, streptavidin, and immunoglobulin) and on surface properties (Laurenceau et al., 2011; Yang et al., in press). Moreover, regarding the biological activity of various immobilized antibodies for the detection of colorectal tumor markers, the analytical performances of the antibody microarrays were also dependent on surface chemistry. Thus, it is essential to optimize the surface chemistry to each type of immobilized proteins. Other parameters which could affect protein immobilization are the pH and ion composition of the spotting buffer (Cacace et al., 1997).

In this paper, we investigated both surface chemistry and protein immobilization conditions to improve the sensitivity of the detection of tumor autoantibodies based on TAAs microarray. Ten proteins including four well-known TAAs (CEA, p53, HER2, NY-ESO-1), two heat shock proteins (Hsp60, Hsp70) and four potential new TAAs (MYCL1, CHEK2, HNRNPK, NME1), were immobilized onto microstructured glass slides functionalized with various surface chemistries. All the surface modifications were home-made according to well-established protocols. The presence and amount of autoantibodies directed against these proteins were evaluated in 29 sera from breast cancer patients and 28 healthy donors. Results evidenced that the sensitivity and specificity depends both on the surface chemistry, and on the panel of TAAs.

2. Experimental

2.1. Materials

All chemicals were of reagent grade or highest available commercial-grade quality and used as received unless otherwise stated. Bovine serum albumin (BSA) lyophilized powder, dimethyl sulfoxide (DMSO, anhydrous, P99.9%), 0.01 M phosphate-buffer saline (PBS, pH 7.4) at 25 °C (0.0027 M potassium chloride and 0.138 M sodium chloride), sodium bicarbonate $NaHCO_3$ (M_r=84.01 g/mol), sodium carbonate Na_2CO_3 (M_r=105.99 g/mol), N-Hydroxysuccinimide (NHS), N, N′-diisopropylcarbodiimide (DIC), 2-(N-morpholino) ethanesulfonic acid (MES), tetrahydrofuran (THF) (purum grade), poly vinylalcohol (PVA), (3-glycidoxypropyl) dimethylethoxysilane (APDMES), and maleic anhydride-alt-methyl vinyl ether (MAMVE, M_w=216,000 g/mol), all were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). Tween 20 was purchased from Roth-Sochiel (Lauterbourg, France). Dextran (M_w=40,000 g/mol) was purchased from Pharmacosmos. Chitosan (M_w=470,000 g/mol, degree of deacetylation (DD) 94%) was kindly provided by Dr. T. Delair (Polymer Materials and Biomaterials Laboratory (LMPB), University Claude Bernard Lyon 1, Lyon, France).

Borosilicate flat glass slides ($76 \times 26 \times 1$ mm^3) were purchased from Schott (Mainz, Germany). Hydrogen peroxide (H_2O_2) solution (30 vol) was obtained from Gilbert Laboratories (Hérouville Saint-Claire, France). Ultrapure water (18.2 MΩ) was delivered by an Elga water system.

Anti-tumor antibodies (anti-CEA, anti-Hsp60) and tumor antigens (CEA, Hsp60) were provided by bioMérieux. Other tumor markers and recombinant protein are commercial products. Myoglobin and p53 was obtained from Sigma-Aldrich. MYCL1, CHEK2, HNRNPK, NME1, glutathione S-transferase P1 (GSTP1), Transglutaminase 1 (TMG1), Ebstein Bar Virus Nuclear Antigen 1 (EBV-NA) were supplied by Abnova Corporation (Taiwan). Hsp70 and Measle Hemagglutinin Protein (MHP) were provided by Abcam plc (UK). All the proteins were stored as aliquot at −20 °C or −80 °C following manufacturer specifications. Her2-Fc antigen was purchased from R&D system (Minneapolis, USA). NY-ESO-1 antigen and anti-Her2-Fc antibody was supplied by Thermo Scientific (USA). Cy3-labeled goat anti-human antibody (IgG+IgA+IgM) and Cy3-labeled streptavidin were purchased from Jackson ImmunoResearch and Sigma, respectively. Amersham dye of Cy3-NHS ester was purchased from GE healthcare (USA).

Myoglobin was labeled according to Alexa Fluor 647 Microscale Protein labeling Kit (A30009) (Molecular Probes, Inc. USA), and the dye/protein ratio was determined to 2.2.

0.01 M PBS or PBS 1X (pH 7.4) was prepared by dissolving the content of one pouch of dried powder in 1 L of ultrapure water. 0.1 M sodium acetate powder was dissolved to obtain the sodium acetate buffer, and pH was adjusted to 4.5. 0.02 M sodium carbonate buffers at pH 10.7 was prepared from 0.1 M sodium bicarbonate ($NaHCO_3$) $NaHCO_3$ and 0.1 M Na_2CO_3 solutions in ultrapure water. 0.1 M sodium bicarbonate ($NaHCO_3$) was