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Evaluation of a New CA15-3 Protein Assay Method: Optical Protein-Chip System for Clinical Application,

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Carbohydrate antigen 15-3 (CA15-3) is frequently measured as a breast cancer marker test. Here we describe a novel type of optical biosensor system, the optical protein chip (OPC), to detect CA15-3 in serum.

The complex formed by interaction between an antibody molecule and its corresponding antigen can be detected on a silicon substrate by an optical sensor, as described in previous reports *(1, 2)*. For processing and modification of the silicon substrate surface, silicon wafers were cut into ~2 × 0.7 cm rectangles and made hydrophilic by immersion in an acidic peroxide solution (300 g/L H_2O_2–980 g/L H_2SO_4; 1:3 by volume) and light shaking in a shaker for 30 min. The solution not only removed contaminants from the silicon surface but also increased the number of silanol groups on the surface. The hydrophilic surfaces were rinsing in distilled water 3 times and in absolute ethanol 3 times, then incubated in a mixture of 3-aminopropyltriethoxysilane and ethanol (1:15 by volume) and shaken lightly in a shaker for 2 h; The mixture liquid was then removed, and the silicon wafers were rinsed in absolute ethanol 3 times and in phosphate-buffered saline (PBS) buffer 3 times, then placed in a mixture of glutaraldehyde and PBS (1:10 by volume), shaken lightly in a shaker for 1 h, and finally, washed in PBS buffer 3 times and left in a beaker with PBS buffer until use. Through the reaction of glutaraldehyde with 3-aminopropyltriethoxysilane, Fc regions of the antibody molecules were covalently immobilized on the chip surfaces.

Protein chip preparation and detection included the following steps: (*a*) CA15-3-specific monoclonal antibody (Biodezign) was concentrated to 0.1 g/L, and then 20 μL of CA15-3 solution was delivered individually to each analytical spot on the chip by a microfluidics system (MFS) at a flow rate of 2 μL/min for 10 min. (*b*) After the entire volume of solution flowed onto each analytical spot on the silicon surface, 40 μL of diluted water was delivered individually to each spot on the chip by the MFS at a flow rate of 8 μL/min for 5 min to remove all nonadsorbed CA15-3 monoclonal antibody molecules on the analytical spot surface. (*c*) After the entire volume of diluted water flowed onto the analytical spots, 20 μL of a 1 g/L bovine serum albumin solution was delivered in the same way at a flow rate of 2 μL/min for 10 min to block nonspecific binding. (*d*) The chip was rinsed with 50 μL of diluted water in the same way at a flow rate of 10 μL/min for 5 min. (*e*) Serum samples were diluted with equal volumes of Tween 20 (20 mL/L) to a final volume of 50 μL, then the diluted samples were delivered individually to each analytical spot on the chip by the MFS at a flow rate of 2 μL/min for 25 min until the entire serum solution had flowed onto the analytical areas. (*f*) The chip was rinsed with 100 μL of diluted water in the same way at a flow rate of 20 μL/min for more than 5 min. (*g*) The chip was removed from the MFS and dried under a stream of nitrogen. The thicknesses of layers in the analytical areas were measured with the biosensor imaging ellipsometry, which produced an ellipsometric image of a surface of each chip with a lateral resolution of 2 μm. The biosensor system used here was developed to visualize antigen–antibody binding on the surface, as described in the literature *(3)*.

The OPC detection procedure was performed at least twice for each sample. Quantitative analysis was performed with use of a calibration curve, which was constructed with a serum sample with a known concentration of CA15-3 that had been determined by an electrochemiluminescence immunoassay (ECLIA). CA15-3 was undetectable in 30 serum samples from healthy blood donors. ROC plot analysis *(4)* was used to assess the accuracy of

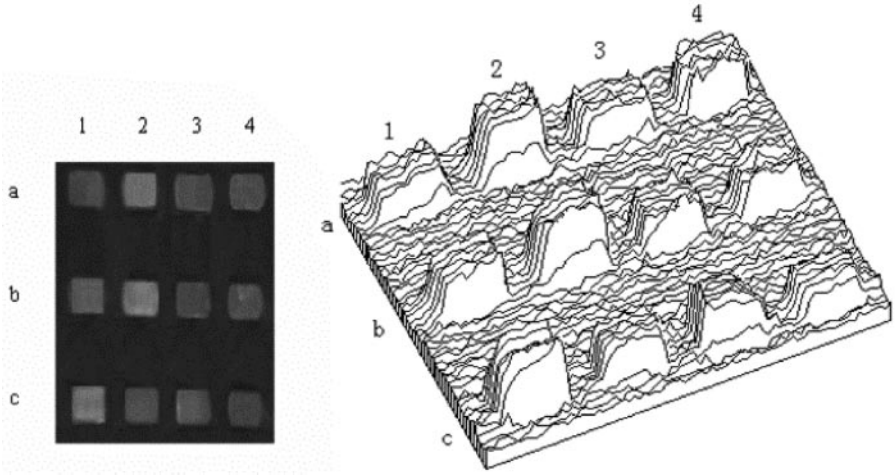

Fig. 1. Results of OPC test based on imaging ellipsometry for detection of CA15-3.

(*Left*), image in grayscale of anti-CA15-3 antibody immobilized after reaction with patient serum containing CA15-3 antigen. (*Right*), image in three dimensions deduced from (the data on the left) according to the principle that the intensity in the image is proportional to the square of the thin layer thickness. *1a* and *4c*, spots containing anti-CA15-3 IgG as a control; the mean thickness of the anti-CA15-3 IgG layer is 6.4 nm. *2a–3c*, spots containing CA15-3/anti-CA15-3 complex formed by different samples from patients with breast cancer.

the OPC test and to compare it with ECLIA detection in 60 serum samples from patients.

The CA15-3 image format determined by the OPC test is shown in Fig. 1. The calibration curve was approximated by the equation: $y = 1 - e^{-}$, which was usable up to ~ 20 kIU/L. Test samples need to be diluted when if their concentrations are ≥20 kIU/L (Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue6/). The within-run imprecision (CV) values were 5.2%, 2.5%, and 4.6% at 5, 10, and 18 kIU/L, respectively (n = 10), and the interassay CVs were 7.5%, 3.8%, and 6.3%. The lower limit of detection was 1 kIU/L at a signal-to-noise ratio of 3. The limit of quantification, defined as the lowest amount detectable with imprecision (CV) <20% (n = 10), was 4 kIU/L.

Because CA15-3 is most useful for monitoring advanced breast cancer *(5, 6)*, we collected 60 serum samples from women with breast cancer and other breast diseases for a preliminary clinical study of our test. The median patient age was 48.5 years (range, 22–75 years). Study patients included 24 women with intraductal carcinoma, 15 women with mucinous carcinoma, 5 women with in situ lobular carcinoma, 2 women with medullary carcinoma, and 14 women with breast diseases but no evidence of cancer. We also collected 30 serum samples from healthy blood donors. Serum was separated from the blood cells and stored at −70 °C until analysis. OPC tests were performed with an optical biosensor system; this immunosensor system is based on imaging ellipsometry developed at the Institute of Mechanics, China Academy of Sciences. For comparison, we measured CA 15-3 by an ECLIA on an Elecsys 2010 system (Roche Diagnostics). Both tests were done without knowledge of the clinical status of the patients or knowledge of the results of the other test. The results obtained by ECLIA detection (kIU/L) and the OPC method (kIU/L) were compared by use of Bland–Altman plots with Analyze-it Software (General+Clinical Laboratory statistics, Ver. 1.71; Fig. 2 in the online Data Supplement). The areas under the ROC curves for differentiating women with breast cancer from healthy women and women with other breast diseases were 0.807 (95% confidence interval, 0.695–0.919) for the OPC test and 0.882 (95% confidence interval, 0.776–0.998) for the ECLIA test (Fig. 3 in the online Data Supplement).

Compared with the Biacore system, a fairly widely applied optical detection method based on surface plasmon resonance, the OPC technology used in this study also allows label-free samples and crude samples to be used directly without previous purification. Both technologies are based on the optical sensor principle, but OPC is a direct optical visualization method based on imaging ellipsometry that offers biomolecular layer visualization with a distinct graph and qualitative and quantitative result analysis. Compared with the Biacore method, the OPC technology has advantages such as (*a*) optical sampling without disturbance; (*b*) identification, detection, and purification of biomolecules not only by antigen–antibody interactions but also by receptor–ligand interactions; and (*c*) real-time detection and monitoring of biomolecular interactions between carbohydrates, proteins, and nuclear acids. The OPC setup used in this study has some unique advantages. The multibioprobe analysis for 1 analyte allows up to 24 bioprobes to be arrayed on a chip at the same time, or multianalyte analysis for 1 bioprobe can be arrayed on a chip allowing up to 24 different samples at the same time. Compared with the Biacore system, a disadvantage of the OPC system is that it is not easy to use because of the complicated physical requirements of the current system.

The power and flexibility of proteomic analysis techniques, which facilitate protein separation, identification, and characterization, should hasten our understanding of processes at the protein level *(7)*. The combination of imaging ellipsometry and protein chip technology provides a new potential biosensor system for detection and monitoring of biomolecular interaction events for the fields of proteomics, clinical laboratory testing, and biomolecular interaction research.

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