J. Biomed. Eng. Res : 122-128, 2009

The Fluorescence Immunoassay of lung Cancer Serum Diomarkers using Quantum dots

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(Received November 5, 2008. Accepted April 13, 2009)

Abstract

Cancer serum biomarkers have advanced our ability to more accurately predict tumor classification, prognostic/metastatic potential, and response potential to novel chemotherapies. Serum amyloid A (SAA) and Vascular endothelial growth factor (VEGF) have potential utility as a serum biomarker for lung cancer. Quantum dots, nanometer-sized crystals, have a high quantum yield, sensitivity, and pronounced photostability. The properties of quantum dots can be efficiently applied to the detection of serum biomarkers in immunoassays as fluorescent probe.

We used quantum dots as fluorescent probes in immunoassays and attempted to detect serum amyloid A and vascular endothelial growth factor as serum biomarkers of lung cancer. This fluorescence immunoassay based on the properties of quantum dots is applicable to the detection of serum biomarkers for lung cancer. The fluorescence immunoassay with quantum dots should allow the efficient and specific detection of serum amyloid A (SAA) for the possible diagnosis of lung cancer.

Key words : Lung cancer, Serum, Biomarkers, Quantum dots, Fluorescence immunoassay

I. INTRODUCTION

Recently identified serum biomarkers have advanced our ability to more accurately predict tumor classification, prognostic/metastatic potential, and response potential to novel chemotherapies.[1-3] Moreover, the identification of biomarkers in blood or serum may have utility in the noninvasive detection and classification of diseases. Biomarker identification would be greatly enhanced by methodological improvements in protein detection.[4] Serum amyloid A (SAA) is a well-known major acute phase reactant and inflammatory marker,[5] and is upregulated in patients with various solid tumors and hematopoietic malignancies.[6-8] Vascular endothelial growth factor (VEGF) is the most potent and specific growth factor for endothelial cells. High levels of VEGF expression are found in many solid tumor types, including in lung cancer.[9] The identification of disease-specific proteins will undoubtedly lead to a better understanding of their basic biology, and potentially to novel prognostic markers and therapeutic targets.

Fluorescently labeled molecules have been used extensively for a wide range of applications in biological detection and diagnosis.[10] Quantum dots are fluorescent nanocrystals, which are developing a reputation as unique fluorescent probes in biomedicine. Semiconductive quantum dots are tiny light-emitting particles on the nanometer scale. They have captivated scientists and engineers over the past two decades because of their fascinating optical and electronic properties.[11] The optical properties of quantum dots are perfectly suited to the simultaneous measurement of biological responses in high-throughput formats, as are required in chemical biological assays.[12] Therefore, the unique properties of quantum dots may provide a new class of biological labels that could overcome the limitations of organic dyes and fluorescent proteins.[13,14] The broad absorption spectra of quantum dots also make it possible to excite quantum dots of all colors simultaneously with a single excitation light source and to minimize sample autofluorescence by choosing an appropriate excitation wavelength.[15,16] Moreover, they can be distingu-

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This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health &Welfare, and Republic of Korea (A040041) and the Samsung Biomedical Research Institute grant, #SBRI CA64081

ished from background and other fluorophores, increasing the sensitivity of detection.[17]

Based on the biotechnology of quantum dots, various platforms have been and will be built in the areas of bioanalysis and diagnostics. In recent years, research into the application of quantum dots to in vitro bioanalysis has been reported.[18,19] Immunoassays typically involve the specific binding of a labeled antibody to an analyte, followed by the physical removal of the unbound antibody to allow the quantification of the bound label. Quantum dots have been conjugated to antibodies for use in an assortment of these fluorescence immunoassays, to detect proteins and small molecules.[20-22] With the availability of new technologies, the identification of molecular biomarkers also enables the development of a new generation of diagnostic products and to integrate diagnostics and therapeutics.[23]

In this study, the possibility of using SAA serum levels as a biomarker of lung cancer was explored with the technical platform based on quantum dots fluorescence immunoassay. We hypothesized that fluorescence immunoassay with quantum dots would be efficient and specific in the detection of serum amyloid A (SAA) in a serum sample. We also detected VEGF as a potential serum biomarker of lung cancer. Here, we show that various serum biomarkers can be reliably detected with quantum-dots-based immunoassay. We also propose that SAA protein levels in serum are potentially useful as a serum biomarker of lung cancer.

II. MATERIALS AND METHODS

A. Sample collection

Approximately 7-10 mL of blood was collected from members of three groups. Serum samples from 19 patients with a diagnosis of non-small-cell lung cancer, 4 patients with a diagnosis of small-cell lung cancer, and 17 healthy individuals with no disease were selected. These serum samples were obtained from patients with lung cancer at the Samsung Medical Center (Seoul, Korea). After clotting, the serum specimens were isolated by centrifugation (3000 rpm) for 10 min at 4 °C and stored at -80 °C. The SMC institutional review board approved the study protocol.

B. Preparation of antibody and quantum dots

Antibody directed against SAA, murine anti-human monoclonal antibody (capture antibody), anti-human SAA biotin tracer antibody, and recombinant human apo-SAA were purchased from Antigenix America (Huntington Station, NY USA). Antibody directed against VEGF was purchased from R&D Systems (Minneapolis, MN USA). Streptavidin-conjugated quantum dots 605 were purchased from Invitrogen (Carlsbad, CA USA).

C. Fluorescence sandwich immunoassay using streptavidin-conjugated quantum dots

This assay system is identical to the enzyme-linked imm-

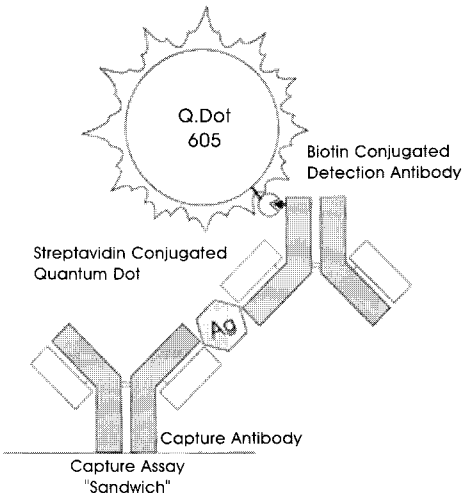

Fig. 1. Scheme of the fluorescence immunoassay to detect serum biomarker.

Our target proteins (antigen) are serum amyloid A and vascular endothelial growth factor. We detected the two antigens using quantum dots with maximum emission at 605 nm and appropriate filter sets (excitation 430 nm/emission 595 nm). We used biotinylated antibody directed against antigen and streptavidin-conjugated quantum dots as fluorescence probes.

unosorbent assay (ELISA), except that quantum dots take the place of the enzyme. We prepared antibody-conjugated quantum dots with maximum emission at 605 nm before the fluorescence immunoassay. The 96-well opaque microtiter plates were coated overnight (4 °C) with capture antibody directed against SAA (3-3.5 μg/ml), a serum biomarker of lung cancer. The coated plates were blocked with 2% (w/v) BSA in PBS. After the samples were washed three times with PBST, each serum sample (from five patients with non-small-cell lung cancer, four patients with small-cell lung cancer, and three healthy individuals) and a dilution series of recombinant SAA protein (for the standard curve) were added to individual wells. They were immobilized at room temperature for 1 h on the 96-well microtiter plates by the antibody-antigen reaction.

After the unbound nonspecific proteins were washed away, the plate was incubated with biotinylated tracer SAA antibody for 1 h at room temperature. After the incubation period, the nonbinding tracer antibody was removed by washing the samples with PBST, and streptavidin-conjugated quantum dots 605 were added to each well as the probes for the immunoassay. After the unbound streptavidin-conjugated quantum dots 605 were washed away, all the liquid was removed from the wells and the plates were dried to allow the measurement of the fluorescence intensities with a Spectrafluor Plus microplate reader (Tecan, Grodig, Austria). The filter set (ex 425 nm/em 595 nm) was chosen for the streptavidin-conjugated quantum dots 605. All experiments were performed in duplicate (Fig. 1). This immunoassay system is identical to that for VEGF.

Ⅲ. WESTERN BLOTTING

To confirm the identification of the proteins by the immunoassay with quantum dots, a monoclonal antibody directed against each identified protein was used to probe a western blot of proteins from the serum samples (from non-small-cell lung cancer patients, small-cell lung cancer patients, and healthy persons), which were identical to the samples used in the fluorescence immunoassay using quantum dots. Each serum sample was diluted 1:10 in PBS, separated on a 15% polyacrylamide gel, transferred to a nitrocellulose membrane, blocked with nonfat dry milk (5% w/v in Tris-buffered saline [TBS]), and then probed with mouse anti-human SAA monoclonal antibody at 1 μg/mL. After four washes in TBS with 0.05% (v/v) Tween 20 (TBST), the blot was incubated with horseradish-peroxidase-conjugated rabbit anti-mouse IgG at 1:5000, washed again in TBST, and developed with a chemiluminescent substrate (Santa Cruz Biotechnology, Santa Cruz, CA).

A. ELISA of Serum Amyloid A as a serum biomarker

We developed an ELISA to verify that the serum protein SAA can be used to differentiate the sera of patients with lung cancer from those of healthy individuals. Serum samples from 14 patients with a diagnosis of lung cancer and 14 healthy individuals were selected from our specimen repository. Briefly, mouse anti-human SAA monoclonal antibody was diluted 1:1000 (3 μg/mL) in coating buffer and allowed to bind well to the ELISA overnight at 4 °C. Each well was incubated in blocking buffer at room temperature for 1 h, then incubated with either serum diluted 1:10 in diluent (PBS, 0.1% BSA, 0.05% Tween 20), or a recombinant SAA dilution series for 2 h at room temperature. Horseradish-peroxidase-conjugated anti-mouse IgG was then added at a dilution of 1:1000 and allowed to bind for 1 h at room temperature. Substrate was added (ABTS, 0.05% v/v H_2O_2), and after a final wash in PBST, the plates were read at 405 nm. The plates were washed three times with PBST between each of the above steps.

This study was reviewed and consented by the Institutional Review Board (IRB) of Samsung Medical Center. The file number is 2005-10-018-002. Samsung Medical Center was confirmed from AAHRPP(Association for the Accreditation of Human Research Protection Program).

Ⅳ. RESULTS

A. Detection of serum biomarkers using quantum dots

The fluorescent labeling of biological materials using small-molecule organic dyes is widely used in biological imaging and clinical diagnosis. Semiconductor nanocrystals have been prepared for use as fluorescent probes in biological staining and diagnostics. We performed the plate-based fluor0 escence immunoassay using an indirect biotin-streptavidin conjugation system (Fig. 1). We used biotinylated antibody directed against SAA and streptavidin-conjugated quantum dots 605 (emission 605nm) as fluorescence probes. We selected appropriate filter sets (excitation 430 nm/emission 595 nm) to minimize the background signal. Then, we selected two target proteins that have potential utility as a serum biomarker for lung cancer. Our target proteins were Serum amyloid A (SAA) and Vascular endothelial growth factor (VEGF), which are overexpressed in the sera of lung cancer patients compared with normal sera [6-10]. We constructed protein standard curves for two marker proteins, SAA and VEGF (Fig. 2). A serial dilution of the recombinant protein was used to generate the standard curve for the quantitation of each protein. We detected serum amyloid A (7.8ng/mL ~ 250ng/mL, R^2 = 0.9988) and vascular endothelial growth

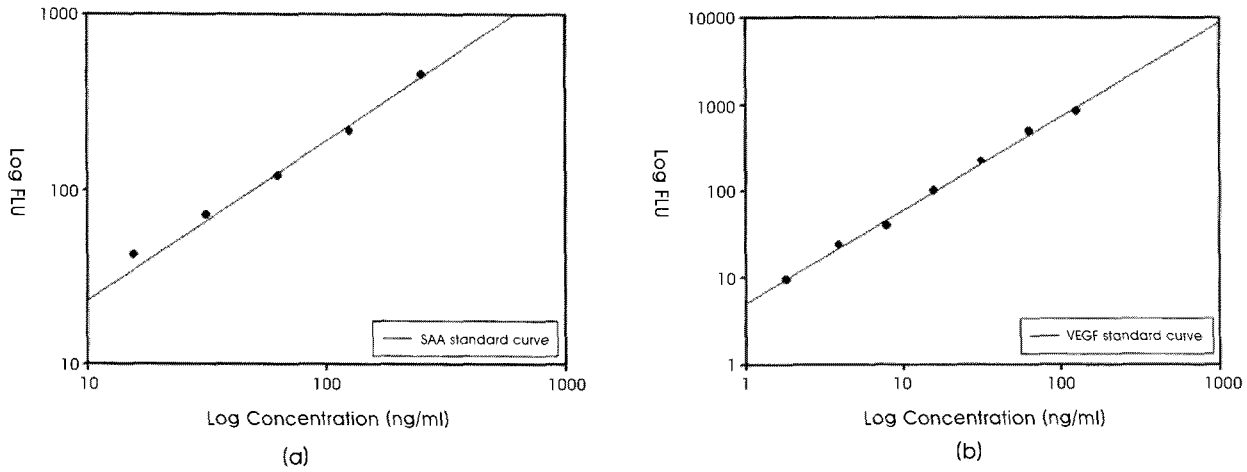

Fig. 2. Detection of serum biomarkers using quantum dots.

The standard curve of the target protein was constructed by the quantum-dot-based fluorescence immunoassay. We detected (a) serum amyloid A and (b) vascular endothelial growth factor as lung cancer serum biomarkers using quantum dots (excitation 430 nm/emission 595 nm) The curves were linear across protein concentrations ranging from 1.8 ng/mL to 250 ng/mL.

factor (1.8ng/mL ~ 125ng/mL, R^2 = 0.9949) as lung cancer serum biomarkers. We detected very low background signals throughout all these experiments. These results indicate that the fluorescence immunoassay with quantum dots is applicable to the detection of proteins, with little nonspecific binding or consequent background signal, and that its sensitivity is at least similar to that of commercial assays.

B. Application of quantum-dot-based fluorescence immunoassay to detection of serum biomarkers

Based on previous standard curves (Fig. 2a), we quantified the protein level of SAA in the sera of patients with non-small-cell lung cancer, patients with small-cell lung cancer, and healthy individuals (Fig. 3a). Our results show that the level of serum amyloid A (SAA) detected by western blotting in each serum was proportional to that observed with

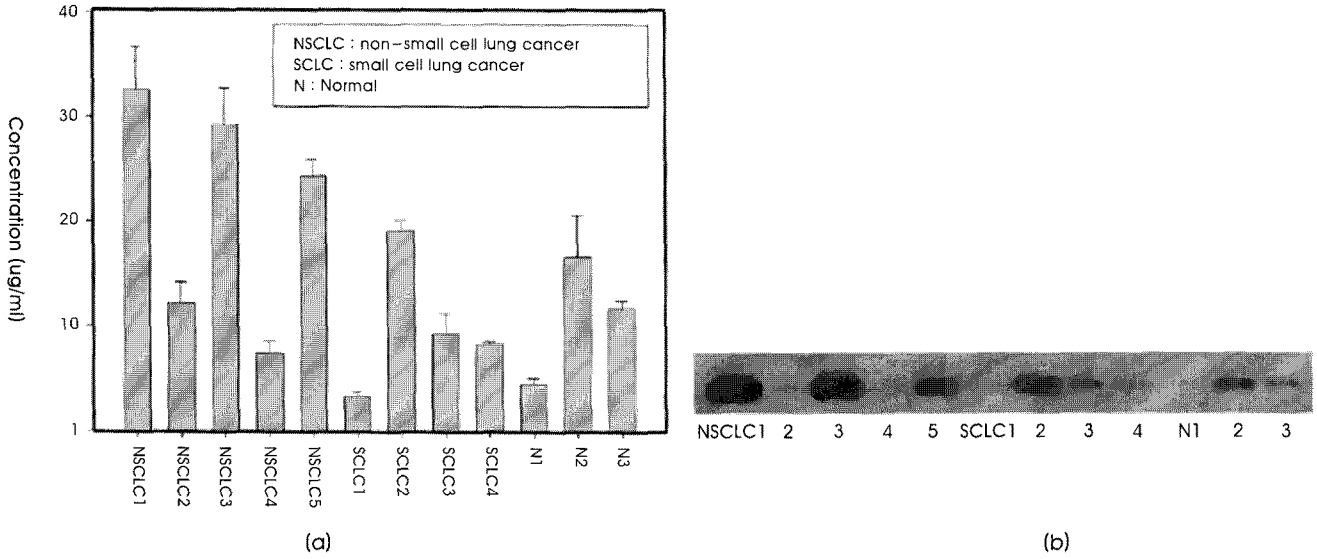

Fig. 3. Application of the quantum-dot-based fluorescence immunoassay to detect serum amyloid A and validation with western blotting.

We performed the fluorescence immunoassay using quantum dots on the sera of three groups of real patients (five patients with a diagnosis of non-small-cell lung cancer, four patients with a diagnosis of small-cell lung cancer, and three healthy individuals). We found that SAA is differentially expressed in each serum sample. (a) We also performed the western blotting to confirm the serum amyloid A level on the sera of three groups of real patients. (b) The data show that the pattern of serum amyloid A in each serum is identical to that observed in the fluorescence immunoassay.

the fluorescence immunoassay with quantum dots (Fig 3b).

C. Validation of serum amyloid A as a potential lung cancer serum biomarker

The ELISA data show that SAA levels were higher in the sera of tumor patients than in those of healthy individuals (mean SAA was 1.78 μg/mL for healthy individuals vs 9.92 μg/mL for cancer patients), providing initial insights into utilizing this as potential serum biomarker of lung cancer (Fig. 4a). The western blotting data also confirmed that the SAA in total serum samples from patients with non-small-cell lung cancer was about 46.5 fold higher than that in samples from individuals without lung cancer (Fig. 4b and c).

V. DISCUSSION

Assays based on fluorescence have the advantage of greater sensitivity than that of colorimetric enzyme immunoassays. Quantum dots have unique properties compared with those of organic fluorescent dyes.[24-26] We hypothesized that the photophysical properties of quantum dots should be advantageous in the fluorescence immunoassays in the detection of serum biomarkers. Based on this hypothesis, we performed a fluorescence immunoassay to detect serum biomarkers in serum samples. The ultimate objective of this procedure is to develop a platform that can reliably measure serum biomarkers, improving their diagnostic accuracy for cancer. The

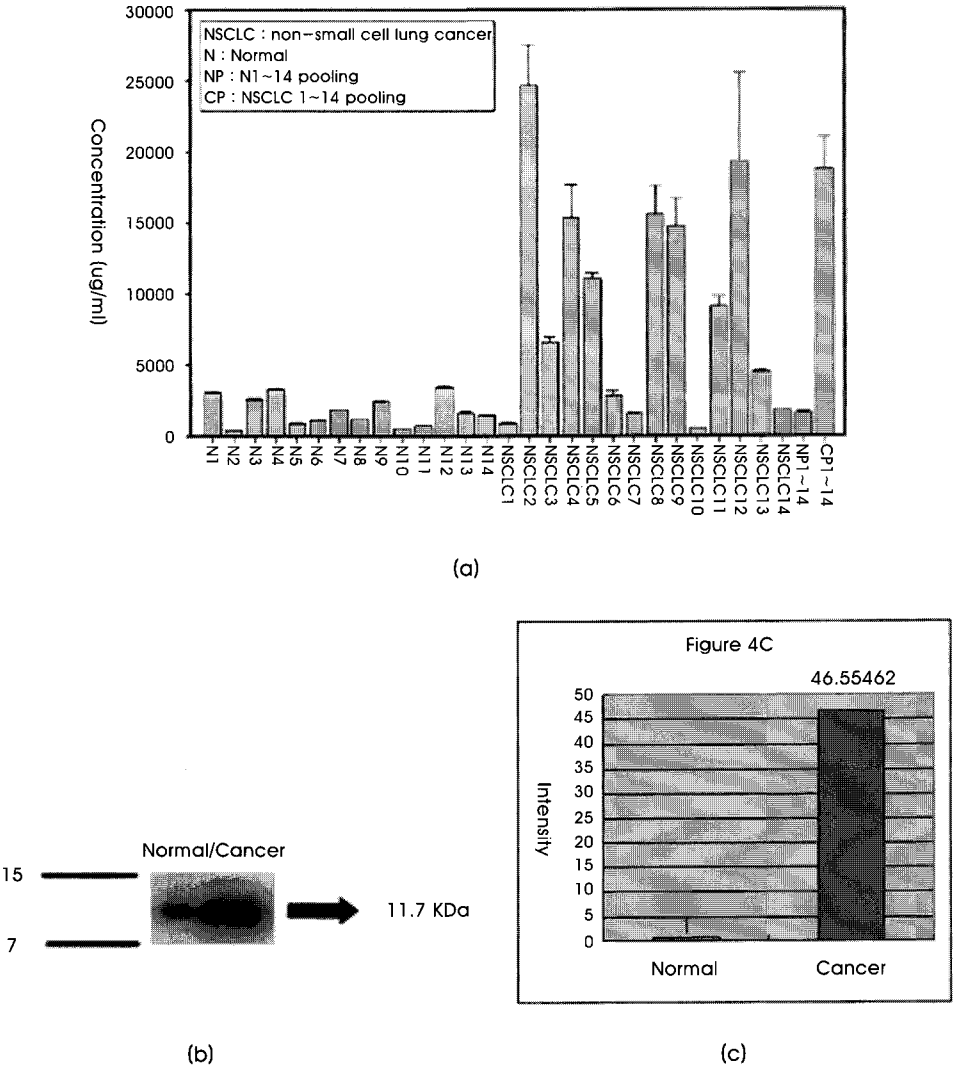

Fig. 4. Validation of serum amyloid A as a potential lung cancer serum biomarker.

We performed an ELISA to validate the potential of serum amyloid A as a serum biomarker in 14 patients with non-small-cell lung cancer and 14 healthy individuals. (P = 0.003, t test) (a) The western blotting data confirm that the serum amyloid A in total serum differs in patients with non-small-cell lung cancer and healthy individuals without lung cancer. (b and c) The serum levels of SAA are significantly higher in the sera of tumor patients than in those of healthy individuals.

properties of quantum dots allow a limit of detection lower than that of other fluorescent dyes, and the assay is simpler than enzymatic methods of plate-based detection when used in a multiplex format.[17] The principle of this assay system is almost identical to that of the enzyme-linked immunosorbent assay (ELISA), except that quantum dots replace the enzyme.

However, this technique may not require an amplification step or an enzymatic reaction, and we may be able to detect serum biomarkers for the diagnosis of lung cancer in a fast, easy, and highly reproducible way. To detect serum biomarkers, we performed a plate-based fluorescence immunoassay using quantum dots. We selected two target proteins, serum amyloid A (SAA) and vascular endothelial growth factor (VEGF), as potential serum biomarkers for the diagnosis of lung cancer. The final results show that the fluorescence immunoassay with quantum dots successfully detects serum biomarkers. We used the quantum-dots fluorescence immunoassay to detect SAA in three groups of real sera (patients with a diagnosis of non-small-cell lung cancer, patients with a diagnosis of small-cell lung cancer, and healthy individuals). We analyzed the SAA level in each serum sample and quantified its concentration. The serum levels of serum amyloid A (SAA) were higher in the sera of tumor patients than in those of healthy individuals.

There was large variation of SAA levels among tumor patients, which was caused by collection of serum samples without concerning for cancer stage. The higher level of SAA was meant that the sample was derived from later stage of cancer.[27] These results indicate that SAA is potentially useful as a serum biomarker for lung cancer. Use of immunoassay for other serum markers should improve its diagnostic accuracy. A set of control experiments included in the current study suggest that various markers in patient sera, in a concentration range of nano-gram/mL, are reliably and accurately quantified by a simple fluorescence-based plate assay. We are currently working on the detection limit of the quantum-dot-based fluorescence assay.

The SAA levels in the sera of lung cancer patients were higher than those in healthy individuals. Recently, SAA has been reported as one of the proteins upregulated in lung cancer.[5-8] SAA has been traditionally regarded as an inflammatory marker. The simple and accurate quantification of tumor biomarkers that does not involve intermediate amplification steps is required for many large-scale clinical association studies between various clinicopathological characteristics and serum biomarkers. We think that the quantum-dot-based fluorescence immunoassay can provide a solution.

In conclusion, the fluorescence immunoassay with quantum dots is efficient and specific in the detection of serum biomarkers in patient samples. The elevated SAA levels in the sera of lung cancer patients are potentially useful as a serum biomarker of the malignancy. Therefore, we suggest that quantum dots have potential utility in a variety of fluorescence bioassays and in other platforms that detect serum biomarkers, improving their diagnostic accuracy for cancer.

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