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Highly sensitive detection of multiple tumor markers for lung cancer using gold nanoparticle probes and microarrays

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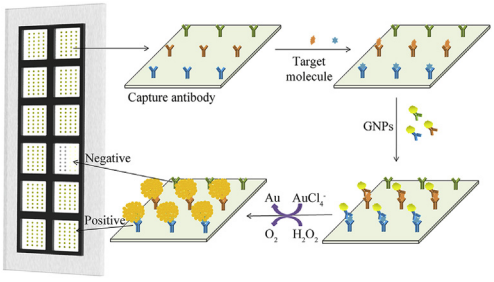
HIGHLIGHTS

- A co-detection method based on gold nanoparticles and microarrays is reported for detecting multiple biomarkers.
- A gold nanoparticle/antibody composite is synthesized as detecting probe.
- The gold deposition staining with $HAuCl_4$ and H_2O_2 produces a color change that can be observed with the naked eye.
- This protein biochip is capable of detecting multiple biomarkers in 12 serum samples simultaneously in 1 h.
- The results confirm the clinical significance of DKK1 as a serologic marker of lung cancer.

GRAPHICAL ABSTRACT



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ABSTRACT

Assay of multiple serum tumor markers such as carcinoembryonic antigen (CEA), cytokeratin 19 fragment antigen (CYFRA21-1), and neuron specific enolase (NSE), is important for the early diagnosis of lung cancer. Dickkopf-1 (DKK1), a novel serological and histochemical biomarker, was recently reported to be preferentially expressed in lung cancer. Four target proteins were sandwiched by capture antibodies attached to microarrays and detection antibodies carried on modified gold nanoparticles. Optical signals generated by the sandwich structures were amplified by gold deposition with $HAuCl_4$ and H_2O_2, and were observable by microscopy or the naked eye. The four tumor markers were subsequently measured in 106 lung cancer patients and 42 healthy persons. The assay was capable of detecting multiple

Abbreviations: NSCLC, non-small cell lung cancer; SCC, lung squamous cell carcinoma; SCLC, small cell lung cancer; AC, adenocarcinoma; GNP, gold nanoparticle probe; SEM, scanning electron microscopy; ECLIA, electrochemiluminescence immunoassay.

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Gold nanoparticles
Gold deposition
Tumor marker
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biomarkers in serum sample at concentration of <1 ng mL^{-1} in 1 h. Combined detection of the four tumor markers highly improved the sensitivity (to 87.74%) for diagnosis of lung cancer compared with sensitivity of single markers. A rapid, highly sensitive co-detection method for multiple biomarkers based on gold nanoparticles and microarrays was developed. In clinical use, it would be expected to improve the early diagnosis of lung cancer.

1. Introduction

Lung cancer is characterized by early development of widespread metastases, a high relapse rate and a 5-year survival rate of less than 16% [1]. Although advances in noninvasive imaging have improved the ability to detect lung cancer, more than 75% of patients have an advanced stage of disease at diagnosis, when therapeutic options are limited [2]. It is thus important to achieve early diagnosis and prompt treatment of lung cancer. Serum tumor markers improve early diagnosis, have prognostic value, and can be used to monitor therapy in patients with advanced disease.

Carcinoembryonic antigen (CEA) is an accepted tumor marker in colorectal cancer, and has been reported as a prognostic marker in lung cancer. Cytokeratin-19 fragment antigen (CYFRA21-1) is a sensitive tumor marker for non-small cell lung cancer (NSCLC), especially lung squamous cell carcinoma (SCC) [3]. High serum level of neuron specific enolase (NSE) suggest a high probability of SCLC, especially with a differential diagnoses including neuroendocrine types [4–7]. Recently, dickkopf-1 (DKK1) has been proposed as a tumor marker for lung cancer. DKK1 is the first member of the dikkopf family to have been identified. It is reported that DKK1 is preferentially expressed in lung cancer, with increasing serum concentrations significantly associated with tumor progression and decreased survival [8]. Serum biomarkers are valuable for diagnosis, prognosis and the treatment of lung cancer, but have relatively low sensitivity and specificity when used alone. Simultaneous assay of multiple tumor markers is frequently chosen in clinical diagnosis.

Protein arrays assist in the performance of high-throughput studies of protein interaction networks, immune reactivity, and the simultaneous assay of a number of proteins [9]. Sandwich immunoassays can comprise functional microarrays of primary “capture” antibodies that specifically bind target proteins. Secondary “detection” antibodies labeled with signaling molecules then bind to the target proteins, forming a sandwich structure [10,11]. Signal amplification is important in such assays because the concentration of biomarkers in the serum is low [12–14]. Nanoparticle-based amplification has proven useful, especially gold nanoparticles, which have surface effects, small size, and biological compatibility that allow noncovalent bonding with biomolecules. Previous studies of antibody-modified gold nanoparticles found that electroless gold deposition provided greater signal amplification than observed with silver staining [15,16]. It was recently shown that gold nanoparticles or nanoclusters can be produced in liquid solution from chloroauric acid ($HAuCl_4$) using H_2O_2 as the reducing agent, and can vary in color [17,18]. In this study, we present a new co-detection method for multiple biomarkers based on gold nanoparticles and microarrays. Gold nanoparticles conjugated to detection antibodies on chip microarrays are amplified by immersing them in a solution of $HAuCl_4$ and H_2O_2. The amplification can produce a color change that can be observed with the naked eye or read microscopically. The performance of this protein chip method for the quantitative testing of these four serum markers (NSE, CEA, CYFRA21-1, and DKK1) will be verified in both healthy people and lung cancer patients. Our aims are to develop a rapid and highly sensitive co-detection method that can be applied for clinical detection of tumor biomarkers and to confirm the clinical significance of DKK1 as a serologic marker of lung cancer.

2. Experimental

2.1. Patients and serum samples

One hundred and six lung cancer patients and a control group of forty-two healthy participants were enrolled at the first affiliated hospital of Zhengzhou University from July 2011 to November 2012. This study protocol was approved by the Ethics Review Committee of the first affiliated hospital of Zhengzhou University. Written informed consent was obtained from all of the patients and healthy donors prior to the collection of blood samples. The diagnoses of lung cancer were confirmed by microscopic examination of the material obtained by bronchoscopy, surgery, and/or biopsy. Healthy participants were outpatients who visited the hospital for routine physical examinations. Sera were collected by standard protocols in anticoagulant-free tubes, allowed to clot for 60–90 min, and centrifuged 10 min at 2500 rpm. The serum fractions were aliquot and stored at −80 °C within 4 h of collection.

2.2. Reagents

We purchased an anti-DKK1 polyclonal antibody, antigen, and DKK1 kit from R&D Systems Inc. Anti-CEA, NSE, and CYFRA21-1 monoclonal antibodies were obtained from Medix Biochemica; CEA and NSE antigens were purchased from Fitzgerald. CYFRA21-1 antigen was from Xema Co.Ltd. We purchased Tween 20, polyethylene glycol (PEG) 8000, polyvinyl-pyrrolidone (PVP), 2 - (N - morpholine) ethyl sulfonic acid (MES) and tetrachloroauric acid ($HAuCl_4$) from Sigma-Aldrich. Gold chloride acid powder was from ACROS Organics and serum diluent was from ImmunoChemistry Technologies. All other reagents were analytical grade and used without further purification.

2.3. Preparation of microarrays

Capture antibodies against CEA, CYFRA21-1, NSE, and DKK1 were fixed by carboxyl–amino reaction as a designed array onto 12 wells of a substrate (BiaO®) by the SPRi-arrayer (Horiba) following the manufacturer's instructions. The chips were kept in a 25 °C incubator for more than 16 h, and then stored at 4 °C until used.

2.4. Preparation and characterization of gold nanoparticle probes (GNPs)

First, we optimized the ratios of the antibody solutions and gold nanoparticles by the direct color signal of the gold nanoparticles. Then, 15-nm-diameter colloidal gold nanoparticles were prepared as previously described [19–21]. Following adjustment of the pH of the gold nanoparticle solution to 8.5–9.0 by adding 0.2 M K_2CO_3,