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ORIGINAL RESEARCH

Comparison of tumor markers using different detection devices

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Background: With the development of proteomics, tumor markers have attracted increasing attention for the early diagnosis and treatment of lung cancer. The biochip technology and nanotechnology continues to grow, rapid and high sensitivity joint detection of multi-tumor markers has become possible.

Methods: Eighty-six patients with lung cancer and 40 healthy controls were recruited for this study. Based on analysis of the detection results, we plotted four standard tumor marker graphs, and compared the results of the highly sensitive nanogold probe and protein chip detection with the results of electrochemiluminescence immunoassay and Dickkopf-1 (DKK1) detection used in the clinic. We then analyzed the relationship between the detection results and our clinical data.

Results: Four plotted standard protein graphs all had stages with sound linear relationships. It was found in a correlation analysis of the detection results that overall the two methods showed consistency.

Conclusion: We developed a detection method for ultra-trace protein that can detect four tumor markers, namely carcinoembryonic antigen, cytokeratin-19 fragments, neuron-specific enolase, and DKK1, in a highly sensitive way within 1.5 hours by magnifying the signal of nanogold deposition based on protein chips and nanogold probes. By comparing the results from the different detection devices, we have developed an experimental basis for detection of tumor markers in the clinic.

Keywords: tumor markers, nanotechnology, electrochemiluminescence immunoassay, lung cancer

Introduction

Lung cancer ranks first in the incidence of malignant tumors worldwide. In the People's Republic of China, the morbidity and mortality has leapt to the first in all types of common tumors.[1] About 600,000 people die of lung cancer every year. Further, the 5-year survival rate of patients with advanced lung cancer is very low.[2] With the development of medical technology, the ability to diagnose and treat the disease has improved, but is still far short of the needs in clinical practice. It is thought that the most effective way to reduce the mortality of lung cancer is to diagnose and treat the disease as early as possible. In this way, the 5-year survival rate of patients with lung cancer could be over 70%. However, no distinctive clinical symptoms are found in the early stages of lung cancer due to the insidious nature of the disease. Most lung cancers are found in the intermediate or advanced stages, when they are more likely to proliferate and metastasize. The prognosis of lung cancer is closely related to the clinical stage at which treatment is started. Therefore, it is important to identify the insidious symptoms of lung cancer, make an early diagnosis, and start treatment immediately. Currently, the iconography detection method, ie, low-dose spiral computed tomography (CT), is widely used in clinical practice to detect early-stage lung cancer. Low-dose spiral

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CT has high sensitivity when scanning for lung cancer nodules. However, it has the shortcomings of a high rate of false-positive results and being very expensive to perform. A long-term follow-up study in volunteers at high risk of lung cancer in the USA showed a false-positive rate of 21% for low-dose spiral CT.[2] Therefore, identifying whether lung cancer nodules are benign or malignant when investigating for early stage-lung cancer is problematic. In the meantime, the diagnosis of lung cancer relies mainly on histopathologic and cytologic examination, which is invasive, impractical for mass screening, and has poor diagnostic accuracy in the early stages of lung cancer. With the development of proteomics, tumor markers have attracted increasing attention for the early diagnosis of the disease.

Tumor markers may be synthesized via gene expression in tumor cells or produced by the response of the organism to a tumor. The appearance and quantitative changes in these markers reflect in occurrence, development, and proliferation of a tumor. These markers include proteins, enzymes, hormones, the product of cancer gene, polyamine. Many studies have shown that tumor markers are of significance in the early-stage detection and diagnosis, clinical staging, pathological typing, response evaluation, monitoring, and prognostication.[3] Further, there are some data showing that tumor markers could be considered as a predictor of the outcome of targeted therapy for lung cancer in more advanced stages.[4] Markers for benign tumors should have high sensitivity and specificity and be simple to test. Currently, there are still no markers for lung cancer with high sensitivity and specificity.[5] In clinical practice, we tend to use joint detection of multiple tumor markers so as to improve the positivity rate for detection of lung cancer. This joint detection requires scientific analysis and strict screening of appropriate tumor markers, so as to avoid wasting of medical resources.[6] However, the joint detection of multiple indicators is quite complicated. The current detection methods are still inadequate in terms of their sensitivity, but with the development of biochip technology and nanotechnology, joint detection of multiple tumor markers with high sensitivity can be achieved.

After the screening of several tumor markers, we selected carcinoembryonic antigen (CEA), cytokeratin-19 fragments (CYFRA21-1), neuron-specific enolase (NSE), and Dickkopf-1 (DKK1) for joint detection. Based on nanogold probe and protein chip technology, the detection antibody was coated on the surface of nanoparticles, and the antigen and antibody were then specifically on the surface of protein chips. After strengthening the signal by deposition of nanogold, a highly sensitive and feasible detection system was developed. The results obtained with this system were then compared with those obtained from the electrochemiluminescence immunoassay (ECLIA) and DDK1 kits presently used in clinical practice.[7] This aim of this work was to provide an experimental basis for future clinical detection of tumor markers.

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Materials and methods

Main reagents and devices

The main reagents used were 2-morpholinoethane sulfonic acid, Tween-20, PEG8000, polyvinylpyrrolidone, bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA), chloroauric acid (Acros Organics, Geel, Belgium), CEA antigen and antibody (Abcam, Cambridge, UK), CYFRA21-1 antigen and antibody (Xeme, Moscow, Russia), NSE antigen and antibody (Medix Biochemica, Kauniainen, Finland), DKK1 antigen and antibody (R&D systems, Minneapolis, MN, USA), quality control antibody immunoglobulin G (Abcam), aldehyde substrate (Shanghai Baio Technology Co Ltd, Shanghai, People's Republic of China), nanogold solution (15 nm, Shanghai BioServe Co Ltd, Shanghai, People's Republic of China), nonfat milk powder (Shanghai Sangon Biological Engineering Technology and Service Co Ltd, Shanghai, People's Republic of China), sucrose (Shanghai Lingfeng Chemical Reagents Co Ltd, Shanghai, People's Republic of China), and trihydroxymethyl aminomethane (Beijing Dingguo Chansheng Biotech Co Ltd, Bainjing, People's Republic of China).

The main devices used were an ultra-low temperature freezer (MDF-U4086S, Sanyo, Osaka, Japan), an ultraviolet-visible spectrophotometer (V670, JASCO, Tokyo, Japan), a fluorescence inverse microscope (BX51, Olympus, Tokyo, Japan), a transmission electron microscope (JEM2100, Olympus), a refrigerated centrifuge (5804R, Eppendorf, Hamburg, Germany), nitrocellulose films with an aperture of 0.22 μm (Corning, New York, NY, USA), a chip sampling instrument (ProSys5510A, Cartesian Technologies, Irvine, CA, USA), a simple water purification system (Millipore, Molsheim, France), a hybridization oven (FYY-3, Xinghua Analytical Instrument Factory, Jiangsu Province, People's Republic of China), and an ordinary freezer (Haier Electronics Co Ltd, Qingdao, People's Republic of China).

Serum samples and clinical data

Serum samples were taken from 86 patients with biopsy-proven lung cancer and 42 healthy controls at Putuo District Center Hospital. All patients signed their informed

consent before entering the study. The study protocol was approved by the medical ethics committee at our institution. The 86 patients with lung cancer comprised 58 males and 28 females of mean age 61 (range 42–82) years, and the 42 healthy controls comprised 25 males and 17 females of mean age 54 (range 31–68) years. All the patients have not been treated by radiotherapy, chemotherapy and immunotherapy before.

Determination of optimal quantity of protein and preparation of nanogold probes

Take 500 μL of nanogold solution and regulate the pH of the solution by K_2CO_3 (0.2 mol/L, to pH 8.5–9.0), and then divide the solution into five tubes equally. Add the CEA detection antibody of 0.4, 0.6, 0.8, 1.0, and 1.2 μL into the tube, respectively. Keep it stable for 10 minutes at room temperature and then add 10 μL of 1 mol/L NaCl into each tube immediately and observe the color of the solution. Take the least protein addition of the solution which remained red, that was, the optimal stable quantity of protein in the 100 μL of nanogold solution (the minimum concentration of protein). Repeat the experiment four times to confirm the reliability of experimental results. In a similar way, the optimal [illegible] quantity of detection antibody CYFRA21-1, NSE, and DKK1 in the 100 μL nanogold solution was availabl[illegible]

Take 2,000 μL of nanogold solution ([illegible] nm) and remo[illegible] the supernatant until the remaining s[illegible]ution [illegible] 400 μL after centrifugation; and then re[illegible]ate th[illegible]H of solution by K_2CO_3 (0.2 mol/L) to a pH [illegible]8.5–9.0. Di[illegible]de the solution into four tubes equally, and th[illegible] add a certain amount of detection antibody [illegible] the four tumo[illegible] markers; shake up and then place them into the hybridization oven at 25°C for around 1.5 hours; a[illegible]l 11 μ[illegible] of 10% PEG8000, respectively, and then leav[illegible] alone overnight at 4°C. Twelve hours later, centrifug[illegible] these s[illegible]lutions [illegible] and remove the supernatant again; [illegible]pply th[illegible] he heavy suspension to set the volume as 50 μL [illegible] finally add 1.5 μL 5 M NaCl, respectively, and keep the[illegible] at 4°C. Transmission electron microscopy was used to observe the size and shape of nanogold probes. An ultraviolet-visible spectrophotometer could be applied for scanning analysis and prediction of the concentration of nanogold probes. The centrifugations above were carried at 4°C and at 9,000 rpm for 50 minutes.

Fabrication of protein chips

Firstly, lay the substrate of aldehyde group at the chip place of the chip sampling instrument, then configure the respective antibodies of CEA, CYFRA21-1, NSE, and DKK1, and configure the quality control antibody immunoglobulin G and spotting solution at the ratio of 1:1 (v/v) on the sample configuration board; next, lay them at the sample board area of chip sampling instrument; turn on the instrument, set the relative programs, and apply spotting according to operational norms. After that, place the protein chips in the incubator and leave for 16 hours at 25°C and finally keep it dry at 4°C.

Immunodetection

Firstly, keep the solution of 5% n[illegible]lk powder sealed on protein chips for 10 minutes; [illegible]end the so[illegible]ion of 5% non-fat milk powder with nanogold [illegible]robes and d[illegible]fferent serum samples together and th[illegible]n add the[illegible] into th[illegible] detection area respectively; next, la[illegible]he solut[illegible]n into [illegible]ybridization oven and keep them i[illegible]cu[illegible]ed f[illegible] 45 min[illegible]tes; wash chips one or two times in [illegible]tions; the[illegible]dd so[illegible] nanogold deposition for dying fo[illegible]minutes (dy[illegible]g can be carried at 37°C or at room temperatur[illegible] After roughly observing the chip detecti[illegible]results by eye, re[illegible]ove the dye solution rapidly and then [illegible]dd some ultrapure water to stop dying. Observe the result [illegible]nd signal, re[illegible]eat dyeing if the signal is too weak.

Analysis of results

It [illegible] considered as positive when there were dark brown points in chips. Usually, the color changes with the diameter of nanogold probes. It tends to be deep when the diameter is large. We could observe the result with the naked eye or by microscopy. Image-Pro Plus 7.0 software was used to analyze the results by calculating optical density. In this way, the indirect quantitative detection of proteins was achieved.

Statistical analysis

Statistical Package for the Social Sciences version 19.0 software (SPSS Inc, Chicago, IL, USA) was used for the statistical analysis. The data are expressed as the median (interquartile range). The chi-squared test was used to compare rates. The non-parametric test was used to test for differences between the groups. These differences were compared using the Wilcoxon rank sum test, and the difference among three or over three groups was by the Kruskal-Wallis test. $P<0.05$ was considered to be statistically significant.

Results

Representation of nanogold probes

Observing the nanogold labeling antibody by transmission electron microscopy, we found that the nanoparticles were of uniform size with an average diameter of 15 nm.

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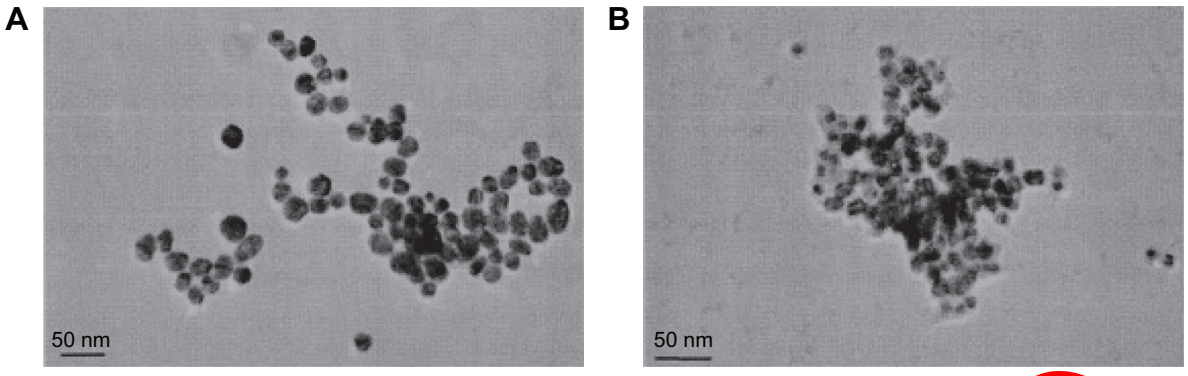

Figure 1 (**A**) Unmarked and (**B**) marked nanogold particles as seen on transmission electron microscopy.

The surrounding interface was quite clear before nanogold marked the antibody (Figure 1A), but after marking the antibody, there were cycles of gray dark aureole in the surrounding interface (Figure 1B), confirming that the nanogold surface had marked the antibody and that the nanoparticles had not become aggregated.

Analysis of specificity of the four tumor markers

We determined the specificity of the four tumor markers on protein chips separately, and the results are shown Figure 2. The tumor markers showed no cross reaction interruption on the protein chips and had a good specificity. We repeated the experiment four times to en re the reliability of our results.

Plotting of standard graph

A standard curve was drawn bas on the optical de ity of the detection results, so as to be able to ect the proteins indirectly. The standard curves obtained afte detecting the antigen standards for CEA, CYFRA 1, NSE, d DKK1 are shown in Figure 3. A un, we repea the experiment four times to ensure the ccurac of our results.

The standard protein c es all ha stages with sound linear correlati The spec linear distributions were 40 pg/mL t 25 ng L (R^2=0.9) for CEA, 70 pg/mL to 25 ng/mL (R^2=0.994) fo YFRA21-1, 90 pg/mL to 25 ng/mL (R^2= 994) for NSE, and 90 pg/mL (R^2=0.996) for DKK1. Det tability wit regard to sensitivity was determined to be 1 g/mL for EA protein, 15 pg/mL for CYFRA21-1, 20 pg/ fo NSE protein, and 30 pg/mL for DKK1. Co ared with ordinary enzyme-linked immunosorbent say detection for CEA, this method greatly improved the sensitivity of detection, reaching 1.65 ng/mL.

Detection and analysis in serum samples

The detection system was tested using serum samples from the 86 patients with lung cancer and the 42 healthy controls, and the results are shown in Figure 4. The concentration of the four tumor markers in the patients with lung cancer is

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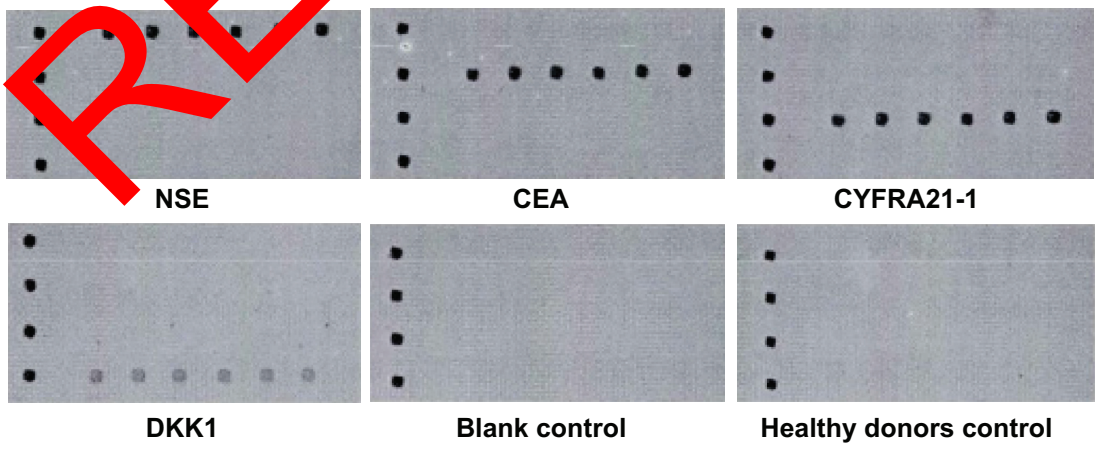

Figure 2 Results of analysis of these four specific tumor markers.
Abbreviations: CEA, carcinoembryonic antigen; CYFRA21-1, cytokeratin-19 fragments; NSE, neuron-specific enolase; DKK1, Dickkopf-1.

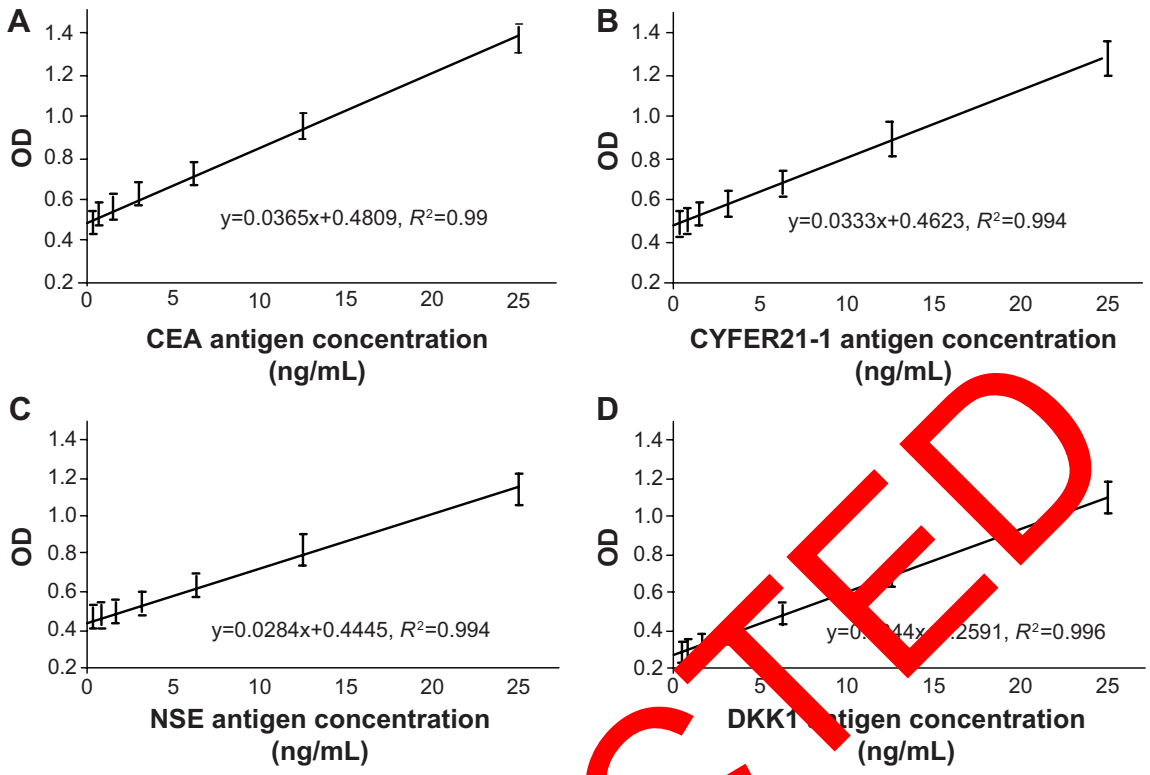

Figure 3 Standard graphs showing the relationship between the four tumor markers a optical density.
Notes: (**A**) Antigen concentration of CEA (ng/mL), (**B**) antigen concentration of CYFR21 ng/mL), (**C**) ant n concentration of NSE (ng/mL), and (**D**) antigen concentration of DKK1 (ng/mL).
Abbreviations: CEA, carcinoembryonic antigen; CYFRA21-1, cytokerati ments; NSE, specific enolase; DKK1, Dickkopf-1; OD, optical density.

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shown in Figure 5A. The relative values wer ared w those from ECLIA used in clinical practi , and a atter di gram for CEA is shown in Figure 5B. C rall s showed that the detection results these t methods were consistent (relative coefficien e r=0.986; FRA21-1: r=0.985; NSE: r=0.978; DKK1: r= 93 respectively).

The four tumor m ers in the 128 um samples were found not to follo a norm d distribution (all $P<0.05$). Comparing the lun an r group and the healthy control group (Table 1), the difference in the four tumor markers was statistically significant (all $P<0.05$), with their concentrations being markedly higher in the lung cancer group than in the control group.

We took 95% of the values for the four tumor markers from the 42 healthy controls as the critical value (4.82 ng/mL for CEA, 3.04 ng/mL for CYFRA21-1, 23.7 ng/mL for NSE, and 14.15 ng/mL for DKKl). We then analyzed the positive detection rate for the four tumor markers in the patients with

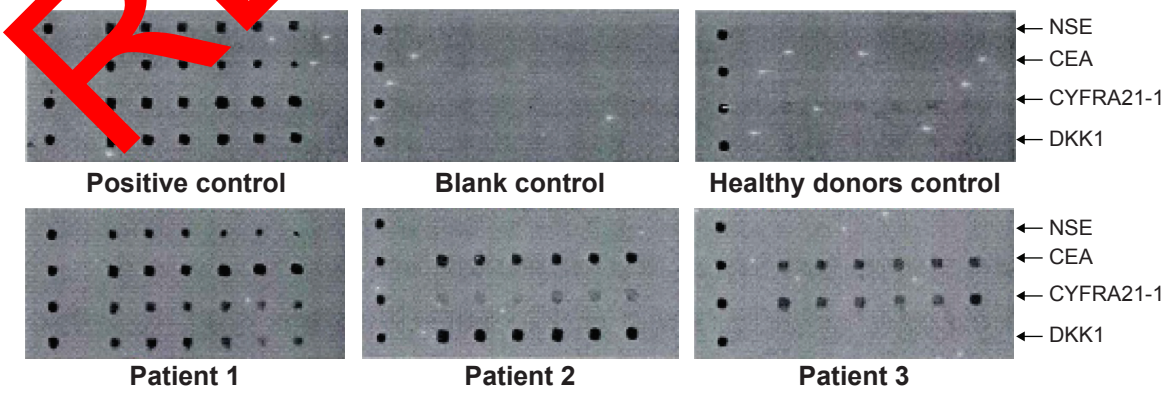

Figure 4 Sample of detection results for the four tumor markers.
Abbreviations: CEA, carcinoembryonic antigen; CYFRA21-1, cytokeratin-19 fragments; NSE, neuron-specific enolase; DKK1, Dickkopf-1.

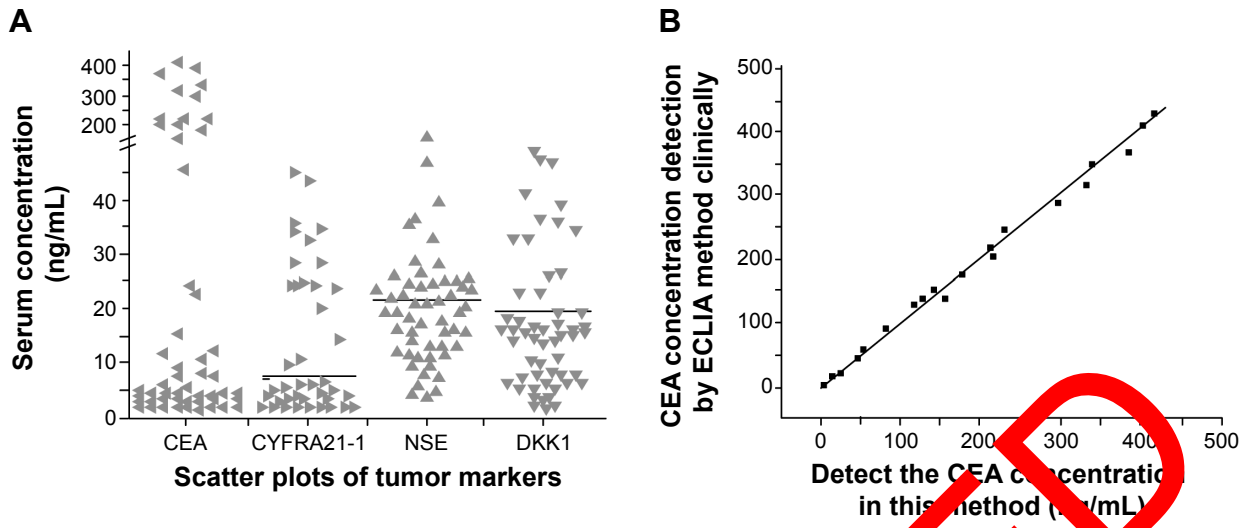

Figure 5 Distribution of the concentrations of four tumor markers (**A**) and comparison of CEA concentrations detected by the method in this study and by electrochemistry method (**B**).
Abbreviations: CEA, carcinoembryonic antigen; CYFRA21-1, cytokeratin-19 fragments; ECLIA, electrochemiluminescence immunoassay; NSE, neuron-specific enolase; DKK1, Dickkopf-1.

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lung cancer. Table 2 shows that in the lung cancer group the sensitivity was 38.37% for CEA, 51.16% for CYFRA21-1, 26.745% for NSE, and 52.33% for DKK1. The joint detection rate for the four tumor markers was 88.37%, which was much higher than any single detection (all $P<0.001$). The sensitivity of CEA in glandular cancer was 54.35%, and that of CYFRA21-1 in squamous cancer and NSE in small cell cancer was 54.84% and 66.67%, respectively.

Discussion

CEA is a glycoprotein that is distributed on the colonic mucosal epithelium of the embryo and is expressed at high levels in several malignancies, including certain gastric, lung, breast, and ovarian cancers. Detection of changes in serum CEA can be useful for early diagnosis of malignant tumors, monitoring the response to treatment, and assisting with the prognosis, which is usually bad for patients showing progressive increases in CEA.[8] CYFRA21-1, a scaffold protein in normal or malignant epithelium, is found in the epithelial monolayers and is present in high concentrations in tumors originating from epithelial tissues. CYFR is also a sensitive tumor marker, and has high specificity in squamous cancer. Serum CYFRA21-1 levels tends to rise with the increasing tumor stage, and can predict the prognosis and determine the effects of treatment. It has been reported that joint detection of CEA and CYFRA-21 could effectively improve the rate of accurate diagnosis of non-small cell lung cancer.[9] NSE plays an important role in the diagnosis of small cell lung cancer and a high serum NSE concentration aids in the diagnosis of small cell lung cancer and in the identification and diagnosis of neuroendocrine tumors. There is some evidence that combined measurement of NSE and pro-gastrin-releasing peptide levels can help to identify.[10] DKK1 is a secretory glycoprotein including a signal peptide sequence and two domains of cysteinamine, which has come to the attention of researchers just recently. It is a secretory protein firstly found by the Dickkopf family. It acts as an inhibitor of wnt/β-catenin signals, which

Table 1 Concentration of four tumor markers in controls and patients with lung cancer

	n	CEA	CYFRA21-1	NSE	DKK1
Healthy donors	42	2.01 (1.29–3.18)	1.36 (1.03–2.03)	13.56 (10.51–15.86)	8.48 (4.91–14.4)
Patients with lung cancer	86	4.15 (2.78–17.06)	3.06 (1.92–5.37)	15.77 (11.43–23.76)	14.20 (5.75–20.33)
Z		–5.633	–6.11	–2.416	–3.311
P		<0.001	<0.001	0.016	0.001

Notes: Values are expressed as the median [interquartile range] in ng/mL.
Abbreviations: CEA, carcinoembryonic antigen; CYFRA21-1, cytokeratin-19 fragments; NSE, neuron-specific enolase; DKK1, Dickkopf-1.

Table 2 Sensitivity of four tumor markers in patients with lung cancer according to pathological typing

	n	CEA	CYFRA21-1	NSE	DKK1	Joint detection
Lung cancer	86	38.37 (33/86)	51.16 (44/86)	26.74 (23/86)	52.33 (45/86)	88.37 (76/86)
Squamous	31	19.35 (6/31)	54.84 (17/31)	25.81 (8/31)	54.84 (17/31)	87.1 (27/31)
Glandular	46	54.35 (25/46)	52.17 (24/46)	19.57 (9/46)	56.52 (26/46)	91.3 (42/46)
Small cell	9	22.22 (2/9)	33.33 (3/9)	66.67 (6/9)	22.22 (2/9)	77.78 (7/9)

Note: Data are shown as sensitivity (the number of samples that have sensitivity of tumor marker/the total amount of samples).

strictly controls the functional status of these signals, and these signals play an important role in the development of stem cells of adults and the regulation system. There have been studies showing markedly decreased serum DKK1 concentrations in patients with gastric cancer, colorectal cancer, ovarian cancer, and cervical adenocarcinoma, and a significant increase in expression in other tumors, such as liver cancer, lung cancer, Wilm's tumor, hepatoblastoma, breast cancer, and multiple myeloma. The increase in DKK1 in patients with lung cancer is closely related to the treatment of tumors.[11]

The microarray of protein chips based on the intensity and parallel processing principle of microelectronics can cure a great number of biological samples with biology significance orderly on solid-phase carrier to specifically arrest the effective ingredients in samples.[12] It also takes advantage of a charge coupled device or laser scanning system to acquire and analyze images, so that a great deal of information can be handled and made available rapidly. It has the features of high flux, high sensitivity, and an ability to perform multivariate analysis. Nanogold particles also called colloidal gold because they have a colloid appearance in aqueous solution. With their unique biological features, they can be marked on the surface of many biomolecules and integrate with them completely. Moreover, they are quite easy to detect because of their physical features, and play an important role in the biomedical detection.

Table 1 shows that the concentrations of these four tumor markers were higher in the lung cancer group than in the control group, indicating that their detection would be helpful in the diagnosis of lung cancer. Table 2 shows that sensitivity of CEA for detection in the lung cancer group was 38.37%, with respective values of 51.16%, 26.74%, and 52.33% for CYFRA21-1, NSE, and DKK1; the joint detection rate for the four markers was 88.37%, which is higher than that for detection of a single marker ($P<0.0011$), indicates that joint detection may be useful for diagnosis of lung cancer. The highest sensitivity of CEA in detecting glandular cancer was 54.35%, with values of 54.8% and 66.67% for CYFRA21-1 in squamous cancer and for NSE in small cell lung cancer, respectively. These findings provide further evidence that CEA is beneficial for detecting glandular cancer, CYFRA21-1 for squamous cancer, and NSE for small cell cancer. DKK1 has no distinct role in the pathological typing of tumors, but it has quite high sensitivity in all tumor stages as a new tumor marker and can greatly improve the sensitivity in the diagnosis of lung cancer. It is important to use joint tumor marker detection when screening for lung cancer and when treating the disease. With continued developments in science and technology, new techniques and methods for biomedical detection based on nanogold probes are constantly emerging. Despite this, few can be applied in clinical practice. This is mainly because these techniques will lead to the increase of false positive signals while improving sensitivity due to to lack of effective control for non-specific signals. Especially during detection of serum samples in clinic, a great number of non-specific signal will appear as there are a lot of unknown proteins. These problems need to be resolved by further studies. Compared with the single antibody, non-specific absorption particles are easier to clean. This study, taking full advantage of this mechanism, marked the detection antibody on nanogold, which greatly reduced the disturbance signal. Using nanogold as a detection carrier, the detection signal of protein chips and nanogold was magnified. High sensitivity and specificity were ensured, thereby meeting present clinical requirements.

Conclusion

Using protein chips and nanogold probes, we developed a detection method for ultra-trace protein by magnifying the signal of nanogold deposition. This method enabled visual testing for proteins in a semi-quantitative way, and could detect four tumor markers, ie, CEA, CYFRA21-1, NSE, and DKK1 within 1.5 hours with high sensitivity. It enables simultaneous and rapid detection of multi-tumor markers, enabling early diagnosis and assessment for lung cancer. In this work, we also performed a correlation analysis between the results in this study and the detection results from ECLIA combined with DDK1 kit in clinical practice, and found consistency. The method described here is considered to have a wide application, given that the experimental results can be analyzed simply with the naked eye or an ordinary

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microscope. Comparison of detection results using different devices provides a sound basis for the detection of tumor markers in clinical practice.

Disclosure

The authors report no conflicts of interest in this work.

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