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Journal of Immunological Methods 287 (2004) 13–20

JIM Journal of Immunological Methods

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Research Paper

Novel amperometric immunosensor for rapid separation-free immunoassay of carcinoembryonic antigen

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Received 1 August 2003; received in revised form 9 December 2003; accepted 5 January 2004

Abstract

A novel immunosensor for rapid separation-free determination of carcinoembryonic antigen (CEA) in human serum is proposed. The immunosensor is prepared by co-immobilizing thionine and horseradish peroxidase (HRP)-labeled CEA antibody on a glassy carbon electrode (GCE) through covalently binding them to GCE with a glutaraldehyde (GA) linkage. The electrochemical behavior of the immobilized thionine displays a surface-controlled electrode process with an average electron transfer rate constant of $4.74 \pm 2.99\ s^{-1}$. It can be used as an electron transfer mediator for enzymatic activity detection of the HRP-labeled antibody to CEA. After the immunosensor is incubated with CEA solution at 23 °C for 40 min, the access of activity center of the HRP to thionine is partly inhibited, which leads to a linear decrease in the catalytic efficiency of the HRP to the oxidation of immobilized thionine by H_2O_2 at −300 mV over two CEA concentration ranges from 0.5 to 3.0 and 3.0 to 167 ng/ml. Under optimal conditions, the detection limit for the CEA immunoassay is 0.1 ng/ml at three times background noise. The immunosensor shows good accuracy and acceptable storage stability, precision and reproducibility with intra-assay CVs of 6.1% and 5.8% at 2.5 and 50 ng/ml CEA, respectively, and an inter-assay CV of 6.3% at 50 ng/ml. This method is economical and shortens the analytical time, making it potentially attractive for clinical immunoassays.

Keywords: Immunosensor; Carcinoembryonic antigen; Thionine; Biosensors; Glassy carbon electrode; Immunoassay; Separation-free; Amperometry

1. Introduction

Immunoassay techniques, based on the property of highly specific molecular recognition of antigens by antibodies (Andrey et al., 1998), have become the main analytical methods in clinical examinations (Itoh and Ichihara, 2001; Trull, 2001; Worwood, 2002), biochemical analyses (Panteghini, 2000; Rossier et al., 2002; Sato et al., 2003) and in other areas such as

Abbreviations: BSA, bovine serum albumin; CEA, carcinoembryonic antigen; CV, coefficient of variation; GA, glutaraldehyde; GCE, glassy carbon electrode; HRP, horseradish peroxidase; HRP-CEA antibody, HRP-labeled CEA antibody; PBS, phosphate buffer solution; SCE, saturated calomel electrode.

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doi:10.1016/j.jim.2004.01.012

environmental (Dietmar, 1995; Van Emon et al., 1998) and food quality control (Ring et al., 2001; Belloque et al., 2002). Currently, most immunoassays are performed with 96-well microtiter plates in which samples can be processed simultaneously. The technique has proved to be sensitive but is difficult to automate (Palmer et al., 1992). The conventional immunoassay methods, including radioimmunoassay, single radial immunodiffusion, immuno-turbidimetry and enzyme-linked immunoassay, etc., are successful but involve complicated, tedious assay processes, requiring long analytical time (1 h to several hours) and specially equipped laboratories and/or skilful personnel (Nilsson et al., 1992; Palmer and Miller, 1995). These limit the application of conventional immunoassay techniques and make them unfit for fast determinations of the analyte. Thus, it would be useful to develop a particular immunosensor for fast and convenient immunoassay (Andrey et al., 1998).

Immunosensors are miniaturized analytical devices, which combine the selectivity of the immunological reaction with the sensitivity and convenience of various detection techniques. Many kinds of immunosensors have been developed, including electrochemical (Cui et al., 2000; Liu et al., 2000a,b; Sarkar et al., 2002), chemiluminescent (Rubtsova et al., 1998; Kaiser et al., 2000), fluorometric (GonzalezMartinez et al., 1997; Penalva et al., 1999), piezoelectric (Wu et al., 2000) and surface plasmon resonance (Lyon et al., 1998) immunosensors, impedance biosensing chips (Ruan et al., 2002) and even multichannel microchips for multianalyte determination (Tang et al., 2002; Yakovleva et al., 2002). Electrochemical detection of the label has several advantages such as high sensitivity and the low cost of the resulting sensors and instrumentation. An electrochemical detector can also be arranged as a microcell that allows miniaturization of the biosensor (Andrey et al., 1998; Skládal and Kalab, 1995) and has therefore received special attention.

Electrochemical immunosensors determine the level of analyte by detecting the changes of potential (Solé et al., 1998; Campanella et al., 1999; Gerdes et al., 2000; Milligan and Ghindilis, 2002), current (Wang and Pamidi, 1998; Campanella et al., 1999; Liu et al., 2001; Darain et al., 2003), conductance (Hianik et al., 1999) or impedance (Alfonta et al., 2001) caused by the immunoreaction. Although a low detection limit can be achieved through amperometric immunosensors, the addition of a mediator, such as hydroquinone (Santandreu et al., 1999), or *o*-aminophenol (Liu et al., 2001) for HRP and H_2O_2, leads to a more complex immunoassay system and increases the analytical time and expense. Simultaneous immobilization of mediator and HRP molecules has been widely used to construct H_2O_2 sensors (Liu et al., 1999; Xiao et al., 1999; Liu et al., 2000a,b; Xu et al., 2003), which simplifies greatly the assay system. In the present work, an amperometric immunosensor has been developed by co-immobilizing mediator and enzyme-labeled antibody on a glassy carbon electrode (GCE).

It has been reported that the active site of an enzyme conjugated to an antigen can be shielded and access of its substrate may be either partially or completely blocked when the labeled antigen reacts with its antibody to form an immunocomplex (Skládal, 1997). This leads to a decrease in detection signal and has been widely used in homogeneous immunoassays (Fonong and Rechnitz, 1984; Broyles and Rechnitz, 1986; Skládal, 1997). Here, we combine the advantages of a mediator-immobilized immunosensor with a heterogeneous immunoassay to design a simple and fast heterogeneous immunoassay method. Using CEA as a model, the immunosensor was prepared by co-immobilizing thionine and HRP-labeled CEA antibody on GCE. The incubation of the immunosensor with CEA solution resulted in the formation of an immunocomplex on the electrode surface, which inhibited the electron transfer between the immobilized thionine and the active center of the immobilized HRP. The assay format avoids the addition of an electron transfer mediator to the solution and the separation step. This significantly simplifies the immunoassay procedure and shortens assay times.

2. Materials and methods

2.1. Reagents

CEA ELISA and IRMA kits were purchased from Diagnostic Products (DPC, USA). The ELISA kits consisted of a series of CEA standard solutions with different concentrations from 0 to 500 ng/ml, and a solution of horseradish peroxidase conjugated mono-

clonal anti-CEA antibody. Bovine serum albumin was obtained from Sigma (St. Louis, MO, USA). Thionine, glutaraldehyde and H_2O_2 of analytical grade were from the Shanghai Biochemical Reagent Company (China). All other reagents were of analytical grade. Double distilled water was used for all experiments. Phosphate buffer solutions (PBS, 0.1 M) at various pH values were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and then adjusting the pH with 0.1 M NaOH and H_3PO_4. All reagents were brought to room temperature (ca. 20 °C) before use.

2.2. Preparation of thionine/HRP-CEA antibody modified GCE

The GCE (4 mm diameter) was first polished using rough and fine sandpapers. It was then polished to a mirror finish with 0.3 and 0.05 μm alumina slurry (Beuhler). After it was thoroughly rinsed with double distilled water, a potential of + 1.75 V was applied to the electrode in 0.1 M pH 5.0 PBS for 300 s. The electrode was then scanned between + 0.3 and + 1.3 V until a steady-state current–voltage curve was obtained (Wang et al., 2001). The pretreated GCE was soaked in 20 mM glutaraldehyde solution for more than 12 h to form glutaraldehyde modified GCE. After the modified GCE was thoroughly rinsed with double distilled water to remove physically adsorbed glutaraldehyde, it was immersed in a mixture of 30 μl HRP-labeled CEA antibody solution and 30 μl 0.2 mM thionine solution for 5 h to yield a thionine/HRP-CEA antibody modified GCE. This procedure is shown in Fig. 1. The prepared immunosensor was soaked in pH 7.0 PBS at 4 °C prior to use.

2.3. Apparatus

Electrochemical measurements were performed on a BAS-100B electrochemical analyzer (Bioanalytical Systems, USA) with a three-electrode system comprising a platinum wire as auxiliary electrode, a

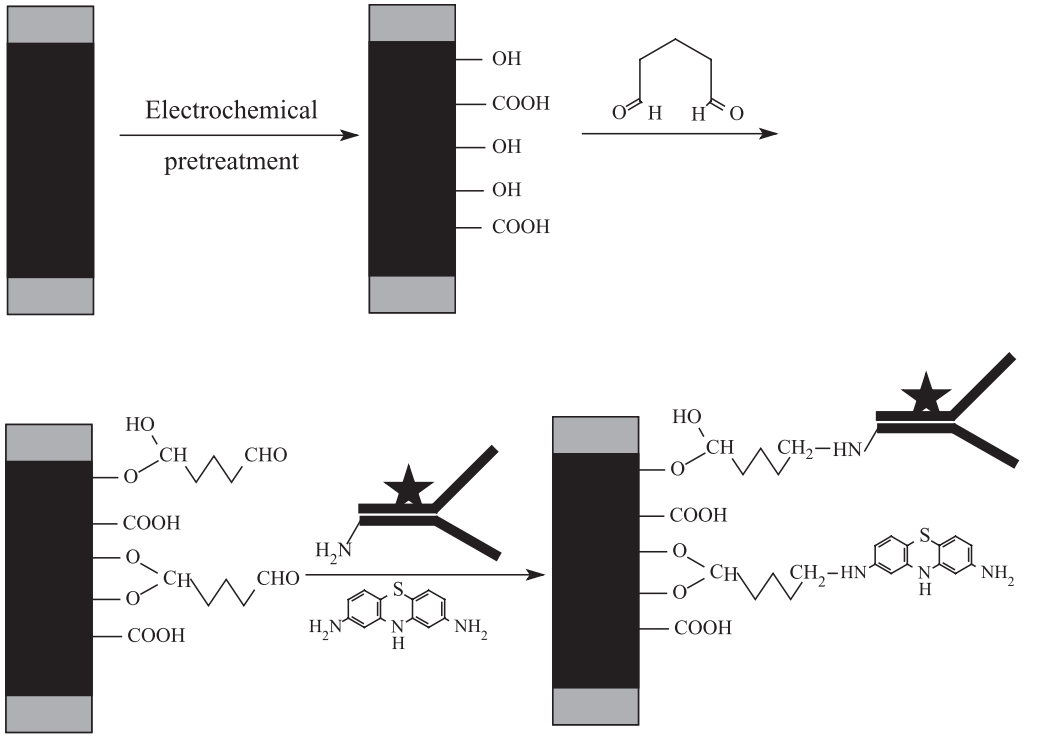

Fig. 1. Procedure for the preparation of the immunosensor.

saturated calomel electrode (SCE) as reference and a thionine/HRP-CEA antibody modified GCE as working electrode. The IRMA procedure was carried out with an FMJ-182 Immunoradiometric Gamma Counter (China) according to the instructions and assay procedures in the operator's manual.

Electrochemical measurements were done in an unstirred electrochemical cell at 20 ± 0.5 °C. All experimental solutions were bubbled thoroughly with high purity nitrogen for 5 min.

2.4. Procedure

The analytical procedure for the immunoassay was based on the inhibition of immunocomplex formation by electron transfer between HRP and thionine. The immunosensor was submitted to a potential of -300 mV in 5 ml anaerobic pH 7.0 PBS with or without the presence of H_2O_2 to record the amperometric response at 80 s. The immunoreaction was performed by incubating the immunosensor in CEA solution at 23 °C for 40 min. The detection of CEA level was performed by detecting the decrease of amperometric response of the immunosensor to H_2O_2 after the immunoreaction was performed.

3. Results and discussion

3.1. Electrochemical behavior of CEA immunosensor

Fig. 2 shows the cyclic voltammograms of different electrodes in 0.1 M pH 7.0 PBS. No peak was observed using both unpretreated and pretreated GCEs (curves a and b) in the potential range from 0 to -0.5 V. The electrochemical pretreatment results in a greater background current which arises from the formation of –OH and –COOH groups on the GCE surface (Wang et al., 2001). When the pretreated GCE is soaked in glutaraldehyde solution, glutaraldehyde molecules bind covalently with the –OH groups resulting from the formation of acetal or semiacetal. The glutaraldehyde modified GCE did not exhibit any redox peak (curve c) in 0.1 M pH 7.0 PBS. After soaking in the solution containing thionine and HRP-labeled CEA antibody for 5 h, a pair of stable and well-defined redox peaks appeared on the cyclic voltammogram obtained in 0.1 M pH 7.0 PBS (curve d). The anodic and cathodic peak potentials attributed to the redox of immobilized thionine were at -0.270 and -0.293 V (vs. SCE) at 50 mV/s, respectively. Both the anodic and cathodic peak currents were proportional to the scan rate in the range from 5 to 500 mV/s (Fig. 3), indicating a surface-controlled electrode process. From the peak-to-peak separation at different scan rates, an average electron transfer rate constant of 4.74 ± 2.99 s^{-1} was obtained (Laviron, 1979). The surface coverage of thionine was calculated from the peak areas of cyclic voltammograms to be $(3.21 \pm 1.62) \times 10^{-11}$ mol/cm^2.

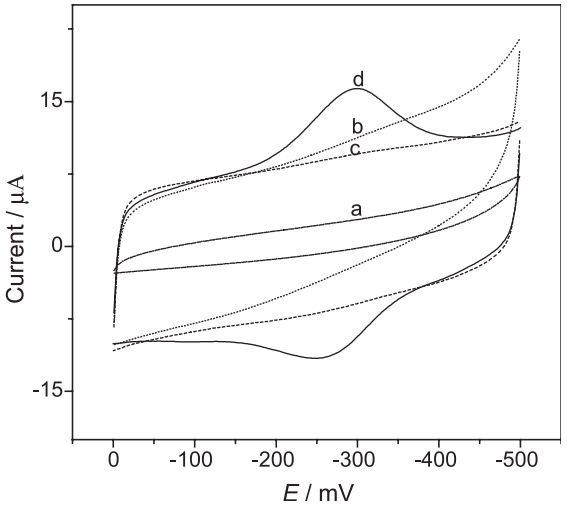

Fig. 2. Cyclic voltammograms of (a) unpretreated GCE, (b) electrochemical pretreated GCE, (c) glutaraldehyde modified GCE and (d) immunosensor in pH 7.0 PBS at 50 mV/s.

3.2. Cyclic voltammetric response of immunosensor to H_2O_2

It has been shown that HRP can catalyze the oxidation reaction of thionine by H_2O_2 (Ruan et al., 1998; Xiao et al., 1999). Using the thionine/HRP-CEA antibody modified GCE as an immunosensor, a pair of redox peaks was observed in pH 7.0 PBS (Fig. 4a). Upon addition of 4.5 mM H_2O_2 to the solution, the reduction peak current increased and the oxidation peak current decreased greatly, at the same time the reduction peak potential shifted slightly to a more negative value (Fig. 4b), suggesting an obvious electrocatalytic process. In contrast, no obvious change was

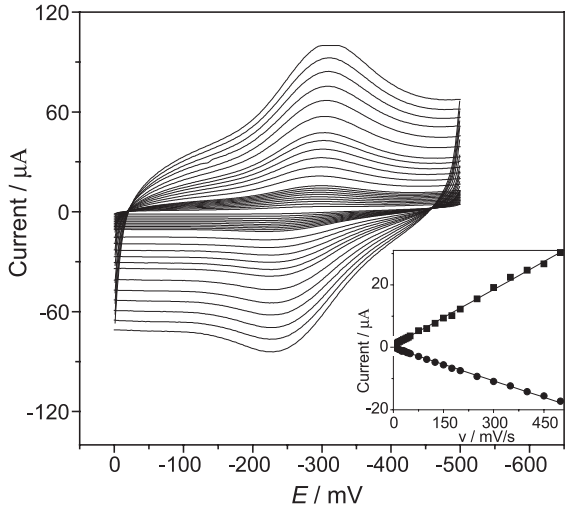

Fig. 3. Cyclic voltammograms of immunosensor in pH 7.0 PBS at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450 and 500 mV/s (from lowest to highest peak currents). Inset: Plots of peak current vs. scan rate.

observed with thionine modified GCE (inset in Fig. 4). Thus, the electrocatalytic reactions involved the participation of the HRP conjugated to immobilized CEA antibody. The mechanism for whole electrode process could be expressed as shown in Fig. 5 (Ruan et al., 1998). After the immunosensor was incubated with 300 ng/ml CEA solution, the electrocatalytic current decreased greatly, while no corresponding decrease was observed when the immunosensor was incubated with 0.04% BSA solution. Thus, the active center of the HRP for the catalytic oxidation of thionine was partly shielded due to the formation of immunocomplex.

3.3. Amperometric detection of CEA levels

The performance of the amperometric immunosensor is usually related to the incubation temperature and time, the pH value of the detection solution and the concentration of H_2O_2. When the incubation temperature increased from 12 to 33 °C the percentage decrease of the amperometric response increased and then reached a maximum value at an incubation temperature of 23 °C, which was selected as the incubation temperature in subsequent experiments. With an increasing incubation time, the percentage decrease increased and tended to a maximum value at 40 min. Thus, the optimal incubation time was 40 min. The acidity of the solution greatly affected the enzyme activity. The maximum peak current of the immunosensor in 0.1 M PBS containing 4.5 mM H_2O_2 occurred at pH 7.0, corresponding to maximum enzyme activity. Thus, the optimal pH value of the enzymatic reaction was pH 7.0 at which the amperometric response of the thionine/HRP-CEA antibody modified GCE to H_2O_2 displayed a curve characteristic of a Michaelis–Menten's mechanism. At H_2O_2 concentrations less than 4.5 mM, the amperometric response increased linearly with the increasing H_2O_2 concentration. When the H_2O_2 concentration was higher than 4.5 mM, the amperometric response tended to a constant value. Therefore, the optimal H_2O_2 concentration for the immunoassay of CEA levels was 4.5 mM.

Under optimal immunoassay conditions, the calibration graph for CEA determinations was shown in Fig. 6. The percentage decrease was proportional to the CEA concentration in two ranges from 0.5 to 3.0 and 3.0 to 167 ng/ml with linear slopes of 54.9 and 1.30 nA/ng/ml and correlation coefficients of 0.993 and 0.992, respectively. The detection limit was

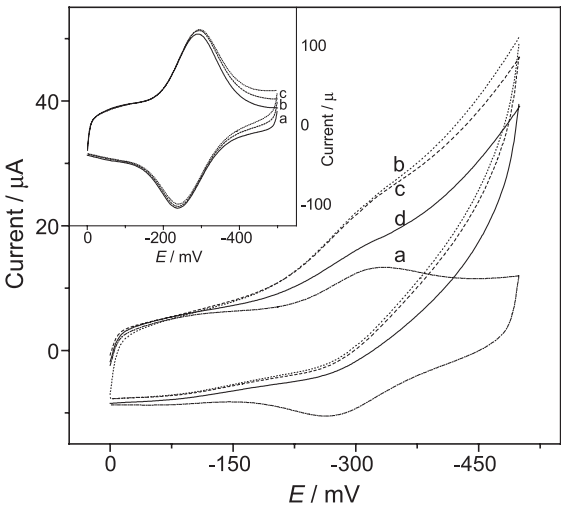

Fig. 4. Cyclic voltammetric responses of enzymatic reaction on immunosensor in (a) pH 7.0 PBS, (b) (a) + 4.5 mM H_2O_2, (c) (a) + 4.5 mM H_2O_2 after immunosensor was incubated with 0.04% BSA in pH 7.0 PBS and (d) (a) + 4.5 mM H_2O_2 after immunosensor incubated with 300 ng/ml CEA at 50 mV/s. Inset: Cyclic voltammetric response of thionine modified GCE in (a) pH 7.0 PBS, (b) (a) + 2.4 mM H_2O_2 and (c) (a) + 4.5 mM H_2O_2.

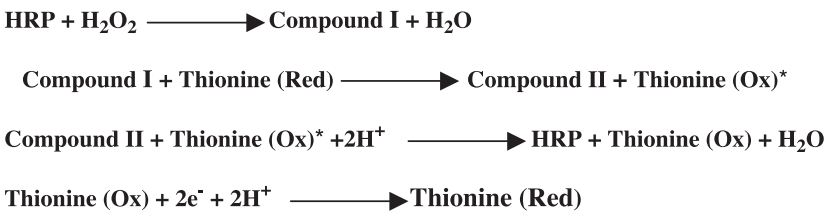

Fig. 5. The mechanism of enzymatic and electrode reactions.

calculated from the slope of 54.9 nA/ng/ml and three times the background noise to be 0.1 ng/ml, which was lower than that of traditional RIA and was almost the same as values characteristic of chemiluminescence immunoassays and fluoroimmunoassays. Thus, the present method could be used in the determination of CEA for clinical diagnosis.

3.4. Precision, reproducibility and stability of the immunosensor

Typical intra-assay and inter-assay precisions for the immunoassay of CEA were estimated from three determinations. The intra-assay coefficients of variation (CVs) of this method were 6.1% and 5.8% at CEA concentrations of 2.5 and 50 ng/ml, respectively, while the inter-assay CV on three immunosensors used independently was 6.3% at 50 ng/ml, indicating acceptable precision and fabrication reproducibility. The stability of this sensor was acceptable with a 10.1% decrease of amperometric response to 4.5 mM H_2O_2 in pH 7.0 PBS after 7 days of storage in pH 7.0 PBS at 4 °C. The present immunoassay method is thus suitable for the determination of CEA in human serum for routine clinical diagnosis.

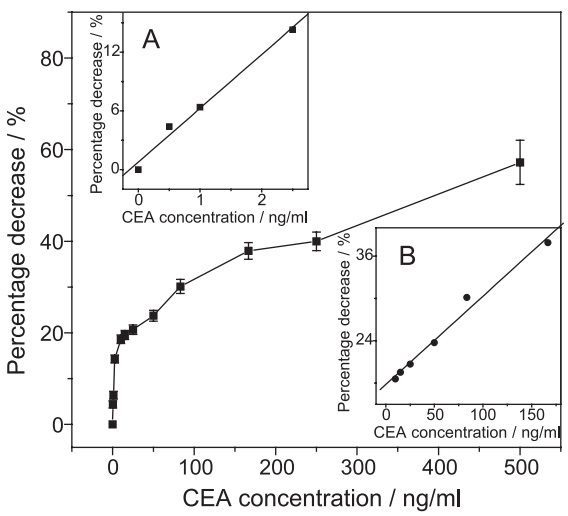

Fig. 6. Calibration curve for CEA determination. Inset: linear relationship between decrease percentage of amperometric response and CEA concentration in ranges of (A) 0.5–3 and (B) 3–167 ng/ml.

3.5. Accuracy and clinical application

The accuracy of the CEA determination was examined by comparison of the results obtained by this method with those from IRMA. The CEA contents in two sera were quantified with both techniques. The mean CEA concentrations obtained by this method in three determinations were 11.7 and 34.2 ng/ml, while the values obtained from IRMA were 10.2 and 35.8 ng/ml, producing relative deviations of 14.7% and −4.5%, respectively. These results of both methods were thus in good agreement.

4. Conclusions

Prepared by co-immobilizing thionine and HRP-labeled CEA antibody on GCE, a novel amperometric immunosensor for the rapid determination of CEA in human serum has been developed. Based on the inhibition of the electron transfer between the activity center of the HRP and immobilized thionine after incubation with CEA solution, the immunosensor directly detects CEA concentrations in the incubation solution without the addition of electron transfer mediator or enzyme-labeled antibody. The immunosensor shows good accuracy and acceptable precision, reproducibility and storage stability. The proposed method could be valuable for clinical immunoassays

and could be extended readily to the preparation of other amperometric immunosensors and the detection of other clinically important antigens.

Acknowledgements

This project was supported by the National Natural Science Foundation of China (No. 20275017, 90206037), the Specialized Research Funds for Excellent Young Teachers from the Chinese Ministry of Education, the Science Foundation of Jiangsu (No.BS2001063) and the Key Project of the Cancer Institute of Jiangsu Province.

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