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RESEARCH ARTICLE

IMMUNOCHROMATOGRAPHIC STRIP BIOSENSOR APPLICATION FOR EARLY DIAGNOSIS OF CANCER BY URINARY CARCINOEMBRYONIC ANTIGEN

***Nermin A. El-Morshedi**

Assistant Professor of Histology, Cell Biology and Genetics-Health Administration Affairs, Ministry of Health, Egypt

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Carcinoembryonic antigen (CEA),
Gold Nanoparticles (Au-NP),
Immunochromatographic strip tests (ISTs).

ABSTRACT

Carcinoembryonic antigen (CEA) is an oncofoetal cell-surface glycoprotein that serves as an important tumor marker for different types of carcinoma. Combining of gold nanoparticle (Au-NP) and antibody-antigen specific molecular recognition was developed for CEA detection specifically as tumor biomarker in human urine. Our test was based on sandwich type immunoreaction principle on the lateral flow test strip as immunochromatographic strip tests (ISTs). We hypothesized that serum CEA level elevation in different cancer types patients reflected in high urine CEA levels also. Urine and serum CEA levels were determined by electrochemiluminescence (ECLIA) sandwich immunoassay test in 308 cancer patients (28 individual for each cancer type group; which subdivided into 4 tumor stages, each stage n=7). Studied Cancer types included: urinary bladder carcinoma, breast cancer, colorectal cancer, lung cancer, hepatome, prostate cancer, uterus cancer, spleen cancer, pancreatic cancer, gastrointestinal carcinoma, ovarian carcinoma and 100 normal control cases (50 male and 50 female). All urinary patients samples were applied by ISTs to detect ISTs ability in different cancer types early diagnosis. A significant difference was detected in serum and urine CEA levels respectively in all patients of the first and second stage compared to control cases, but a high significant difference was detected in serum and urine CEA levels respectively in all patients of the third and fourth stage compared to control cases. There was a definite relationship between serum CEA, urine CEA and patients malignancy stage; the higher stage, the higher serum and urine CEA levels and vice versa. ISTs showed red band test zone which its color intensity was proportionally to the patients malignancy stage, and agreed with patient urinary CEA level. In conclusion, urinary CEA is more useful in early different cancers types detection and its level is also correlated with tumor stage. ISTs thus provides a rapid, sensitive, low cost, individually usage clinical diagnosis tool for the detection of cancer by protein biomarkers (CEA) in human biological samples.

INTRODUCTION

Carcinoembryonic antigen (CEA), a glycoprotein first described in 1965 by Gold and Freedman, that contains about 60% carbohydrates. CEA was originally thought to be specific product of neoplasia derived from the body endoderm (Jezerse *et al*., 1996). So it is one of the most widely used tumor makers, and is used in the clinical diagnosis of urinary bladder cancer (Saied *et al*., 2007), breast cancer (Sahin *et al*., 1996), colon cancer (S. Hammarstrom *et al*., 1989), prostate cancer (K. Nagao *et al*., 2002), uterus cancer (Hall *et al*., 1973), spleen cancer (Hall *et al*., 1972), pancreatic cancer (Neville *et al*., 1973), gastrointestinal carcinoma (Kleisbauer *et al*., 1996), lung cancer (Alsabti and Kamel, 1979), hepatoma (Turner *et al*., 1977) and ovarian carcinoma (Kazuya *et al*., 1999). CEA upper normal range in nonsmoker serum 2.5ng/ml and urine is 3ng/ml, but its normal range in adult smoker is less than 3ng/ml and in urine is less than 3.5ng/ml (Kato *et al*., 2004), and its level exceeds 100ng/ml upon cancer development (Holyoke *et al*., 1972). A rising CEA level indicates progression or cancer recurrence, which can be used to diagnose and monitor cancer at its early stage. The techniques used for quantitative determination of tumor markers are immunological methods, which have become the predominant analytical techniques in the fields of clinical diagnoses studies including: radioimmunoassay, fluoroimmunoassay, chemiluminescence immunoassay and enzyme-linked immunosorbent assays (ELISAs) (Alumanda and Minoru, 1995). Although these conventional strategies provide accurate, sensitive detection of CEA (Yuan *et al*., 2001), there are still some inconveniences exist, as radioactive substances utilization, time-consuming sample purification,

****Corresponding author: Nermin A. El-Morshedi,***
Assistant Professor of Histology, Cell Biology and Genetics-Health Administration Affairs, Ministry of Health, Egypt.

incubation, washing steps before analysis and enzymatic reactions, technical expertise as well as the specialized equipment (Blackburn *et al*., 1991). Immunosensor is an alternative tool to replace CEA detection by traditional immunoassay (P. Aguilar *et al*., 2002), and have simplified the operations, shortened assay time, and provided a good sensitivity, but their applications stay in the laboratory-development level and have not been widely used for clinical diagnosis (Limbut *et al*., 2006).

Immunochromatographic strip tests (ISTs) are simple, rapid, in-field and cheap assays and their results providing simple qualititative detection without the need of skilled personnel (Cui *et al*., 2000). In the present study, IST principle relies on test samples migration and antibody-gold nanoparticle (Ab-Au-NP) conjugates along membrane strips on which the binding interactions take place (Fernandez-Sanchez *et al*, 2005). We present a simple and convenient diagnosis tool based on the lateral flow technology for detection of CEA in human urine as early cancer diagnosis.

MATERIALS AND METHODS

Apparatus: Airjet AJQ 3000 dispenser, Biojet BJQ 3000 dispenser, Clamshell Laminator and Guillotine cutting module CM 4000 were from Biodot LTD (Irvine, CA) which used for CEA strip biosensor preparation.

Reagents: CEA, polyclonal CEA antibody (poly-anti-CEA), monoclonal CEA antibody (anti-CEA, clone #:M111147) were purchased from Fitzgerald (USA); Goat anti-mouse IgG, rabbit IgG and human IgM were purchased from Thermo scientific; $Na_3PO_4 \cdot 12H_2O$, $HAuCl_4$, trisodium citrate, sucrose, Tween 20, sodium chloride-sodium citrate buffer (pH 7.0), phosphate buffer saline (PH 7.4, 0.01 M), bovine serum albumin were purchased from Sigma-Aldrich. Glass fibers (GFCP000800), cellulose fiber sample pads (CFSP001700), laminated cards (HF000MC100) and nitrocellulose membranes (HFB18004) were purchased from Millipore (Billerica, MA). All chemicals used in this study were analytical reagent grade. All other solutions were prepared with ultrapure (>18 MΩ) water from Millipore Milli-Q water purification system (Billerica, MA). Human urine samples were collected from National Cancer Institute (Cairo, Egypt).

Preparation of Gold Nanoparticles (Au-NP) and Au-NP-anti-CEA Conjugates: Au-NP with average diameter 20nm± 3.5nm used in this work were prepared according to citrate reduction of $HAuCl_4$ (K. Grabar *et al*., 1995). All glassware used in this preparation was thoroughly cleaned in aqua regia (3 parts HCl, 1 part HNO_3), rinsed with double distilled H_2O, and oven-dried prior to use. 250ml aqueous solution of 0.01% $HAuCl_4$ was added to 500ml round-bottom flask and heated to boiling with vigorously stirring, and then added 4.5ml of 1% trisodium citrate to this solution quickly. The solution turned deep blue within 20sec and finally changed to wine-red 60sec later. Boiling was continued for an additional 10min, the heating source was removed, and the colloid was stirred for another 15min. The colloids solution were stored in dark bottles at 4^{o}C and were used to prepare Au-NP-anti-CEA conjugate. The conjugation was carried out by adding 50µl of 4mg/ml anti-CEA monoclonal Ab to 1ml of 5-fold concentrated Au-NP solution (pH 8.2) followed by incubation at room temperature with periodic gentle mixing for 1h. Then certain volume of 10% bovine serum albumin was slowly added to the mixture solution to a final concentration of 1%. After gentle stirring for 30min, the solution was centrifuged at 13,000×g for 15min. Two phases can be obtained: a clear to pink supernatant of unbound antibodies and a dark red loosely packed sediment of Au-NP-anti-CEA conjugates. The supernatant was discarded and the soft sediment of Au-NP-anti-CEA conjugates was rinsed by re-suspending in 1ml of phosphate buffer saline/bovine serum albumin and collected after a second centrifugation at 13000×g/15min. Finally, conjugate was re-suspended in 1ml of buffer containing 20mM sodium phosphate, 0.25% Tween-20, 10% sucrose and 5% bovine serum albumin (G. Frens, 1973).

Preparation of CEA Strip Biosensors: CEA strip biosensor schematic diagram is shown in Figure 1. Briefly, the biosensor consists of four components: sample application pad, Au-NP-anti-CEA conjugate pad, nitrocellulose membrane and absorbent pad. The sample application pad (17mm×30cm) was made from cellulose fiber (CFSP001700, Millipore) and saturated with a buffer (pH 8.0) containing 0.25% TritonX-100, 0.05M Tris-HCl and 0.15mM NaCl. Then it was dried and stored in desiccators at room temperature. The conjugate pad (8mm×30cm) was prepared by dispensing a desired volume of Au-NP-anti-CEA conjugate solution onto the glass fiber with the dispenser Airjet AJQ 3000, and then drying it in oven at 35^{o}C and stored in desiccators at 4^{o}C. Test zone and control zone at the nitrocellulose membrane (25mm×30mm) was prepared by dispensing poly-anti-CEA and goat anti-mouse IgG with Biojet BJQ 3000, respectively. The distance between two zones is around 0.2cm. The membrane was dried at room temperature for 1h and stored at 4°C. Finally, all of the parts were assembled on a plastic adhesive backing layer (typically an inert plastic, e.g., polyester) using the Clamshell Laminator (BioDot, CA, USA). Each part overlapped 2mm to ensure the solution migrating through the strip during the assay. ISTs with 4.7mm width were cut by using Guillotin cutting module CM 4000.

Sample assay Procedure: during optimizing experimental parameters of the biosensor, 150µl (3 drops) of sample solution containing a desired concentration (e.g. 200:250mg/ml) of urinary CEA (prepared in phosphate buffer saline+1% bovine serum albumin buffer) was added onto the sample application pad. After waiting for 5min, 50µl (1 drop) of buffer (phosphate buffer saline+1% bovine serum albumin buffer) was applied to the strip. Both the test zone and control zone were visualized within 10min. The optical intensities of the bands are proportional to the amount of Au-NPs, then CEA concentration in the sample solutions.

Clinical Materials: We have prospectively collected blood and urine of 308 cancer patients (National Cancer Institute, Cairo, Egypt) (28 individual for each cancer type group; which subdivided into 4 tumor stages, each stage n=7). Studied Cancer types included: urinary bladder carcinoma, breast cancer, colorectal cancer, lung cancer, hepatome, prostate cancer, uterus cancer, spleen cancer, pancreatic cancer, gastrointestinal carcinoma, ovarian carcinoma and 100 normal control cases (50 male and 50 female) as control group to evaluate CEA detect ability by ISTs and its normal levels in healthy volunteers.

Methods: Samples (serum and urine) were analyzed for their CEA levels using commercial assays (National Cancer Institute, Egypt) that were ran on immunochemistry analyzer (COBAS E411, Roche diagnostics, Mannheim, Germany). Method of detection is based on an electrochemiluminescence (ECLIA) sandwich immunoassay (R. Ben-Ishay *et al*., 2016).

Statistical Methods: Statistical analysis was performed as means±standard deviations (S.D). Analysis of variance for two variables (Two Way-ANOVA) was used and Student t-test. Significant analysis of variance results were subjected to post hoc Tukey's test (R. Fisher, 1970). Statistical significance was set at $p < 0.05$ and high significance was set at $p \leq 0.01$.

spleen cancer, pancreatic cancer, gastrointestinal carcinoma, ovarian carcinoma and 100 normal control cases (50 male and 50 female). A significant difference was detected in serum and urine CEA levels respectively in all patients of the first and second stage compared to control cases, but a high significant difference was detected in serum and urine CEA levels respectively in all patients of the third and fourth stage compared to control cases. Median levels of serum and urine CEA of healthy controls were normal and median serum and urine CEA levels of cancer patients were higher in all patients which was increased with patient tumor stage increasing (Table 1).

Table 1. CEA levels in serum and urine (ng/ml) of all cancer patients groups (each group n=28) and each group divided into 4 tumor stages (each stage n= 7) and healthy (control) group (50 male and 50 female). Values expressed as means ± standard deviation. (*): Significance; $0.01 \leq P \leq 0.05$; (**): high significance; $p \leq 0.01$. UB.C.: urinary bladder carcinoma, B.C.: breast cancer, C.C.: colorectal cancer, L.C.: lung cancer, H.: hepatome, Pr.C.: prostate cancer, U.C.: uterus cancer, S.C.: spleen cancer, Pc.C.: pancreatic cancer, GIT.C.: gastrointestinal carcinoma, O.C.: ovarian carcinoma M: male and F: female

			Pr.C.	GIT.C.	UB.C.	B.C.	O.C.	C.C.	L.C.	H.	S.C.	Pc.C.	U.C.
Patient	Serum	S1	18	14	16	11	15	14	11	12	13	12	16
	ng/ml		±0.54*	±0.42*	±0.48*	±0.33*	±0.45*	±0.42*	±0.36*	±0.38*	±0.41*	±0.37*	±0.47*
		S2	38	26	40	23	32	28	24	27	25	26	22
			±0.56*	±0.39*	±0.61*	±0.35*	±0.48*	±0.42*	±0.36*	±0.41*	±0.37*	±0.39*	±0.33*
		S3	78	85	83	63	68	88	61	74	76	75	77
			±0.62**	±0.67**	±0.66**	±0.51**	±0.54**	±0.69**	±0.48**	±0.59**	±0.6**	±0.59**	±0.61**
		S4	145	151	157		139	142	125	138	132	133	146
			±0.91**	±0.94**	±0.98**		±0.87**	±0.88**	±0.78**	±0.86**	±0.82**	±0.84**	±0.91**
	Urine	S1	21	22	25	15	24	18	13	23	26	27	29
	ng/ml		±0.63*	±0.67*	±0.75*	±0.45*	±0.72*	±0.54*	±0.39*	±0.69*	±0.74*	±0.81*	±0.87*
		S2	56	62	60	45	52	54	42	63	58	55	61
			±0.85*	±0.94*	±0.91*	±0.68*	±0.78*	±0.82*	±0.64*	±0.95*	±0.88*	±84*	±0.92*
		S3	165	155	162	138	149	137	131	153	135	132	157
			±1.31**	±1.23**	±1.28**	±1.09**	±1.18**	±1.08**	±1.04**	±1.21**	±1.07**	±1.05**	±1.24**
		S4	235	228	242	218	230	223	215	232	226	220	238
			±1.46**	±1.42**	±1.51**	±1.36**	±1.46**	±1.39**	±1.34**	±1.45**	±1.41**	±1.38**	±1.49**
Control	Serum	M						0.68±0.2					
	ng/ml	F						0.64±0.019					
	Urine	M						0.72±0.21					
	ng/ml	F						0.76±0.23					

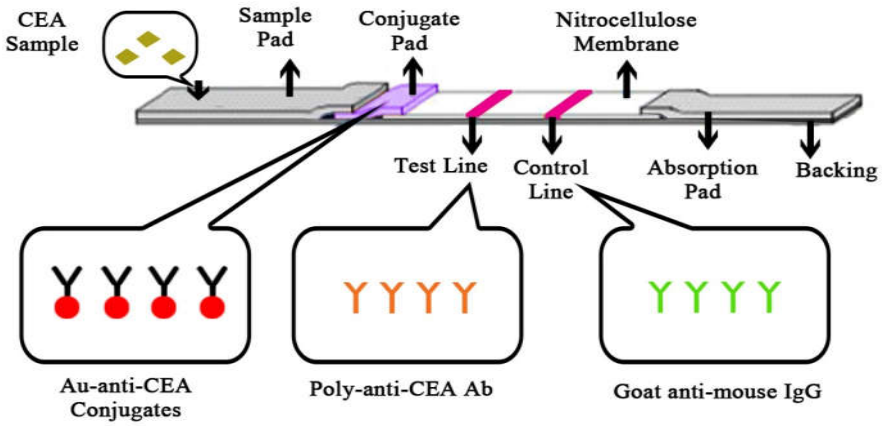

Figure 1. Schematic illustration of CEA strip biosensor

RESULTS

In our study, serum and urine of patients in all cancer types groups and all stages had detectable serum and urine levels of CEA in those body fluids which was feasible (Table 1). Urine and serum CEA levels were determined in 308 cancer patients (28 individual for each cancer type group; which subdivided into 4 tumor stages, each stage n=7). Studied Cancer types included: urinary bladder carcinoma, breast cancer, colorectal cancer, lung cancer, hepatome, prostate cancer, uterus cancer,

All patient's groups showed a positive linear correlation between CEA serum levels and urinary CEA levels, with patient tumor stage. On the other hand, when we applied the urinary CEA samples of all patients individually to ISTs, red band on test zone was appeared and its color intensity was proportionally to analytical results (regarding CEA the lower limit of detection is 0.5ng/ml).

DISCUSSION

Diagnosis at an early stage is important for successful therapeutic intervention and are essential for cancer patient

recovery. Monitoring cancer biomarkers in blood, urine and other body fluids is an important method for early detection, as allow for identification of the disease at its very early stage, even before its symptoms can be recognized by a patient. Here, we analyzed urinary and serum CEA tumor marker in different types of cancer patients, and compared the results with the impacted red band appearance of ISTs to ensure its sensitive as an easy and fast biosensor for CEA tumor biomarker.

Principle of CEA Measurement in the Strip Biosensor: The biosensor principle is based on sandwich-type immunoreactions in the lateral flow test strip (Z. Qingxiang *et al*., 2009) (Figure 2). Ploy-anti-CEA Ab and goat anti-mouse IgG solutions were dispensed on different locations of nitrocellulose membrane to form test zone and control zone, respectively. Au-NPs were used as tags to label monoclonal anti-CEA Ab, the resulting Au-NP-anti-CEA conjugates were dispensed onto the glass fiber as conjugate pad. A sample solution containing CEA desired concentration was applied to sample application pad. The solution migrates along the strip by capillary force and rehydrates Au-NP-anti-CEA in conjugate pad. Then immunoreactions between CEA and Au-NP-anti-CEA conjugates occurred and the formed Au-NP-anti-CEA-CEA complexes continue to migrate along the strip. When reached test zone, complexes were captured by poly-anti-CEA Ab immobilized on test zone via secondary immunoreactions between poly-anti-CEA Ab and CEA. A characteristic red band could be observed because of Au-NPs accumulation on test zone. The capillary action caused liquid sample to migrate further. Once the solution passed through control zone, the excess Au-NP-anti-CEA conjugates were captured on control zone via binding between goat anti-mouse IgG antibody (pre-immobilized on control zone) and anti-CEA Ab, thus forming a second red band. In CEA absence only red band is observed in control zone and no red band is observed in test zone. In this case, the red band in control zone (control line) shows that the biosensor is working properly. Qualitative analysis is simply performed by observing test zone color change. Red band intensity is proportional to captured Au-NPs amount in test zone, which is proportional to CEA concentration in sample solution, and then conflict the patient tumor stage.

Optimization of Parameters

- Sandwich-type immunoreactions were performed on lateral flow test strip biosensor, immunoreaction time, which depends on buffer migration time in the nitrocellulose membrane, plays important role for biosensor sensitivity. HFB18004 nitrocellulose membrane (Millipore) was used to biosensor preparation, as its migration time is 3min and assay time was 10min (Liu *et al*., 2007).
- Biosensor response is relevant to poly-anti-CEA Ab amount immobilized on test zone. If its concentration from commercial vendor is 2.4mg/ml, so S/N ratio of biosensor increased with dilution times increasing from 2 to 6 times, further dilution led to S/N decreasing ratio. There are two factors resulted low S/N ratio at high concentration of poly-anti-CEA Ab on test zone: (1) High Ab concentration in test zone caused nonspecific adsorption of conjugates, resulting a high background signal; (2) Ab excess amount may increase stereo-hindrance effect which may decreasing immunoreactions efficiency (Mao *et al*., 2008).
- Produced red bands intensities in test and control zone depend on Au-NP-anti-CEA conjugates amount captured on them, which corresponds to conjugates amount on conjugate pad. Here, conjugates amount on the pad was controlled by dispensing volumes of conjugate solution, by dispensing the conjugate solution with various cycles on conjugate pad. Maximum S/N was obtained with 4 dispensing times on conjugate pad. So, 4 dispensing times was selected as optimal dispensing time for most of experiments (Freeman *et al*., 1999).
- Running buffer components affected biosensor response greatly. Appropriate buffer exhibited the best performance, minimize nonspecific adsorption, increase biosensor sensitivity and reproducibility is Phosphate buffer saline+1% bovine serum albumin (Kamin, 1973).

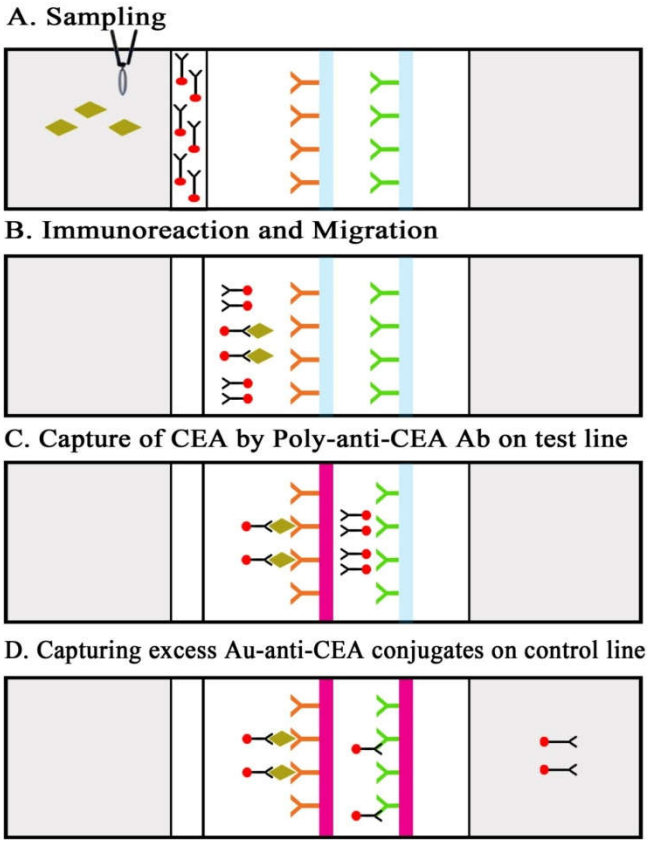

Figure 2. Principle of detection of CEA in the strip biosensor

Conclusion and Recommendation

We have successfully developed an immunochromatographic strip biosensor for rapid, low-cost, individually usage of urinary CEA detection. This strip biosensor shows great promise for point-of-care or in-field of early detection of cancer protein biomarkers. It is available for easy individual usage. ISTs assay time (10min) is shorter than laboratory analytical tests (mainly not less 1.5h). Future work will aim to improve ISTs sensitivity by developing enzyme and Au-NP dual labels based ISTs, to detect multiple protein biomarkers simultaneously with ISTs array.

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