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Research Paper

Rapid and sensitive immunomagnetic-electrochemiluminescent detection of p53 antibodies in human serum

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Abstract

The mutation of tumor suppressor p53 gene is common in malignant tumor. p53 antibodies are products of immunoresponse against abnormal p53 protein. It has been found that p53 antibodies are of importance in tumor's diagnosis, prognosis and relapse monitoring. However, current method for detecting p53 antibodies, i.e. enzyme-linked immunosorbent assay (ELISA), requires a long time with multiple steps, and the assay is only semi-quantitative. In this work, a protocol for quantitative detection of p53 antibodies in human serum using immunomagnetic electrochemiluminescence (IM-ECL) was devoloped. The immunoassay format consisted of a three antibody sandwich in which a biotinylated capture antibody, was banded with the commercial p53 protein. A detector antibody was added to bind the p53 protein at another site. Then, secondary antibody, labeled with ruthenium(II) tris-bipyridal, was added and, when bound to the bead immunocompiex, generated light in the presence of an excess of tripropylamine. The light was detected and measured by the analyzer made by us. Our experimental results indicate that the sensitivity of this assay was 10 pg of p53 antibodies per ml of reference serum (normal human serum). A stable calibration curve with a wide dynamic range was established. The calibration curve was linear from 0.01 to 1000 ng/ml, thus, making quantitation possible. An immunologic prozone effect was observed above 1000 ng p53 antibodies per milliliter of serum. Serum samples from lung and nasopharyngeal carcinoma patients were tested using the IM-ECL assay. The positive rate of p53 antibodies were 28.6% in lung carcinoma and 8.33% in nasopharyngeal carcinoma, respectively. p53 antibody concentration in the carcerous human sera were quantified from the calibration curve. In the case of lung carcinoma, a trend was found that a higher p53 antibody concentration in the serum was likely linked to a higher stage of the cancer. This trend was not found in nasopharyngeal carcinoma. The assay uses only 50 μl of sample per test and requires a 30-min incubation period in addition to a 50 s acquisition time. This assay has several advantages over the commonly used ELISA method in terms of sensitivity, linear range, and assay time. Results of the study suggest that IM-ECL is a feasible method for rapid and sensitive detection of p53 antibodies in human serum.

Keywords: Electrochemiluminescence; Immunomagnetic; p53; Serum antibody

Abbreviations: ECL, electrochemiluminescence; ELISA, enzyme-linked immunosorbent assay; IM-ECL, immunomagnetic electrochemiluminescence; IMS, Immunomagnetic separation; $Ru(bpy)_3^{2+}$, ruthenium(II) tris-bipyridal; TPA, tripropylamine; biotin-NHS ester, N-hydroxysuccinimidobiotin; $Ru(bpy)_3^{2+}$-NHS ester, $Ru(bpy)_3^{2+}$ N-hydroxysuccinimide ester; PMT, photon multiplier tube; cps, counts per second.

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1. Introduction

The p53 tumor suppressor is of special interest to the oncologic diagnosis. The p53 gene is altered in about 50% of human cancers (Hollstein et al., 1991). The most common forms of p53 gene alterations are missense point mutations (Soussi, 1996). This leads to abnormal p53 protein accumulation inside the nucleus of the tumor cells. Some patients develop an immunoresponse against p53 protein. p53 antibodies in the serum, detected with enzyme-linked immunosorbent assay (ELISA), correlate with a detectable p53 mutation in the tumor (Wild et al., 1995). Although the production mechanism is unclear, serum p53 antibodies are an indirect result of p53 gene missense point mutations and independent of exact positions of the mutation on the p53 gene.

Several studies have described the detection of antibodies against p53 protein in the sera of patients with various malignant diseases (Gadducci et al., 1996; Angelopoulou and Diamandis, 1997; Volkmann et al., 1993; Galle and Volkmann, 1994). p53 gene alterations can be found very early during the course of oncogenesis (Bennet et al., 1993; Shin et al., 1994). In patients with hepatocellular carcinoma p53 antibodies were found independent of the alpha-fetoprotein (AFP) status (Volkmann et al., 1993). p53 antibodies are also detected in asymptomatic patients (Raedle et al., 1995; Ryder et al., 1996).

The p53 antibody status is an independent diagnostic marker. In cancer patients, temporal changes of p53 antibody titer can be correlated with disease progression or regression (Angelopoulou et al., 1994). The presence of such antibodies is often associated with more aggressive tumors. The antibody status is also an independent prognosis marker (Houbiers et al., 1995). These antibodies were more frequently present in patients who suffered relapse after surgery, subsequently, reduced disease-free survival.

The most common technique for the detection of p53 antibodies is ELISA, which is available in commercial kits. It requires multiple steps and long incubation periods ($\geqslant$1 h per step). Furthermore, the assay is semi-quantitative.

Immunomagnetic separation (IMS) and electrochemiluminesence (ECL) have been developed recently (Yu and Bruno, 1996; Kijek et al., 2000; Gatto-Menking et al., 1995; Shelton and Karns, 2001). IMS offers the advantage of antigen or antibody capture from a variety of complex biological matrices. ECL detection has an increased sensitivity over other chemiluminescece techniques. It is initiated by a voltage potential, thus, can be better controlled. IM-ECL has been used in analyzing many kinds of antigen, antibody and hapten, such as carcinoembryonic antigen and alpha-fetoprotein, etc (Kijek et al., 2000; Gatto-Menking et al., 1995; Shelton and Karns, 2001; Blackburn et al., 1991; Yang et al., 1994). ECL detection is accomplished by ruthenium(II) tris-bipyridal ($Ru(bpy)_3^{2+}$) conjugated to an antibody (Blackburn et al., 1991). Initially, $Ru(bpy)_3^{2+}$ and tripropylamine (TPA) are oxidized at the surface of an anode. $TPA^{+\cdot}$ immediately loses a proton and becomes a powerful reducer. When $TPA^{\cdot}$ and $Ru(bpy)_3^{3+}$ react, the latter enters an excited state by a high energy electron transfer from the electron carrier, $TPA^{\cdot}$. Relaxation of $Ru(bpy)_3^{2+}$* to the ground state results in a light emission, detectable at 620 nm. $Ru(bpy)_3^{2+}$ is not consumed during the reaction and may be oxidized and excited again if there is excessive TPA used in the buffer.

We hypothesize that IM-ECL will provide a more efficient and quantitative assay for p53 antibody detection. To test this hypothesis, we used IM-ECL to detect an extremely low concentration p53 antibodies introduced in normal human sera. The calibrated assay was then used to quantitatively evaluate the presence of p53 antibodies in cancerous human sera that was determined with conventional diagnosis.

2. Materials and methods

2.1. Materials

$Ru(bpy)_3Cl_2 6H_2O$, N-hydroxysuccinimidobiotin (biotin-NHS ester), TPA and the chemicals to synthesize the $Ru(bpy)_3^{2+}$ N-hydroxysuccinimide ester ($Ru(bpy)_3^{2+}$-NHS ester) were purchased from Sigma (Louis, MO, USA). Streptavidin microbeads (2.8 μm diameter) and beads separation device (Dynal MPC-1) were products of Dynal Biotech Company (Lake Success, NY, USA). Mouse anti human p53 monoclonal antibodies and rabbit anti human p53 polyclonal antibodies were purchased from DAKO

(Copenhagen, Danmark) and Serotec (Oxford, UK), respectively. Secondary antibodies (goat anti rabbit IgG and rabbit anti human IgG) were products of SABC (Luoyang, Henan). Recombinant human p53 protein was purchased from BD (San Diego, CA). All buffer solutions were made of tri-distilled water, 0.01M phosphate-buffered saline (PBS, pH 7.4). Human sera were obtained from Cancer Hospital of Sun Yat-sen University, Guangzhou, China.

2.2. Labeling of antibodies and protein

The chemosynthesis of the $Ru(bpy)_3^{2+}$-NHS ester is described by Terpetschnig's paper (Terpetschnig et al., 1995). The assay procedure is illustrated in Fig. 1. Firstly, a biotinylated capture antibody was banded with the commercial p53 protein (shown as A). Then, a detector antibody was added to bind the p53 protein at another site (shown as B). At last, secondary antibody, labeled with ruthenium(II) tris-bipyridal, was added and, when bound to the bead immunocompiex, generated light in the presence of an excess of tripropylamine (shown as C). p53 antibodies (mouse monoclonal antibody, DAKO) were labeled with biotin-NHS at a 1:10 molar ratio for 1 h at room temperature. Secondary antibodies were labeled with fifty-fold excess of $Ru(bpy)_3^{2+}$-NHS ester (Terpetschnig et al., 1995). Microcon centrifugal filter devices (Millipore, Watertown, MA, USA) were used to remove the unbound biotin and $Ru(bpy)_3^{2+}$-NHS ester. Biotin labeled p53 antibodies were pre-bound to Streptavidin microbeads (10 μg antibodies per mg of beads). Beads and antibodies were incubated together, with agitation, for 1 h at room temperature. Unbound antibodies were removed by washing the antibody-coated beads twice with PBS containing 1.5% Tween-20 and twice with PBS in the beads separation device MPC-1. Beads were reconstituted with PBS. Then, the beads were incubated with recombinant human p53 protein for 30 min with agitation at room temperature. Unbound protein was removed by washing the beads with PBS again. These labeled antibodies and protein were stored at 4 °C until used in the assay.

2.3. ECL detection

The assay reaction system was developed in our laboratory. Fig. 2 is a diagram of the essential components of the instrument. The heart of the system is the electrochemical reaction cell, containing a working electrode, a counter electrode and an Ag/AgCl reference electrode. The working electrode (disk) and the counter electrode (ring) are made of platinum. Photon emission from the ECL reaction is collected by an optical fiber-bundle then detected by a single photon multiplier tube (PMT, MP-962, Perkinelmer, Wiesbaden, Gemany). The signal from the PMT is amplified and discriminated. The output Transistor–Transistor Logic (TTL) pulses are con-

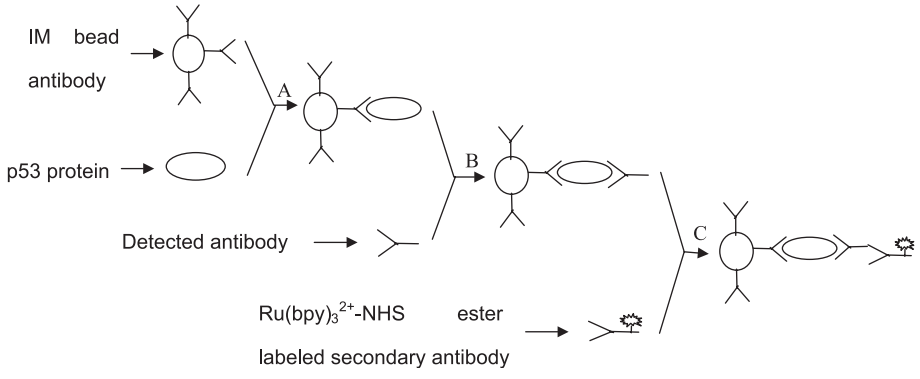

Fig. 1. Diagram of assay procedure. Firstly, a biotinylated capture antibody was banded with the commercial p53 protein (shown as A). Then, a detector antibody was added to bind the p53 protein at another site (shown as B). At last, secondary antibody, labeled with ruthenium(II) tris-bipyridal, was added and, when bound to the bead immunocompiex, generated light in the presence of an excess of tripropylamine (shown as C).

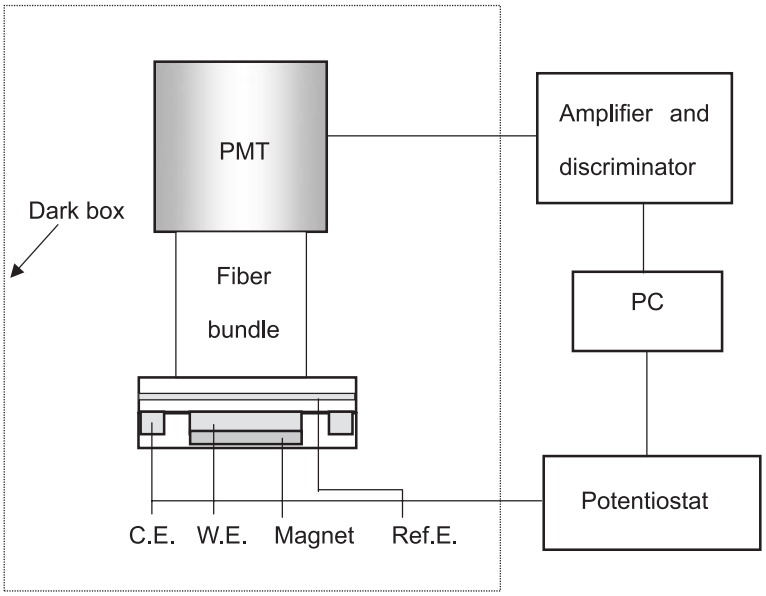

Fig. 2. Diagram of the essential components of IM-ECL assay system. C.E.: count electrode; W.E.: working electrode; Ref.E.: reference electrode. The heart of the system is the electrochemical reaction cell containing a W.E., a C.E. and a Ref.E. Photon emission from the ECL reaction is collected by an optical fiber-bundle then detected by a single photon multiplier tube.

verted with a multi-function acquisition card (PCL-836, Advantech, Taiwan) and analyzed with Labview software. A computer controls the signal collection and a potentiostat (HDV-7C, Sanming, Fujian) to supply voltage to the electrodes. The detection limit of the analyzer is 10^{-18} mol/l $Ru(bpy)_3Cl_26H_2O$. The dynamic range extends over five orders of magnitude, from 0.1 pmol/l to 10 nmol/l. These specifications are established in a present research (unpublished data).

Pre-prepared protein coated beads (36 μg/tube), as described in Section 2.2, and 50 μl sera sample were incubated for 15 min with agitation at room temperature. The beads were washed twice with PBS. $Ru(bpy)_3^{2+}$-NHS ester labeled secondary antibodies (10 μg/tube) were then added and the contents of the tubes were incubated for an additional 15 min. Unbound antibodies were removed by washing the beads twice with PBS. The beads and TPA were added to the reaction cell, where the magnetic beads were captured and temporarily immobilized on the working electrode by a magnet under it. A voltage of 1.25 V was applied across the electrode and the photon signal was measured.

2.4. Calibration of IM-ECL assay for p53 antibodies

Twenty normal human serum samples from individuals were collected. The samples were analyzed with the IM-ECL assay. Based on Kijek's study (Kijek et al., 2000), a cutoff value is calculated based on the average ($V_{negative}$) and standard deviation ($V_{stdev(neg)}$) of the ECL reading from normal human sera, shown as formula (1), to define if a sample was positive of p53 antibodies.

$$V_{cutoff} = V_{negative} + 2.5V_{stdev(neg)} \quad (1)$$

To prepare the calibration standards, the mixtures of all 20 normal human sera were spiked with 10-fold concentrations of p53 antibodies (rabbit polyclonal antibody, Serotec) ranging from 0.01 to 10,000 ng/ml. Three separate samples were prepared and evaluated for each antibody concentration. To further evaluate the reproducibility of the assay, each sample was measured 50 times with one-second data integration, and the averages and standard deviations were calculated. The calibration curve was plotted as the ECL reading (counts per second, cps) against the p53 antibody concentrations in the sera.

2.5. Quantitative assay of p53 antibodies in cancerous human

Serum samples were collected from seven patients clinically diagnosed with lung carcinoma and 12 patients with nasopharyngeal carcinoma. Each sample was detected from IM-ECL assays twice. In the ECL assay, the sample with a reading value higher than cutoff value was considered to be positive. The calibrated assay was then used to quantitatively evaluate the presence of p53 antibodies in positive samples.

3. Results

3.1. Baseline

Twenty normal human serum samples from individuals were analyzed with the IM-ECL assay. The mean reading value is 93.72, with a standard deviation of 25.21. Based on formula (1), we estimated the cutoff value for positively identifying p53 antibody is 156 cps ECL reading.

3.2. System stability

The system stability of our assay technique was tested by repeated measurements from either the same sample or samples prepared from the same batch of human serum. As shown in Fig. 3, each bar represents an average value based on fifty 1-s measurements from the same sample, with the error bar (standard deviation) reflecting the measurement variation of the system. With the lowest p53 antibody concentration, 0.01 ng/ml, we observed an ECL reading approximately the same as the projected cutoff value, calculated from the baseline variation of normal individuals. It is reasonable to assume this value as the limiting p53 concentration for the detection system. The variations in ECL reading, among the three separately prepared samples at each p53 concentration level, reflect the variations introduced by an operator during each assay. It can be concluded, based on the data, that the system stability is rather consistent.

3.3. Calibration

Fig. 3 shows that ECL value increases with the p53 antibody concentration in the samples. The dynamic range of the ECL reading covers p53 antibody concentrations between 0.01 and 1000 ng/ml. When the spiked p53 antibody concentration is greater than 1000 ng/ml, a prozone effect (or hook effect) occurs. The ECL reading does not rise with the increased p53 antibody concentration, possibly due to an overloaded amount of the antibodies.

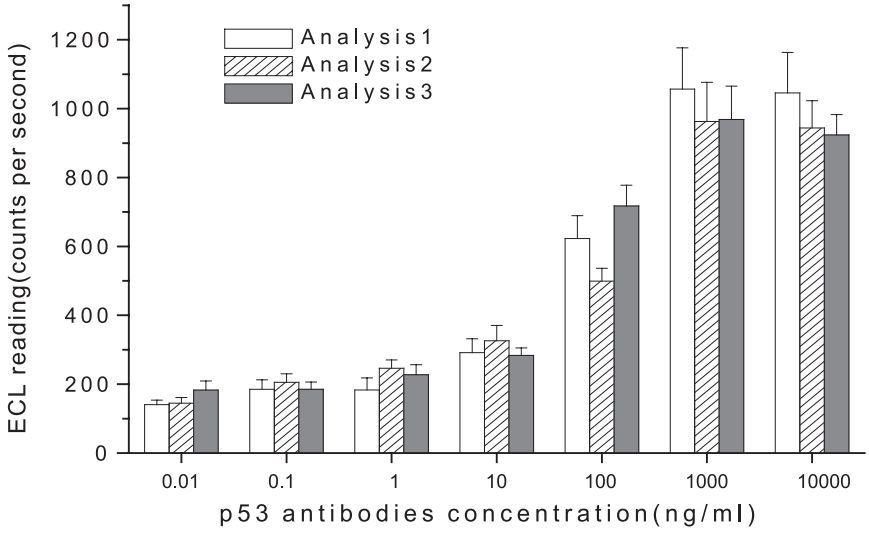

Fig. 3. Reproducibility of p53 antibodies IM-ECL assay. Each analysis represents a single run with 50 s acquisition time of each concentration on each day. Error bars indicate standard deviations from 50 s acquisition time.

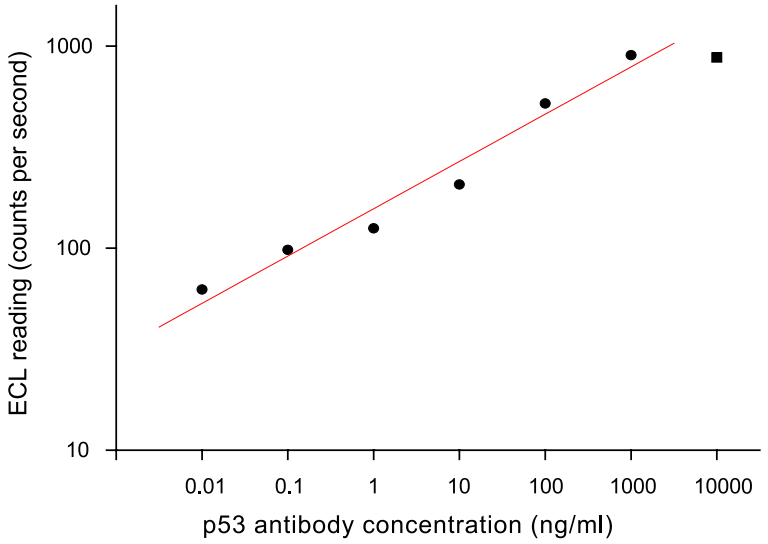

Fig. 4. A standard linear regression (log–log) curve. The linear regression is come from solid circles. The solid circles represent the means ECL reading from three independent assays. $R^2 = 0.9655$.

The calibration curve is plotted in a double log scale, as shown in Fig. 4. The linear regression is come from solid circles. The solid circles represent the means ECL reading from three independent assays, with the average ECL reading from those normal human sera subtracted. Linear regression of the data is performed for p53 concentrations between 0.01 and 1000 ng/ml ($R^2 = 0.9655$).

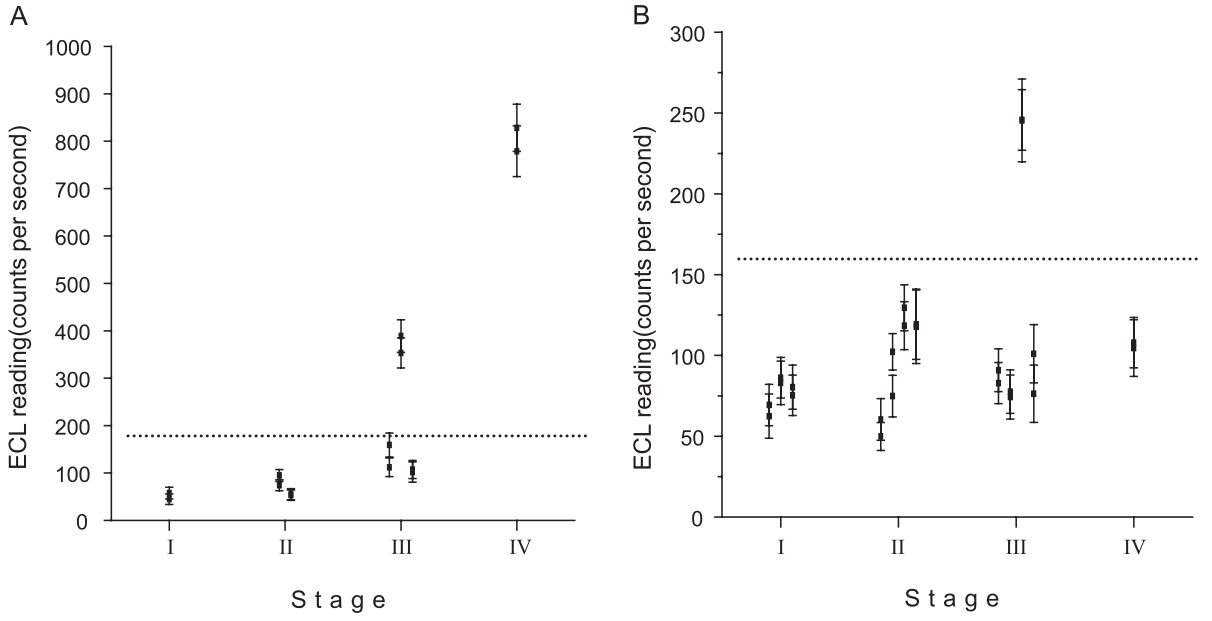

Fig. 5. P53 antibodies IM-ECL assay results of lung carcinoma sera (A) and nasopharyngeal carcinoma sera (B). The cutoff value is indicated as dashed lines. ECL values and error bars represent the reading from independent assays and standard deviations from 50 s acquisition time. In each stage, the point at same line represents two independent detected values from the same sample.

3.4. Evaluation of p53 antibodies in cancer patients

Samples from a total of seven patients, diagnosed with lung carcinoma were evaluated for p53 antibodies. The clinical diagnosis for the patients range from Stages I to IV (I:1, II:2, III:3, IV:1). Samples from 12 patients clinically diagnosed with nasopharyngeal carcinoma (stages I:3, II:4, III:4, IV:1) were also evaluated for p53 antibodies. Fig. 5A shows the results from the lung carcinoma sera. Two of the seven (28.6%) sera tested positive. One of them was in stage IV, with higher alpha-feto-protein concentrations. Another was in stage IV, with metastasis to liver and stomach. Based on the calibration curve, the p53 antibody concentrations in these two samples are 11.504 and 638.202 ng/ml, respectively. ECL reading of one of the stage III samples is 159.15, which is in the critical range (defined as $V_{\text{cutoff}}+20\%$). From the limited cases of lung carcinoma, p53 antibody concentration is corresponded with the disease progression. There is a trend that, a higher p53 antibody concentration in the serum is likely linked to a higher stage of the cancer, although this is not tested statistically verified in our experiments due to the limited sample size. There is no distinct correlation between p53 antibody concentration and the progression of nasopharyngeal carcinoma in our experiments as shown by the results in Fig. 5B. There is only one case of stage III patient tested positive for p53 antibody among a total of 12 patients. The other 11 patients have ECL readings randomly distributed in the negative range. From the calibration curve, we determined the p53 antibody concentration of the positive serum as 0.877 ng/ml.

4. Discussion

An IM-ECL assay was developed consisting of capture antigen-coated magnetic beads, ruthenium-labeled secondary antibodies and an ECL detection system. With the assay, p53 antibodies can be reproducibly detected in human sera at concentration as low as 10 pg/ml. This assay is faster than ELISA; it can be completed in less than 1 h. Using this method, many other antibodies can be detected rapidly.

A stable calibration curve with a wide dynamic range was established. The calibration curve was linear from 0.01 to 1000 ng/ml, as shown in Fig. 4. The saturated ECL signal at the highest antibody concentration represented a prozone effect, which is not uncommon and has been observed with other agents and assay (Kijek et al., 2000; Shelton and Karns, 2001). For our particular application, the dynamic range of the assay covers all p53 antibody levels, used for evaluating human cancerous sera. If the ECL reading is higher or equal the highest point of the linear portion, the unknown samples can be diluted for a proportional measurement.

In IM-ECL assay, a magnet should be set closely under the working electrode. When the PMT is close to the magnet, the normal working condition of PMT will be seriously affected by the strong magnetic filed. But if the PMT is set far from the magnet, the detectable signal will decrease. In order to solve this problem, a quartz fiber bundle was used in our IM-ECL detection system between the working electrode and PMT to make a high efficiency coupling of photons. Thus, the detection efficiency was obviously improved.

Anti-p53 monoclonal antibodies were chosen for immunomagnetic capture to avoid locking too many epitopes of the p53 protein, so the detected antibodies can immunoreact with p53 protein easily. Anti-p53 polyclonal antibodies were chosen, as the detected antibody for the calibration curve, because p53 antibodies in human sera are also polyclonal antibody, thus, would maximize the sensitivity of the assay.

Although our preliminary clinical investigation was limited due to the restricted access to the human samples, the results of the carcinoma sera in this assay are in accord with the results from what reported by others. In our study, the positive rate of p53 antibodies was 28.6% in lung carcinoma by IM-ECL assay, close to the 24% and 21.7% positive rates as tested with ELISA (Schlichtholz et al., 1994; Zhao et al., 2000). Definitive statistics were not obtained, but a trend that the higher the cancer stage is, the higher the p53 antibody concentration in the serum was observed. Xie et al. (1997) reported that overexpression of p53 existed in lung cancer and nasopharyngeal carcinoma. It was related to gene mutation in lung cancer, but not in nasopharyngeal carcinoma. This is consistent with our IM-ECL results of low positive rates of p53 antibodies in the sera of nasopharyngeal carcinoma patients. This result is also in accord with that of reported previously (Chang et al., 2002).

The p53 antibodies IM-ECL assay was simple, rapid, sensitive and reproducible. The assay was able to analyze clinical samples and could provide either qualitative or quantitative results. The IM-ECL assay, thus, may provide a new means for quantitatively evaluating the production of p53 antibodies.

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