High sensitive approach for point mutation detection based on electrochemiluminescence

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Abstract

An electrochemiluminescence–polymerase chain reaction (ECL–PCR) method for point mutation detection has been developed. The target is amplified using a tris (bipyridine) ruthenium (TBR)-labeled forward and a biotinylated reverse primer. The amplification products are digested with specific restriction enzyme, then captured onto streptavidin-coated paramagnetic beads, and detected by measuring the ECL signal of the TBR label. The established technique was further applied to detect a specific point mutation in H-ras oncogene in T24 cell line. The results show that the system has a low detection limit of 100 fmol and a linear range of more than 3 orders of magnitude for H-ras amplicon; the two genotypes can be reliably discriminated. In summary, the mutant specific ECL–PCR method can be used to detect a point mutation that creates or destroys a restriction site in any gene. It is useful in single nucleotide polymorphism (SNP) and mutation detection due to its safety, high sensitivity and simplicity.

1. Introduction

The ability to detect single nucleotide polymorphism (SNP) is of great importance in molecular genetics. Specific identification of point mutations in the human genome plays a major role in diagnosis of genetic diseases, identification of mutations within oncogenes, tumor suppressor genes and mutations associated with drug resistance (Cordon et al., 1997; Diamandis, 1997; Karmochkine and Descamps, 1999; Kwok, 2002; Igloi, 2003; Popescu, 2000).

Single base variations have been analyzed by a variety of techniques, such as restriction fragment length polymorphism (RFLP) (Chen and Viola, 1991), denaturing gradient gel electrophoresis (DGGE) (Uitterlinden and Vijg, 1990; Borresen et al., 1988; Takahashi et al., 1990), chemical cleavage of mismatch (CCM) (Smooker and Cotton, 1993; Bateman et al., 1993; Deeble et al., 1997–1998), enzyme mismatch cleavage (EMC) (Deeble et al., 1997–1998), single strand conformation polymorphism (SSCP) (Suzuki et al., 1990; Yandell, 1991), allele-specific oligonucleotide hybridization (ASO) (Conner and Reyes, 1983), amplification refractory mutation system (ARMS) (Newton et al., 1989), ligase chain reaction (LCR) (Kalin et al., 1992; Laffler et al., 1993) and competitive oligonucleotide primer (Miller et al., 1991). A common shortcoming of these methods is that they are all tedious and laborious, involving handling of large numbers of test tubes, time consuming blots and hybridizations. Furthermore, they all require the use of radioactive isotopes or other hazardous materials. Thus, a highly sensitive, yet simple and safe approach for point mutation detection is expected.

Electrochemiluminescence (ECL) is a chemiluminescent reaction of species generated electrochemically at an electrode surface. Initially, $Ru(bpy)_3^{2+}$ and tripropylamine (TPA) are oxidized at the surface of an anode. $TPA^{\bullet+}$ immediately loses a proton and becomes a powerful reducer. When $TPA^{\bullet}$ and $Ru(bpy)_3^{3+}$ react, the latter enters an excited state by a high energy electron transfer from the electron carrier, $TPA^{\bullet}$. Relaxation of $Ru(bpy)_3^{2+*}$ to the ground state results in a light emission, at 620 nm. Noticeable, $Ru(bpy)_3^{2+}$ is not consumed during the reaction and may be oxidized and excited repeatedly, if there is excessive

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TPA in the buffer (Blackburn et al., 1991; Yang et al., 1994; Deaver, 1995). Since Kenten et al. (1991) first used ECL in DNA probe assay, this method has been used in DNA quantification (Zhu et al., 2003).

We hypothesize that ECL–PCR will provide a more efficient and safe assay for point mutation detection. To test the hypothesis, we used ECL–PCR method to detect a point mutation in H-ras gene. DNA extracted from T24 bladder carcinoma cell line (Bubenik et al., 1973) with a point mutation at codon 12 was used as mutant sample; DNAs extracted from ASTC-a-1 lung cancer cell line and from blood cells were used as wild-type samples. Codon 12 of T24 cell was mutated from GGC (Gly) to GTC (Val) (Chang et al., 1985).

2. Materials and methods

2.1. General

The basic principle of the method is outlined in Fig. 1. Briefly, a 106-bases DNA fragment spanning the mutation was amplified with a TBR-labeled forward primer and a biotin-labeled reverse primer. After amplification, the sample was split into two aliquots. One aliquot was digested with a kind of restriction enzyme, *Hpa*II, which has a specific restriction site (5′-C*CGG-3′). The enzyme cut the fragment only if the sample was wild-type and thus removed the TBR-labeled 5′ part of the fragment from the biotinylated 3′ part. The other aliquot remained unaltered. Biotinylated DNA bound via streptavidin to paramagnetic beads (2.8 μm diameter, Dynal Biotech, Lake Success, NY, USA). This kept the biotinylated DNA in the electrochemical reaction cell of the instrument, while all other components were washed away. The amount of TBR bound to the biotinylated DNA was determined for both samples by measuring the ECL signal generated by the TBR in the detection chamber. The genotype was determined by comparing the signal intensities between the digested and the undigested samples.

Fig. 2. Diagram of the essential components of ECL assay system. C.E.: counter electrode; W.E.: working electrode; Ref. E.: reference electrode.

2.2. Experimental set-up

The ECL detection system was built in our laboratory. Fig. 2 is a diagram of its essential components. The heart of the instrument was an electrochemical reaction cell with a working electrode, a counter electrode and an Ag/AgCl$_2$ reference electrode. The working electrode (disk) and the counter electrode (mesh) were constructed with platinum. A single photon counting module (PMT, MP-962, Perkinelmer, Wiesbaden, Gemany) was used to collect the signal during ECL reaction. To position the samples to the proper locations for ECL detection, a magnet had to be set closely under the working electrode. If the PMT was too close to the magnet, its operation would be seriously hampered by the strong magnetic field. Yet, the PMT positioned far from the magnet would result in less collected signal and reduced sensitivity. To solve the problem, an optical fiber bundle was used to efficiently couple the ECL signals from the working electrode to the PMT. This technical arrangement greatly improved the

Fig. 1. The principle of H-ras mutation assay: (A) forward primer, (B) reverse primer; (✴) TBR, (○) biotin.

efficiency of signal collection, consequently, increased the detection sensitivity. The signal from the PMT was amplified and discriminated. The transistor–transistor logic (TTL) pulses were counted every second over a period of 30 s with a multi-function acquisition card (PCL-836, Advantech, Taiwan) controlled by Labview software. The voltage applied to the electrodes was controlled with a potentiostat (HDV-7C, Sanming, Fujian). The signal collection process and data analysis were accomplished with a personal computer. The detection limit of the analyzer was 10^{-18} mol/L Ru(bpy)$_3$Cl$_2$6H$_2$O (Sigma, Louis, MO, USA). The dynamic range for Ru(bpy)$_3$Cl$_2$6H$_2$O extended over five orders, from 0.1 pmol/L to 10 nmol/L. These specifications were established in a prevenient research (unpublished data).

2.3. Cell preparation and DNA extraction

T24 bladder carcinoma cell line (China Center for Type Culture Collection, Wuhan) and ASTC-a-1 lung cancer cell line (gift from the Medical College of Jinan University) were cultured at 37 °C, 5% CO$_2$, in RPMI 1640 medium supplemented with 10–15% fetal calf serum.

The blood samples were obtained from healthy donors. ACD decoagulant 1 ml (0.48% citric acid, 1.32% sodium citrate, 1.47% glucose) was added to each 6 ml blood. 500 µl blood was diluted by adding 1 ml sterile distilled water, then was centrifuged at 5000 rpm for 2 min (25 °C). The sediment was resuspended with 200 µl Tris–EDTA (TE) buffer (pH 8.0) for DNA extraction.

All the genome DNAs were extracted according to the protocol of the UNIQ-10 column genome DNA extraction kit (Shanghai Shenggong).

2.4. Polymerase chain reaction

pRaichu-Ras plasmid (gift from Michiyuki, 2001), blood cell DNA and ASTC-a-1 cell DNA all contained wild-type H-ras gene (Mochizuki et al., 2001). The nucleotide sequence of the coding regions of pRaichu-Ras was deposited in the DDBJ/EMBL/GenBank data (accession nos. AB046925 and AB051846). T24 cell DNA contained mutant H-ras gene with a point mutation at codon 12 (Reddy et al., 1982; Taparowsky et al., 1982).

The primers for PCR amplification were designed using Primer3 software and synthesized by Shanghai Shenggong. The sequence of forward and reverse primers were 5′(TBR)GGAATATAAGCTGGTGGTGGTG and 5′(biotin)CTCTATAGTGGGGTCGTATTCGT, respectively. The TBR–NHS ester, synthesized in our laboratory according to Terpetschnig's paper (1995), was introduced to forward primer as described by Kenten et al. (1991). The biotin was introduced to reverse primer by Shanghai Shenggong.

All the templates were amplified under the same cycling condition: cycle 1: 95 °C for 4 min, 58 °C for 1 min and 72 °C for 1 min; cycles 2–34: 95 °C for 40 s, 58 °C for 1 min, and 72 °C for 1 min; cycle 35: 95 °C for 40 s, 58 °C for 1 min,

and 72 °C for 5 min. All amplification reactions were carried out in a total volume of 50 µl. The sample amplified without templet was used as negative control; the sample amplified from pRaichu-Ras plasmid was used as positive control.

2.5. HpaII digestion

One microliter of HpaII (10 U/µl, Shanghai Shenggong), 2 µl of buffer and 7 µl of sterile water were added to 10 µl of samples from each PCR products. The samples were digested for 60 min at 37 °C and 20 min at 65 °C.

2.6. Calibration of ECL assay for H-ras amplicon

Amplicon generated from H-ras with biotin- and TBR-labeled primers was purified using UNIQ-10 column PCR product purification kit (Shanghai Shenggong), and then, quantified by absorbance measurement at 260 nm. The products were then serially diluted, bound to streptavidin-coated beads and detected. Each sample was measured 30 times with 1-s data integration. The averages and the standard deviations were calculated using Microsoft Excel spread sheet function. The calibration curve was plotted as the ECL intensity (counts per second, cps) against the H-ras concentration.

2.7. ECL assay for H-ras point mutation

TBR was used as a label of amplification products, in combination with streptavidin-coated paramagnetic microbeads. Both the digested and the undigested amplicons were incubated with streptavidin magnetic beads for 20 min, respectively. Unbound components, such as TBR-labeled forward primers, TBR-labeled 5′ part of the fragment, were removed by washing the beads in a magnetic field twice with TE buffer (pH 7.4). Then, 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. The amplicons with or without HpaII digestion were detected in turn. Each sample was detected three times.

3. Results and discussion

3.1. Specificity of the amplification

After 35 cycles, 5 µl of the reaction mixture was loaded on a 2% agarose gel. As shown in Fig. 3, the first lane was 100 bp DNA ladder (Shanghai Shenggong), the second and the third lanes were negative and positive control, the fifth, the sixth and the seventh lanes were amplicons from T24, ASTC-a-1 and blood DNAs, respectively. The primer pair gave rise to only one band of the expected size, demonstrating a high specificity of the amplification reaction.
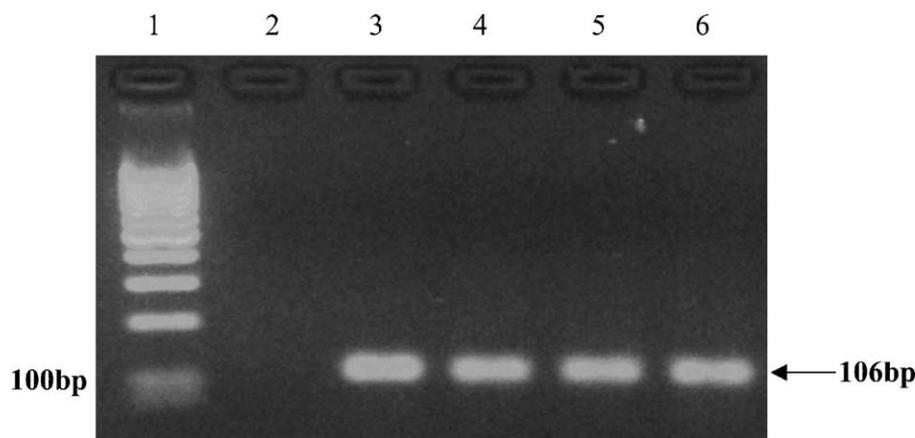
Fig. 3. Agarose gel electrophoresis of the amplified H-ras.

3.2. Calibration of ECL assay for H-ras amplicons

The sensitivity of the system for H-ras amplicon was approximately 100 fmol, which is equivalent to radioisotopic DNA detection assays, but without using any radioisotope agents (DiCesare et al., 1993). And this technique is more sensitive than other conventional DNA detection methods. So, the high level of sensitivity is not only ensures accuracy of the intended detections, but also allows reductions of the PCR cycle number and the required sample size.

Fig. 4 is a calibration curve of ECL assay for H-ras amplicon. The curve is plotted in a double log scale, with the averaged background ECL reading subtracted from the signals. The results show that, the H-ras amplicon could be detected over a linear range of more than 3 orders of magnitude. Linear regression of the data is performed for H-ras quantity between 0.1 and 500 pmol ($R^2 = 0.996$). This wide dynamic range is useful in developing quantification assay.

3.3. H-ras point mutation assay

Fig. 5 shows the comparison of ECL intensities between wild-type and mutant samples, with or without digestion, respectively. Each datum represents a mean value based on 30 1-s measurements from the same sample, with a standard deviation reflecting the measurement variation of the system. The background signal was obtained by measuring the ECL signal from the blanks (PCR without template). The mean background signal is 8.4 ± 2.1 cps (mean $\pm$ S.D.). Threshold value is defined as that, when measured ECL value is above it, the sample is considered to be positive. The threshold value can be derived as the mean $+$ three times the standard deviation of the background signal (DiCesare et al., 1993; Motmans et al., 1996), or, 14.7 cps ECL reading for our system. The data show that for the wild-type samples, the mean signals of the digested amplicons from blood cells and ASTC cell are 7.6 ± 1.5 cps and 9.2 ± 2.4 cps, respectively. Both of these values are under the threshold value.

Fig. 4. A standard linear regression (log–log) curve for H-ras amplicon. Each ECL value represents the mean from three parallel concentration assays. $R^2 = 0.995$.
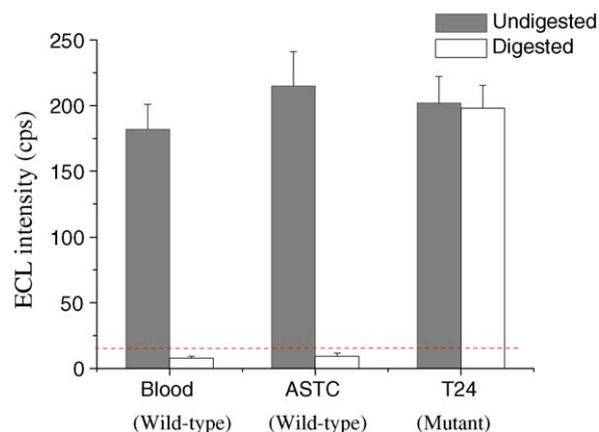
Fig. 5. Point-mutation assay for the H-ras oncogene. Each bar represents an average value based on 30 1-s measurements from three parallel samples, respectively, with the error bar (S.D.) reflecting the measurement variation of the system. The dash line represents the threshold value for positive results.

The mean signals of the undigested amplicons from blood cells and ASTC cell are 182 ± 19 cps and 215 ± 26 cps, respectively. Both of them are over 10 times higher than the threshold value. The difference in signal intensities between undigested and digested amplicons are statistically significant ($P < 0.05$). For the mutant, the mean signals of the undigested and the digested amplicons are 202 ± 20.3 cps and 198 ± 17.4 cps, respectively. The difference in signal intensities between undigested and digested amplicons is negligible ($P > 0.05$).

The results can be explained by the appearance of *Hpa*II restriction site ($5'$-C*CGG-$3'$). The 106 bp fragment amplified from wild-type H-ras can be cut by *Hpa*II, generating 27- and 79-bp fragments, thus removing the TBR label from the biotinylated part of the fragment. The TBR label can react with TPA to produce light emission, so the digested wild-type samples cannot be detected by ECL assay. If mutant H-ras is amplified, *Hpa*II will not cut the fragment and the TBR label is not removed. Thus, the digested mutant samples can be detected by ECL assay. Given the distinctive different results that among the signal intensities, we consider that the point mutation can be detected using this method.

Based on the calibration curve, the quantity of undigested wild-type H-ras amplicons from blood cells and ASTC cell are 1.5 ± 0.08 pmol and 1.7 ± 0.1 pmol, respectively, both the quantity of undigested and digested mutant H-ras amplicons from T24 cell are 1.6 ± 0.05 pmol.

4. Conclusion

In summary, The ECL–PCR technique we describe here for the detection of point mutation is very efficient for the accurate quantification of PCR product. As show in the study, the ECL based system provides a sensitive detection for H-ras amplicon with concentration as low as 100 fmol and a linear response range over more than 3 orders of magnitude. Post-PCR manipulations are minimal and easily automated so that this technique will be very useful when analyzing multiple samples in a short period of time. The new approach has been tested with Gly12Val mutation in H-ras gene. The mutant and wild-type H-ras gene can be reliably discriminated using this method. The mutant specific ECL–PCR method can be used to detect a point mutation that creates or destroys a restriction site in any gene. It is useful in SNP and mutation detection due to its safety, high sensitivity and simplicity.

Acknowledgements

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