A method to quantitatively detect H-ras point mutation based on electrochemiluminescence

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Abstract

Conventional methods for point mutation detection are usually multi-stage, laborious, and need to use radioactive isotopes or other hazardous materials, and the assay results are often semi-quantitive. In this work, a protocol for quantitative detection of H-ras point mutation was developed. Electrochemiluminescence (ECL) assay was coupled with restriction endonuclease digestion directly from PCR products. Only the wild-type amplicon containing the endonuclease's recognition site can be cut off, and thus cannot be detected by ECL assay. Using the PCR–ECL method, 30 bladder cancer samples were analyzed for possible point mutation at codon 12 of H-ras oncogene. The results show that the detection limit for H-ras amplicon is 100 fmol and the linear range is more than three orders of magnitude. The point mutation was found in 14 (46.7%) out of 30 bladder cancer samples. The experiment results demonstrate that the PCR–ECL method is a feasible quantitative approach for point mutation detection due to its safety, high sensitivity, and simplicity.
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Keywords: Point mutation; Electrochemiluminescence; Restriction endonuclease digestion; Polymerase chain reaction; H-ras oncogene; Bladder cancer

Genome mutations often underlie human heritable diseases, and they determine, in many cases, the predisposition of organism to certain pathology. Majority of cancer cases are closely related to the activation of oncogenes or the inactivation of tumor suppressor genes, which was mostly caused by point mutation of these genes [1]. Mutations in genomic DNA are the basis for genome polymorphism. Polymorphous sequences of genomic DNA may be exploited as effective markers in genetic analysis [2]. Therefore, the development of reliable methods for the detection of point mutations is an important issue for up-to-date medical diagnostics.

There are some methods suggested for point mutation detection: methods based on the analysis of DNA fragments after restriction by endonucleases (restriction fragment length polymorphism [3], mutant-enriched polymerase chain reaction (PCR) [4]); methods utilizing hybridization with an allele-specific oligonucleotide (ASO) [5,6]; and method of molecular beacons [7], allele-specific amplification (ASA) [8,9], single nucleotide primer extension [10,11], and oligonucleotide ligation assay (OLA) [12,13]. These methods could be implemented in routine clinical diagnostics, though all of them have some advantages and drawbacks. A common shortcoming of these methods is that they are usually multi-stage, 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. And the analysis results are often semi-quantitative. Thus, a highly sensitive, yet simple, safe, and quantitative approach for point mutation detection is expected.

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Electrochemiluminescence (ECL) has been developed recently. It has an increased sensitivity over other chemiluminescence (CL) techniques. It is initiated by a voltage potential, thus, can be better controlled. Since Kenten et al. [14] first used ECL in DNA probe assay, this method has been widely used in DNA quantification assay [15–17]. ECL detection is accomplished by $Ru(bpy)_3^{2+}$ (TBR) conjugated to DNA fragment. 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^+ 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 [18].

We hypothesize that ECL assay coupled with restriction endonuclease digestion directly from PCR products will provide a more efficient and quantitative method for point mutation detection. To test this hypothesis, 30 bladder cancer samples plus ten healthy human blood samples were analyzed for possible point mutation at codon 12 of H-ras oncogene. H-ras oncogene is often activated by point mutation in human bladder cancer cases. Codon 12 is the major "hot spots" for its activation [19–22]. A simple and accurate method to detect the point mutation may be clinically useful for early diagnosis, prognosis, and monitoring of bladder cancer.

Materials and methods

Materials. Tripropylamine (TPA) and the chemicals to synthesize the $Ru(bpy)_3^{2+}$ *N*-hydroxysuccinimide ester (TBR–NHS ester) were purchased from Sigma (Louis, MO, USA). Streptavidin microbeads (2.8 μm diameter) were products of Dynal Biotech (Lake Success, NY, USA). The human bladder carcinoma cell line T24 was purchased from China Center for Type Culture Collection. Codon 12 of T24 cell was mutated from GGC (Gly) to GTC (Val) [23]. Healthy human blood samples were obtained from the hospital of South China Normal University, Guangzhou, China. The paraffin-embedded bladder cancer tissues were gift from the fifth attached hospital of the medical college of Jinan University, Guangzhou, China. The primers were designed using Primer3 software and synthesized by Shanghai Sangon Biological Engineering and Technology Services (SSBE). The sequences of forward and reverse primers were 5′TBR-ggaata-taagctggtggtggtg and 5′biotin-ctctatagtggggtcgtattcgt respectively. The TBR-NHS ester, synthesized in our laboratory according to Terpetschnig's paper [24], was introduced into forward primer as described by Kenten et al. [14]. The biotin was introduced into reverse primer by SSBE.

Equipment. The ECL detection system was built in our laboratory. Fig. 1 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/AgCl₂ reference electrode. The working electrode (disk) and the counter electrode (mesh) were con-
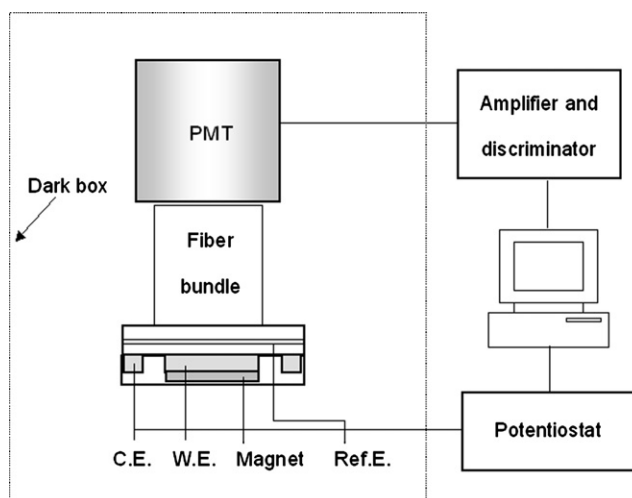

Fig. 1. Diagram of the essential components of ECL assay system. C.E., counter electrode; W.E., working electrode; and Ref.E., reference electrode. The heart of the system is the electrochemical reaction cell containing a W.E., a C.E., and a Ref.E. Photon emission from the ECL reaction is collected by an optical fiber-bundle and then detected by a single photon multiplier tube.

structed with platinum. An optical fiber-bundle receives the light emitted during the ECL reaction and conducts it to an ultrahigh sensitivity single photon counting module (PMT, MP-962, Perkin–Elmer, Wiesbaden, Gemany). 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, China). The signal collection process and data analysis were accomplished with a personal computer.

Method. The basic principle of the method is outlined in Fig. 2. Briefly, a 106-base DNA fragment harboring codon 12 is amplified with a TBR-labeled forward primer and a biotin-labeled reverse primer. After amplification, the sample is digested with a restriction endonuclease, *Hpa*II, which has a specific restriction site (5′-C*CGG-3′). The *Hpa*II restriction site is lost when the point mutation occurs at codon 12. So, the enzyme cuts the fragment only if the sample is wild-type, and thus removes the TBR-labeled 5′part of the fragment from the biotinylated 3′part, while the mutant sample cannot be cut off. Biotinylated DNA binds via streptavidin to paramagnetic beads, which keep the biotinylated DNA in the electrochemical reaction cell of the instrument, while all other components are washed away. The amount of TBR bound to the biotinylated DNA is determined by measuring the ECL signal generated by the TBR in the reaction cell. The quantity of mutant samples is then evaluated according to the calibration curve.

PCR amplification. Genomic DNAs extracted from T24 cells, from healthy human bloods, and from paraffin-embedded bladder cancer tissues were amplified using the labeled primers specific for H-ras oncogene. The amplification condition was: cycle 1: 95 °C for 4 min, 56 °C for 1 min, and 72 °C for 1 min. Cycles 2–34: 95 °C for 40 s, 56 °C for 1 min, and 72 °C for 1 min. Cycle 35: 95 °C for 40 s, 56 °C for 1 min, and 72 °C for 5 min. All of the reactions were carried out in a total volume of 20 μl. The sample amplified without template was used as negative control; the sample amplified from genomic DNA of T24 cells was used as positive control.
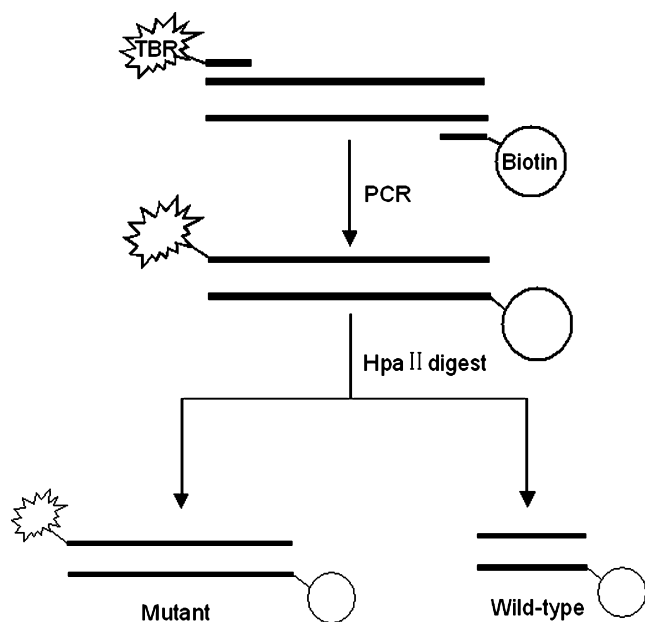
Fig. 2. The principle of H-ras point mutation assay. A 106 bp DNA fragment spanning the mutation is amplified from genomic DNA with a TBR-labeled forward primer and a biotin-labeled reverse primer. After amplification, the sample is digested with *Hpa*II which cuts the fragment only if the sample was wild-type, and thus removes the TBR-labeled 5′part of the fragment from the biotinylated 3′part. Biotinylated DNA binds via streptavidin to paramagnetic beads, which keep the biotinylated DNA in the electrochemical reaction cell of the instrument, while all other components are washed away. The amount of TBR bound to the biotinylated DNA is determined by measuring the ECL signal generated by the TBR in the reaction cell.

HpaII digestion. One microliter of *Hpa*II (10 U/μl, SSBE), 2 μl of buffer, and 7 μl of sterile water were added to 10 μl of PCR products. The mixture was digested for 60 min at 37 °C and 20 min at 65 °C.

Calibration of ECL assay for H-ras amplicon. According to DiCesare's study [25], a cutoff value is calculated based on the average (V_{blank}) and standard deviation ($V_{stdev(bla)}$) of the ECL reading from the blank control (PCR without template), shown as Formula (1), to define if a sample was positive for H-ras point mutation.

$$V_{cutoff} = V_{blank} + 3V_{stdev(bla)}. \tag{1}$$

To prepare the calibration standards, the H-ras amplicon generated from T24 DNA was purified using UNIQ-10 PCR product purification kit (SSBE), and then, quantified by absorbance measurement at 260 nm. The samples 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 reading (counts per second, cps) against the quantity of H-ras amplicon.

ECL assay for H-ras point mutation. All of the bladder cancer samples and healthy human blood samples were detected three times using ECL assay. The digested amplicon was incubated with streptavidin magnetic beads for 20 min. 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 into 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 sample with a reading value higher than cutoff value was considered

positive for point mutation. The calibrated assay was then used to evaluate the quantity of mutant H-ras amplicon in positive samples.

Results

Specificity of PCR

The specificity of PCR was confirmed by 2% agarose gel electrophoresis. As shown in Fig. 3, the first lane was 100 bp DNA ladder (SSBE), the second to the eighth lanes were H-ras amplicons from seven bladder cancer samples, and the ninth lane was positive control. The primer pair gave rise to only one band of the expected size, demonstrating a high specificity of the amplification reaction.

Cutoff value

The average ECL reading value of blank control is 8.4 cps, with a standard deviation of 2.1 cps. Based on Formula (1), we estimated that the cutoff value for point mutation positive of H-ras is 14.7 cps reading.

Calibration curve and linearity

Fig. 4 shows that ECL reading increases with the quantity growth of H-ras amplicon. The dynamic range of the ECL reading covers the quantity of H-ras amplicon between 0.1 and 500 pmol. The calibration curve is plotted in a double log scale. Linear regression of the data is performed for H-ras quantity between 0.1 and 500 pmol ($R^2 = 0.996$).

H-ras point mutation analysis

Thirty bladder cancer samples and 10 healthy human blood samples were evaluated for H-ras point mutation by ECL assay. The pathological grade of the bladder cancer samples range from Grade I to III (I , 8; II, 11; and III, 11). Fig. 5 shows the results of the assay. Four-
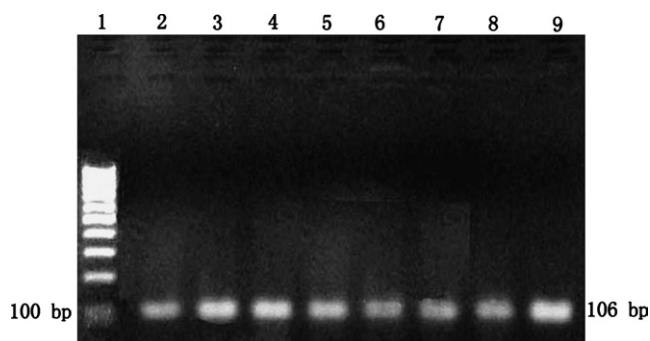

Fig. 3. Agarose gel electrophoresis of the H-ras amplicon. 1, 100 bp DNA ladder; 2–8, bladder cancer samples; and 9, positive control.
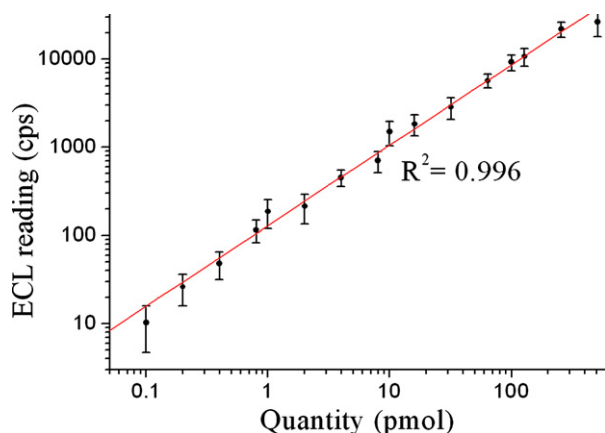
Fig. 4. A standard linear regression (log–log) curve. Each ECL value represents the mean from three independent assays. $R^2 = 0.996$.
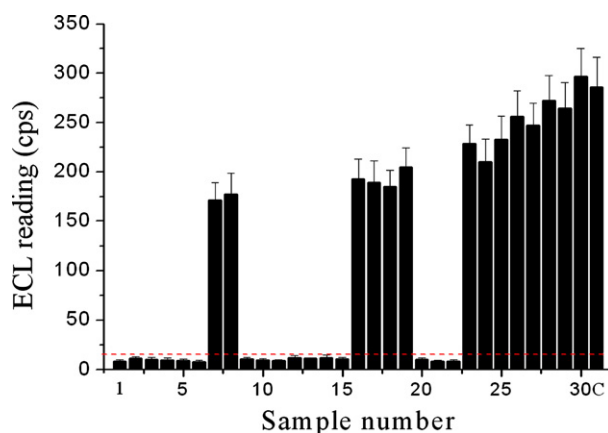

Fig. 5. Point mutation assay for the H-ras oncogene in bladder cancer. 1–30, bladder cancer samples; C, positive control. The cutoff value is indicated as dashed line. ECL values and error bars represent the reading from independent assays and standard deviations from 30 s acquisition time.

teen of the 30 samples (46.7%) were positive for H-ras point mutation, two of which were in grade I, four in grade II, and eight in grade III. None of the blood samples were found to be point mutation positive for H-ras oncogene. Positive samples were then quantified according to the calibration curve. The quantitative results are shown in Table 1.

Discussion

A PCR–ECL method consisting of PCR technique, restriction endonuclease digestion, and ECL assay was developed for point mutation detection. The biotin label was used for selective immunomagnet capture by streptavidin-coated paramagnetic beads in the detection cell, thus, ensuring the specificity of the assay. TBR label was used to react with TPA to emit light for ECL detection. Only the DNA samples labeled with both biotin and TBR can be detected in the detection cell. Using this method, H-ras amplicon can be reproducibly detected at a quantity as low as 100 fmol. The approach does not need to use any dangerous material and is faster than electrophoresis-based conventional methods. It can be completed in less than 1 h. The PCR–ECL method is useful in routine screening of a large amount of clinical samples.

A stable calibration curve with a wide dynamic range was established. The calibration curve was linear from 0.1 to 500 pmol, as shown in Fig. 4. For our particular application, the dynamic range of the assay covers all the H-ras amplicon levels used for evaluating the point mutation.

In ECL assay, a magnet had to be set closely under the working electrode to position the samples to the proper locations for ECL detection. 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 efficiency of signal collection, consequently, increased the detection sensitivity.

The previously reported frequency of H-ras point mutation at codon 12 in bladder cancer ranges from 34.5% to 62.5% in Chinese [26–29]. In our study, the positive rate for H-ras point mutation was 46.7%, which was consistent with previous research. No false-positive results have been found in the blood samples corresponding to the healthy donors, demonstrating a high accuracy of the method.

Table 1
Quantitative results for mutant H-ras amplicon

Grade	ECL reading ($\chi \pm s$) (cps)		Quantitative results (pmol)	
I	170.9 ± 8.2	177 ± 21.4	1.42	1.49
II	192.4 ± 20.5	188.9 ± 22.0	1.59	1.56
	185 ± 16.8	204.8 ± 19.2	1.55	1.69
III	228.6 ± 18.8	210.0 ± 23.2	1.87	1.72
	232.8 ± 24.0	256.0 ± 26.2	1.91	2.16
	246.8 ± 23.0	272.1 ± 25.9	2.05	2.34
	264.5 ± 26.2	296.7 ± 28.6	2.24	2.62

The proposed PCR–ECL method can also be applied to detect the point mutation at codon 12 of K-ras and N-ras oncogenes using *Bst*NI restriction endonuclease [30,31]. The only application limiting for this method is to choose the appropriate enzymatic restriction site.

In summary, the quantitative PCR–ECL method is a safe, sensitive, specific, robust, and cost-effective technique suitable for fast, high-throughput testing and offers distinct advantages over other conventional methods. It can be used to detect any point mutation that creates or destroys a restriction site in genes.

Acknowledgments

References

[1] A.V. Demchinskaya, I.A. Shilov, A.S. Karyagina, V.G. Lunin, O.V. Sergienko, O.L. Voronina, M. Leiser, L. Plobner, A new approach for point mutation detection based on a ligase chain reaction, J. Biochem. Biophys. Methods 50 (2001) 79–89.

[2] C. Lavebratt, S. Sengul, M. Jansson, M. Schalling, Pyrosequencingt-based SNP allele frequency estimation in DNA pools, Hum. Mutat. 23 (2004) 92–97.

[3] S.J. Hong, T. Lee, Y.S. Park, K.O. Lee, B.H. Chung, S.H. Lee, A PCR-RFLP method for the detection of activated H-ras oncogene with a point mutation at codon 12 and 61, Yonsei Med. J. 37 (1996) 371–379.

[4] J.R. Conejo, T. Parra, M. Cantero, A. Jiménez, V. Granizo, G. de Arriba, F. Carballo, Detection of H-ras mutations in urine sediments by a mutant-enriched PCR technique, Clin. Chem. 44 (1998) 1570–1572.

[5] R.B. Wallace, M.J. Johnson, T. Hirose, T. Miyake, E.H. Kawashima, K. Itakura, The use of synthetic oligonucleotides as hybridization probes: II. Hybridization of oligonucleotides of mixed sequence to rabbit beta-globin DNA, Nucleic Acids Res. 9 (1981) 879–894.

[6] B.J. Conner, A.A. Reyes, Detection of sickle cell beta S-globin allele by hybridization with synthetic oligonucleotides, Proc. Natl. Acad. Sci. USA 80 (1983) 278–282.

[7] S.A. Marras, F.R. Kramer, S. Tyagi, Multiplex detection of single-nucleotide variations using molecular beacons, Genet. Anal. 14 (1999) 151–156.

[8] D.Y. Wu, L. Ugozzoli, B.K. Pal, R.B. Wallace, Allele-specific enzymatic amplification of beta-globin genomic DNA for diagnosis of sickle cell anemia, Proc. Natl. Acad. Sci. USA 86 (1989) 2757–2760.

[9] R.A. Gibbs, P.N. Nguyen, C.T. Caskey, Detection of single DNA base differences by competitive oligonucleotide priming, Nucleic Acids Res. 17 (1989) 2437–2448.

[10] S.S. Sommer, A.R. Groszbach, C.D. Bottema, PCR amplification of specific alleles (PASA) is a general method for rapidly detecting known single-base changes, BioTechniques 12 (1992) 82–87.

[11] B.P. Sokolov, Primer extension technique for the detection of single nucleotide in genomic DNA, Nucleic Acids Res. 18 (1989) 3671.

[12] A.M. Alves, F.J. Carr, Dot blot detection of point mutations with adjacently hybridizing synthetic oligonucleotide probes, Nucleic Acids Res. 16 (1988) 8723.

[13] U. Landegren, R. Kaiser, J. Sanders, L. Hood, A ligase-mediated gene detection technique, Science 241 (1988) 1077–1080.

[14] J.H. Kenten, J. Casadei, J. Link, S. Lupold, J. Willey, M. Powell, A. Rees, R. Massey, Rapid electrochemiluminescence assays of polymerase chain reaction products, Clin. Chem. 37 (1991) 1626–1632.

[15] R.A. Collins, L.S. Ko, K.Y. Fung, L.T. Lau, J. Xing, A.C.H. Yu, A method to detect major serotypes of foot-and-mouth disease virus, Biochem. Biophys. Res. Commun. 297 (2002) 267–274.

[16] D.B. Zhu, D. Xing, X.Y. Shen, G.H. Yan, High sensitive detection of presenilin-1 point mutation based on electrochemiluminescence, Chin. Sci. Bull. 48 (2003) 1741–1744.

[17] D.B. Zhu, D. Xing, X.Y. Shen, J.F. Liu, Q. Chen, High sensitive approach for point mutation detection based on electrochemiluminescence, Biosens. Bioelectron. 2004 (in press).

[18] G.H. Yan, D. Xing, S.C. Tan, Q. Chen, Rapid and sensitive immunomagnetic-electrochemiluminescent detection of p53 antibodies in human serum, J. Immunol. Methods 288 (2004) 47–54.

[19] J. Fujita, S.K. Srivastava, M.H. Kraus, J.S. Rhim, S.R. Tronick, S.A. Aaronson, Frequency of molecular alterations affecting ras protooncogenes in human urinary tract tumors, Proc. Natl. Acad. Sci. USA 82 (1985) 3849–3853.

[20] J.L. Bos, The ras gene family and human carcinogenesis, Mutat. Res. 195 (1988) 255–271.

[21] K.V. VisVanathan, R.D. Pocock, I.C. Summerhayes, Preferential and novel activation of H-ras in human bladder carcinomas, Oncogene Res. 3 (1988) 77–81.

[22] B. Czerniak, D. Deitch, H. Simmons, P. Etkind, F. Herz, L.G. Koss, Ha-ras gene codon 12 mutation and DNA ploidy in urinary bladder carcinoma, Br. J. Cancer 62 (1990) 762–763.

[23] E.H. Chang, P.L. Morgan, E.J. Lee, K.F. Pirollo, E.A. White, D.H. Patrick, P.N. Tsichlis, Pathogenicity of retroviruses containing either the normal human c-Ha-ras1 gene or its mutated form derived from the bladder carcinoma EJ/T24 cell line, J. Exp. Pathol. 2 (1985) 177–189.

[24] E. Terpetschnig, H. Szmacinski, H. Malak, J.R. Lakowicz, Metal–ligand complexes as a new class of long-lived fluorophores for protein hydrodynamics, Biophys. J. 68 (1995) 342–350.

[25] J. DiCesare, B. Grossman, E. Katz, E. Picozza, R. Ragusa, T. Woudenberg, A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantitation, BioTechniques 15 (1993) 152–157.

[26] Q.H. Yang, T.J. Yang, Detection of point mutation of Ha-ras oncogene and ras P21 expression in bladder cancer, Chin. J. Clin. Oncol. 23 (1996) 407–411.

[27] Q. Ding, Y.F. Zhang, X. Sun, D.R. Shi, H-ras gene mutation and expression of P21 protein in human bladder cancer, Chin. J. Histochem. Cytochem. 5 (1996) 162–166.

[28] L. Zou, H. Han, H.Y. Song, M.S. Yu, G.X. Shao, Point mutation of ras oncogene and bladder cancer, Chin. J. Urol. 17 (1996) 216–218.

[29] S.B. Xu, P. Guo, Y.F. Tao, Z.H. Li, Z.Q. Yang, J.Y. Zhu, Detection of H ras gene mutation in bladder carcinoma by PCR SSCP siliver staining, Chin. J. Cancer Prev. Treat. 9 (2002) 151–153.

[30] T. Nishikawa, K. Maemura, I. Hirata, R. Matsuse, H. Morikawa, K. Toshina, M. Murano, K. Hashimoto, Y. Nakagawa, O. Saitoh, K. Uchida, K. Katsu, A simple method of detecting K-ras

point mutations in stool samples for colorectal cancer screening using one-step polymerase chain reaction/restriction fragment length polymorphism analysis, Clin. Chim. Acta 318 (2002) 107–112.

[31] I.N. Mammas, A. Zafiropoulos, E. Koumantakis, S. Sifakis, D.A. Spandidos, Transcriptional activation of H- and N-ras oncogenes in human cervical cancer, Gynecol. Oncol. 92 (2004) 941–948.