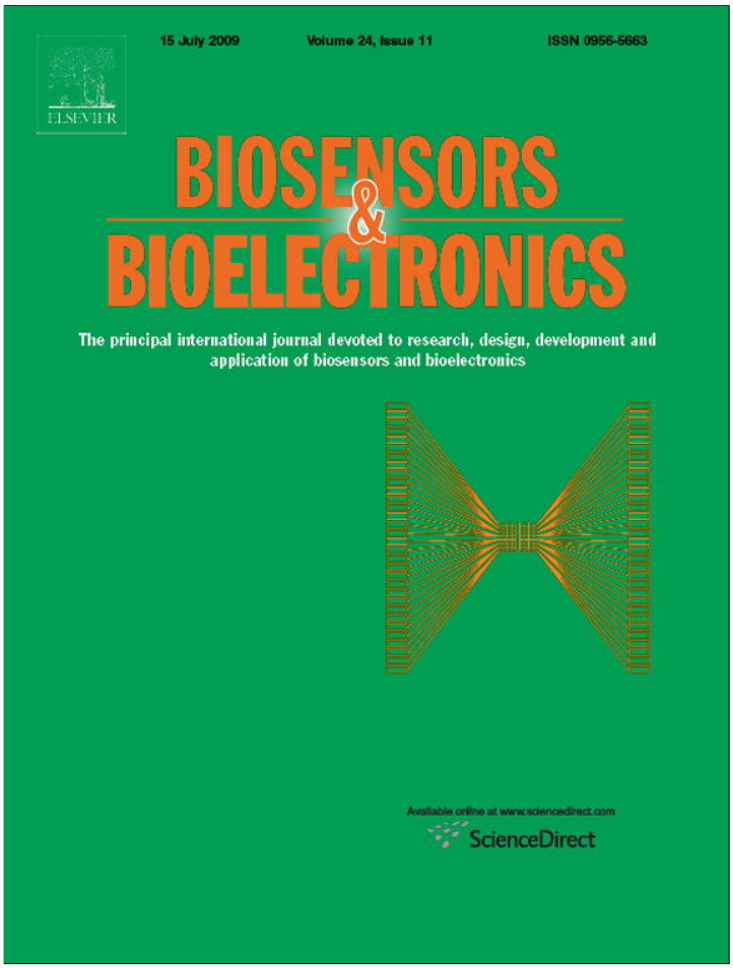

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A novel mutant allele specific amplification and electrochemiluminescence method for the detection of point mutation in clinical samples

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ABSTRACT

A novel mutant allele specific amplification (MASA) and electrochemiluminescence (ECL) method for point mutation detection is proposed. Briefly, the target gene was amplified by a biotinylated mutant specific sense primer and a $Ru(bpy)_3^{2+}$ (TBR)-labeled universal antisense primer. Only the mutant allele can be selectively amplified by the mutant specific primer pair. Then, the MASA product was captured onto the streptavidinylated magnetic beads through biotin–streptavidin linkage and detected by measuring the ECL emission of TBR. The method was applied to detect a possible point mutation at codon 12 of *K-ras* oncogene in 30 colorectal cancer (CAC) clinical samples. The experimental results show that the method can detect *K-ras* mutant in a 5000-fold excess of wild-type allele. Furthermore, different kinds of mutations can be clearly discriminated. The point mutation was found in 15 (50%) out of 30 CAC samples. This novel MASA–ECL method could potentially become a sensitive, specific, simple, rapid and safe approach for point mutation detection.

1. Introduction

Point mutations in genomic DNA are closely related to some human diseases, such as cancer, Alzheimer's disease and sickle cell anemia (Erichsen and Chanock, 2004; Schellenberg et al., 2000; Zhang et al., 2008a,b). Therefore, it is greatly important to detect point mutations in clinical diagnostics. Up to now, many methods have been developed for point mutation detection. However, conventional electrophoresis-based methods, like restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) are complicated and time-consuming. Furthermore, they need to use hazardous materials such as radioactive isotopes and ethidium bromide (EB) (Borresen et al., 1988; Chen and Viola, 1991; Yandell, 1991; Dieterle et al., 2004). To overcome these shortcomings of the electrophoresis-based methods, we have developed an electrochemiluminescence–polymerase chain reaction (ECL–PCR) method for point mutation detection recently (Zhu et al., 2004). Though this ECL–PCR method provides a safe, highly sensitive and simple means for point mutation detection, it needs to digest PCR product with a restriction enzyme. This digestion process costs more than 1 h, which greatly prolongs the detection time. Further more, the method cannot discriminate different kinds of mutations. Therefore, an approach with less detection time and can discriminate different kinds of mutations will be more useful in point mutation detection.

Recently, mutant allele specific amplification (MASA) has been used for point mutation detection (Tada et al., 1993; Paranavitana, 1998; Fox et al., 1998; Clayton et al., 2000). The basic principle of MASA is that the mutant specific primer pair can selectively amplify the corresponding mutant allele. Therefore it provides a highly specific assay protocol with single-base mismatch discrimination capability. We hypothesize that MASA combines with ECL detection will provide an efficient approach for point mutation detection. To test this hypothesis, a possible point mutation at codon 12 of *K-ras* oncogene associated with colorectal cancer (CAC) was analyzed in 30 CAC clinical samples by the MASA–ECL method.

2. Experimental

2.1. Reagents and samples

Tripropylamine (TPA) and the chemicals to synthesize $Ru(bpy)_3^{2+}$ N-hydroxysuccinimide ester (TBR-NHS ester) were purchased from Sigma (Louis, MO, USA). The TBR-NHS ester was synthesized by our laboratory according to Terpetschnig's work (Terpetschnig et al., 1995). Streptavidinylated magnetic beads (2.8 μm diameter) were product of Dynal Biotech Company (Lake Success, NY, USA). Human embryo kidney (HEK) 293 cell line that harbors a homozygous wild-type *K-ras* was purchased from Experimental Animal Center of Sun Yat-sen University, Guangzhou, China. The human CAC SW480 cell line that harbors a homozygous

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GGT-to-GTT mutation at codon 12 (G12V) of *K-ras* was purchased from China Center for Type Culture Collection, Wuhan, China. Thirty paraffin-embedded CAC tissue samples were a gift from the first attached hospital of Guangzhou University of Chinese Medicine, Guangzhou, China. UNIQ-10 column clinical sample genomic DNA isolation kit and other reagents used in this study were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (SSBE).

2.2. DNA sequences

The GAT, GTT and CGT mutations at codon 12 of *K-ras* oncogene were reported in most cancer cases of Chinese patients (He et al., 2002; Pan et al., 2002; Zhang et al., 2002; Gao et al., 2005). These mutations seem to play an important role in tumorigenesis. Therefore, the detection of these mutations may be clinically useful in diagnosis and prognosis of cancer. In this study, we designed three mutant specific primer pairs corresponding to these mutations according to Tada's paper (Tada et al., 1993). The 3′-nucleotide of the sense primers were only complementary to one of the mutant codons (GAT, GTT or CGT). The sequences of R1 = 5′ biotin-GTGGTAGTTGGAGCTCA-3′, R2 = 5′-biotin-TGTGGTAGTTGGAGCTCT-3′, R3 = 5′ biotin-GTGGTAGTTGGAGCAC-3′. The sequence of the universal antisense primer was: R4 = 5′-TBR-CTATTGTTGGATCATATTCG-3′. Primer pairs used were R1–R4 for GAT mutation, R2–R4 for GTT mutation and R3–R4 for CGT mutation. The primers were synthesized by SSBE. The sense primers were all labeled with biotin by SSBE and the universal antisense primer was labeled with TBR by our laboratory.

2.3. Apparatus

A custom-built ECL detection system was described in our previous studies (Zhu et al., 2004; Liu et al., 2004). It is composed of an electrochemical detection cell, a potentiostat (Fujian Sanming HDV-7C), an ultra high-sensitive single photon counting module (Channel Photomultiplier, PerkinElmer MP-962), a multi-function acquisition card (Advantech PCL-836), a computer and LabView software. The electrochemical detection cell contains a working electrode (platinum), a counter electrode (platinum) and a reference electrode (Ag/AgCl). A magnet is placed under the working electrode for collecting the magnetic beads.

2.4. MASA–ECL detection of K-ras point mutation

Genomic DNA was extracted from the cell line and the CAC sample using UNIQ-10 column clinical sample genomic DNA isolation kit, and then amplified by the three mutant specific primer pairs respectively. MASA was performed for 35 cycles (95 °C for 40 s, 56 °C for 30 s and 72 °C for 30 s) according to the procedure reported by Tada with some modification (Tada et al., 1993). The primer pairs directly amplified an 88- or 89-base pair (bp) product from the exon 1 of *K-ras* oncogene. One positive control (using genomic DNA of SW480 cells that harbors a homozygous GGT-to-GTT mutation at codon 12 of *K-ras* as template), one negative control (using genomic DNA of 293 cells that harbors a homozygous wild-type *K-ras* as template) and a water negative control (as control for contamination) were processed in parallel with each batch of samples.

The MASA product was added into the electrochemical detection cell and incubated with streptavidinylated magnetic beads for 20 min, to form the biotin–streptavidin linkage. Unbound components were removed by washing the beads in the magnetic field twice with TE buffer (pH 7.4). Then, TPA was added into the electrochemical detection cell, where the magnetic beads were captured and temporarily immobilized on the working electrode by the magnet under it. A voltage of 1.25 V was applied across the electrode and the photon signal was measured.

Three threshold values to define a positive sample for one kind of *K-ras* point mutations is calculated based on the averages ($V_{negative}$) and the standard deviations ($V_{stdev(neg)}$) of the ECL values of the three negative controls (three MASA products amplified by the three

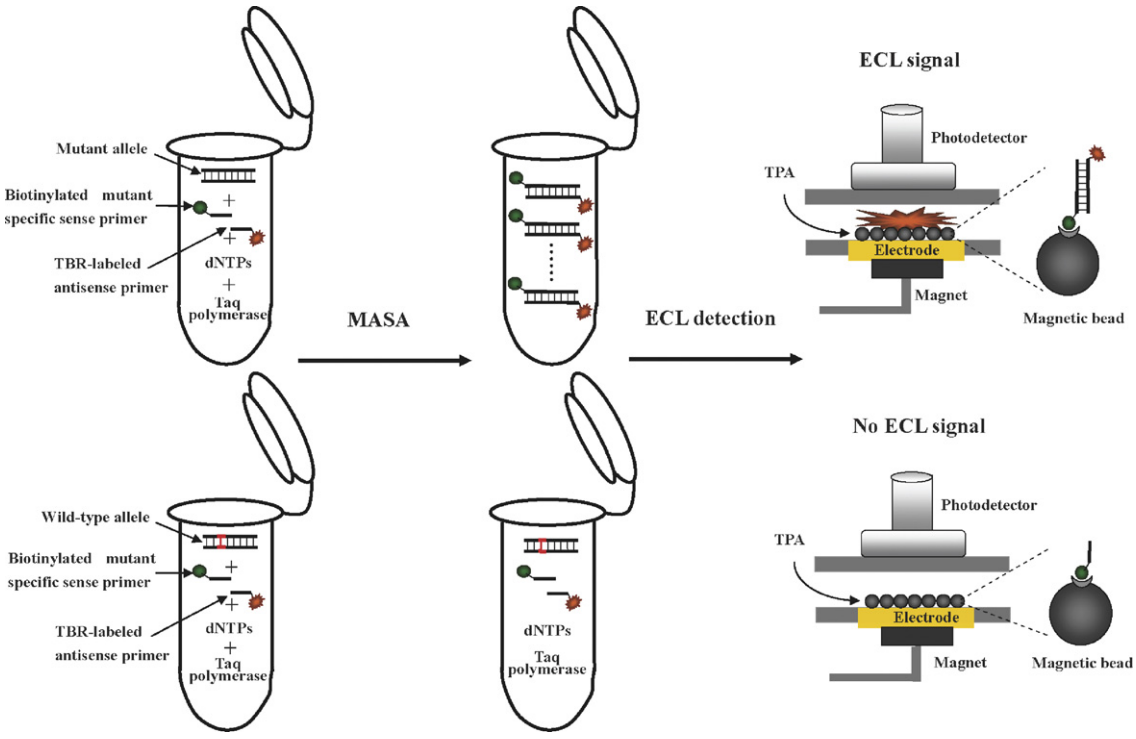

Fig. 1. The basic principle of the MASA–ECL method for the detection of *K-ras* point mutation.

mutant specific primer pairs using genomic DNA of 293 cells as templates), shown as formula (1). A sample with an ECL value higher than one of the threshold values was considered to be positive for *K-ras* point mutation.

$$V_{threshold} = V_{negative} + 3V_{stdev(neg)} \quad (1)$$

2.5. Dilution experiment

Three separate samples with different GTT mutant *K-ras* concentrations were prepared by diluting the genomic DNA of SW480 cells into the genomic DNA of 293 cells with different ratios (from 1:1 to 1:5000). Each sample was measured 30 s with one-second data integration, and the averages and standard deviations were calculated using Microsoft Excel spread sheet function.

3. Results

3.1. Experimental approach

The basic principle of the MASA–ECL method for the detection of *K-ras* point mutation is outlined in Fig. 1. Briefly, *K-ras* target DNA fragment harboring codon 12 is amplified by the mutant specific primer pair. Only the corresponding mutant *K-ras* allele is selectively amplified. Then, the MASA product is captured onto the streptavidinylated magnetic beads through biotin–streptavidin linkage. The beads are immobilized on the working electrode of the electrochemical detection cell by the magnetic field. The detection of MASA product is realized by direct measuring the ECL emission of TBR. Only the mutant *K-ras* amplification product labeled with both biotin and TBR can be detected by ECL assay.

3.2. Specificity of the MASA–ECL method

To evaluate the specificity of the MASA–ECL method, the genomic DNA of 293 cells (wild-type) and the genomic DNA of SW480 cells (GTT mutant) were amplified by the three mutant specific primer pairs (GAT, GTT and CGT) respectively, and then detected by ECL assay in turn. Results show that: in the case of 293 cells, all of the ECL values of MASA products are close to the value of ECL buffer background (Fig. 2A); in the case of SW480 cells, the ECL values of MASA products amplified by GAT and CGT mutant specific primer pairs are near to the value of ECL buffer background, while the ECL value of MASA product amplified by GTT mutant specific primer pair is significantly higher than the value of ECL buffer background (Fig. 2B). These results demonstrate a highly specificity of the MASA-ECL method.

3.3. Repeatability and reproducibility of the MASA–ECL method

To estimate the repeatability and reproducibility of the MASA–ECL method, three separate samples for each GTT mutant *K-ras* concentration were prepared and each sample was measured 30 s with one-second data integration. As shown in Fig. 3, each bar represents an average value based on 30 s measurements from the same sample, with the error bar (standard deviation) reflecting the measurement variation of the system. The variations in ECL values, among the three separately prepared samples at each GTT mutant *K-ras* concentration level, reflect the variations introduced by an operator during each assay. It can be concluded, based on the data, that the approach could be used for point mutation analysis with excellent repeatability and reproducibility.

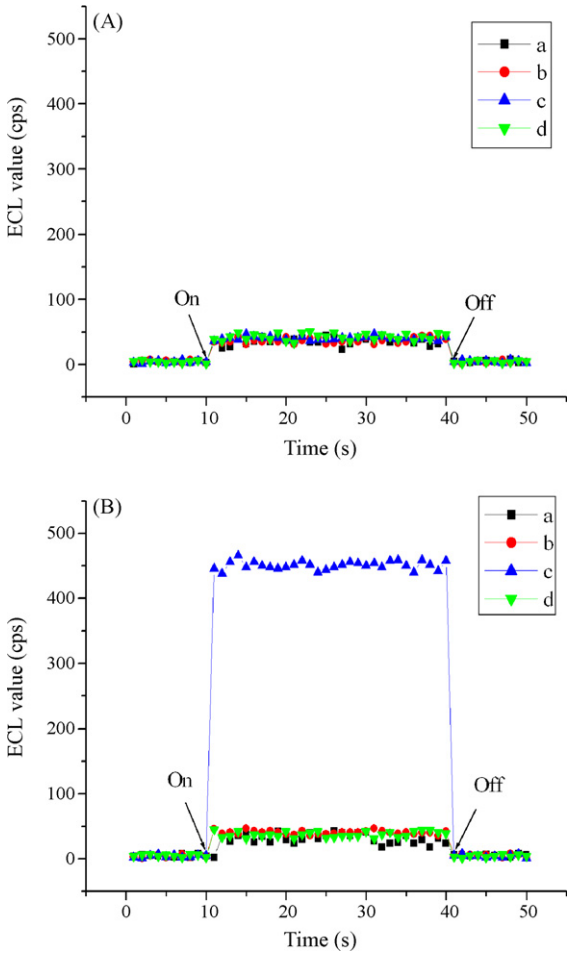

Fig. 2. Detection of 293 cells (A) and SW480 cells (B) showing the specificity of the MASA–ECL method. The genomic DNA of 293 cells (wild-type) and the genomic DNA of SW480 cells (GTT mutant) were amplified by the three mutant specific primer pairs (GAT, GTT and CGT) respectively, and then detected by ECL assay. ECL values corresponding to (a) ECL buffer background (TE + TPA), (b) genomic DNA amplified by the GAT mutant specific primer pair, (c) genomic DNA amplified by the GTT mutant specific primer pair and (d) genomic DNA amplified by the CGT mutant specific primer pair. On: potentiostat on. Off: potentiostat off.

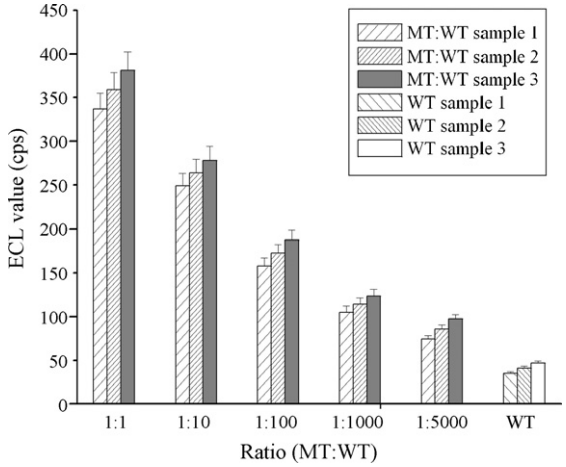

Fig. 3. Serial dilution experiment showing the sensitivity, repeatability and reproducibility of the MASA–ECL method. Three separate samples at each GTT mutant *K-ras* concentration were prepared by diluting genomic DNA of SW480 cells containing homozygous GTT mutant *K-ras* into genomic DNA of 293 cells containing homozygous wild-type *K-ras* with different ratios, and detected by the MASA-ECL method. Each analysis represents a single run with 30 s acquisition time of each concentration on each day. Error bars indicate standard deviations from 30 s acquisition time. MT: mutant type; WT: wild-type.

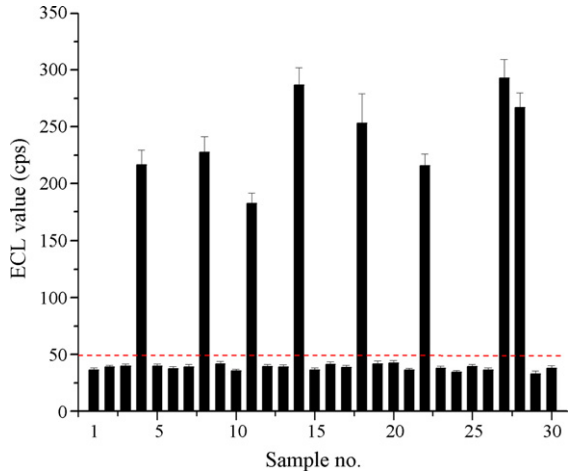

Fig. 4. Detection of GAT mutation in CAC samples by the MASA–ECL method. The genomic DNAs of thirty CAC clinical samples were amplified by the GAT mutant specific primer pair followed by ECL detection. ECL values and error bars represent the reading from independent assays and standard deviations from 30 s acquisition time. The dashed line represents the threshold value for GAT mutation.

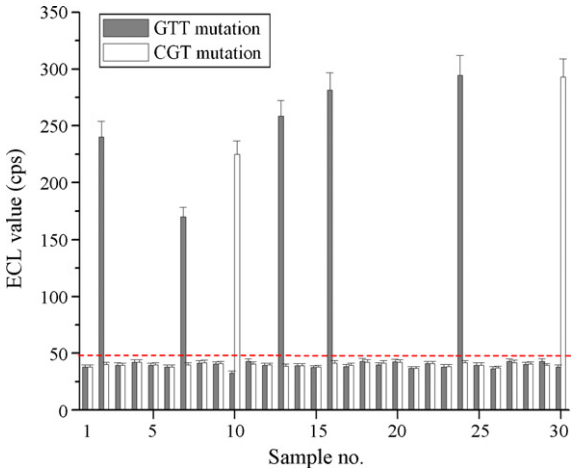

Fig. 5. Detection of GTT and CGT mutations in CAC samples by the MASA–ECL method. The genomic DNAs of thirty CAC clinical samples were amplified by the GTT and the CGT mutant specific primer pairs respectively, followed by ECL detection. ECL values and error bars represent the reading from independent assays and standard deviations from 30 s acquisition time. The dashed line represents the threshold value for GTT mutation.

3.4. Sensitivity of the MASA–ECL method

The sensitivity of the MASA–ECL method is evaluated by dilution experiment using different concentrations of GTT mutant *K-ras* (genomic DNA of SW480 cells) diluted into wild-type *K-ras* (genomic DNA of 293 cells). The result shows that the method can detect *K-ras* mutant in a 5000-fold excess of wild-type allele (Fig. 3).

3.5. MASA–ECL detection of K-ras point mutation in CAC samples

To set the threshold values for point mutation detection, the genomic DNA of 293 cells amplified by the three mutant specific primer pairs were used as negative controls. The average ECL values of the negative controls are 38.9 ± 2.1 counts per second (cps) for GAT mutation, 41.0 ± 2.3 cps for GTT mutation and 43.4 ± 2.4 cps for CGT mutation. Based on the formula (1), we estimated the threshold values are 45.2 cps for GAT mutation, 47.9 cps for GTT mutation and 50.6 cps for CGT mutation.

Thirty CAC clinical samples were evaluated for *K-ras* point mutation by the MASA–ECL method. Results show that fifteen of the thirty samples (50%) were detected positive for *K-ras* point mutation, eight of which were GAT mutation (Fig. 4), five were GTT mutation and two were CGT mutation (Fig. 5).

4. Discussion

Recently, we have developed a highly sensitive, safe and simple ECL–PCR method for point mutation detection. In the ECL–PCR assay, the biotin label was used to selectively immunomagnet capture the target DNA by the streptavidinylated magnetic beads. TBR label was used to react with TPA to emit light for ECL detection. For discrimination of the wild-type allele and the mutant alleles, the assay needs to digest PCR product with a restriction enzyme, which only cut the wild-type allele containing its specific restriction site and thus removed the TBR-labeled 5′ part of the PCR product from the biotinylated 3′ part. This digestion process greatly prolongs the detection time (more than 1 h). Further more, the method cannot discriminate different kinds of mutations (Zhu et al., 2004). In this study, a novel MASA–ECL method which can overcome the shortcomings of the ECL–PCR assay has been developed. Compared with ECL–PCR method, the MASA–ECL method is faster and simpler by avoiding digestion of PCR product with restriction enzyme. The whole detection process can be completed in less than 1.5 h. Moreover, it can discriminate different kinds of mutations.

The MASA–ECL method does not need to use any hazardous materials or any complicated and time-consuming steps that are commonly used in the electrophoresis-based analysis. Therefore, it is faster and safer than the electrophoresis methods.

MASA is based on the rationale that a DNA primer with a 3′ end complementary to a specific gene mutation will preferentially allow chain elongation of mutant DNA sequence during PCR (Tada et al., 1993; Paranavitana, 1998; Fox et al., 1998; Clayton et al., 2000). If appropriate mutant specific primer pair is designed, it will only allow corresponding mutant allele being selectively amplified. Therefore, it can distinguish different mutants. When developing the mutant specific sense primers to detect *K-ras* point mutations, we found that the wild-type allele was also amplified by the mutant specific primer pairs. We therefore included an extra mismatch at position-2 of the sense primers to further destabilize the annealing of primers to target sequence and obtained a satisfying result (Fig. 2). Different mutations (GAT, GTT and CGT) at codon 12 of *K-ras* oncogene were clearly discriminated (Figs. 4 and 5), demonstrating a high specificity of this method.

The use of sensitive molecular techniques to detect rare cells in a population is of increasing interest to the molecular pathologist, but detection limits are often poorly defined in conventional methods. Recently, ECL has become an important and powerful analytical tool due to its sensitivity (Xu and Dong, 1999; Chen et al., 2003; Bard, 2004; Richter, 2004; Lin et al., 2007; Wei et al., 2007; Zhang et al., 2008a,b; Zhu et al., 2008; Miao, 2008; Dai et al., 2009). The MASA–ECL method takes on the sensitivity of ECL and the specificity of MASA. Therefore, it is highly sensitive, which can detect mutant allele in the presence of a very large excess of normal allele, as demonstrated by detecting *K-ras* mutant in a 5000-fold excess of wild-type allele in our study.

It is well documented that malignant transformations are a result of the accumulation of carcinogenic steps corresponding to activation of oncogenes and inactivation of tumor suppressor genes. Among the available candidates, the *K-ras* oncogene is the most well-studied cellular gene whose alterations seem to have an important role in the pathogenesis of human cancer (Bos, 1988). The gene, which encodes 21-kDa GTP-binding protein, controls the mechanisms of cell growth and differentiation. Point mutations in the *K-ras* oncogene lead to uncontrolled stimulation of *ras*-related

functions by the altering p21 *ras* protein-related pathway, locking it in the "on" position for signal transduction (Adjei, 2001; Chen et al., 2004). *K-ras* oncogene mutations have been reported as early events in colorectal tumorigenesis (Zhu et al., 1997; Hardy et al., 2000). The point mutations reside mainly in the first two nucleotides of codon 12 (Chen et al., 2004). As mutant *K-ras* alleles seem to be clinically useful tools for diagnostic and prognostic purposes, rapid and reliable methods are needed to analyze this potential molecular tumor marker.

Up to now, *K-ras* mutations are detected mainly by mutation-specific oligonucleotide hybridization, RFLP (Dieterle et al., 2004), SSCP, MASA and direct sequencing (Nollau and Wagenera, 1997). These methods are multiple steps, time-consuming and need to use hazardous materials. They are impractical for routine clinical use. Therefore, a highly sensitive, yet simple and safe approach for *K-ras* point mutation detection is expected. In this study, a sensitive, simple, rapid, safe and specific MASA–ECL method was developed. Its capability for point mutation detection was demonstrated by detecting the possible point mutation at codon 12 of *K-ras* oncogene in CAC.

Though our preliminary clinical investigation is limited due to the restricted access to more CAC samples, the results of our study are in accord with the results from what reported by others. The positive rate of *K-ras* point mutation detected by MASA–ECL assay in CAC (50%) is consistent with previously reported frequency (30%–60%) (Zhu et al., 1997; He et al., 2002; Pan et al., 2002; Zhang et al., 2002; Prix et al., 2002; Dieterle et al., 2004; Gao et al., 2005), demonstrating a high accuracy of the approach. Furthermore, different kinds of mutations are clearly differentiated. This information is useful in pathological analysis.

5. Conclusions

Compared with electrophoresis-based methods, the MASA–ECL method is faster and safer. It does not use any hazardous materials or any complicated and time-consuming steps that are commonly used in the electrophoresis analysis. It is also faster and simpler than the ECL–PCR approach by avoiding digestion of PCR product with restriction enzyme. It can detect *K-ras* mutant in a 5000-fold excess of wild-type allele. Different kinds of mutations can be clearly discriminated. Therefore, the MASA–ECL method could potentially become a sensitive, specific, simple, rapid and safe approach for point mutation detection.

Acknowledgments

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