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Simultaneous genotyping of multiplex single nucleotide polymorphisms of the K-ras gene with a home-made kit

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Abstract

Accurate and fast genotyping of single nucleotide polymorphisms (SNPs) is important in the human genome project. Here an automated fluorescent method that can rapidly and accurately genotype multiplex known SNPs was developed by using a homemade kit, which has lower cost but higher resolution than commercial kit. With this method, oncogene K-ras was investigated, four known SNPs of K-ras gene exon 1 in 31 coloerctal cancer patients were detected. Results indicate that mutations were present in 8(26%) of 31 patients, and most mutations were localized in codon 12. The presence of these mutations is thought to be a critical step and plays an important role in human colorectal carcinogenesisas.

Keywords: K-ras gene; Single nucleotide polymorphism; SNaPshot

1. Introduction

Point mutation in human genome may result in occurrence of heritable genetic diseases and most cancers, searching for these mutations is becoming more and more important. Recently, capillary electrophoresis (CE) has become an attractive method for DNA analysis and mutation detection. In CE, to get an efficient separation of DNA, the quality of the sieving matrix is a key to success. Many kinds of polymers have been applied to DNA analysis, including linear polyacrylamide (LPA) [1–4], poly(dimethylacrylamide) (PDMA) [5] and some other copolymers. To our knowledge, most commercial polymers have various limitations, such as high cost, poor resolution, long analysis time and so on.

In our previous study [6], a new automated fluorescent method that can rapidly and accurately genotype multiplex known as single nucleotide polymorphisms (SNPs) was developed by using a commercial kit and substantially improving a commercially available protocol (SNaPshot, ABI). The mismatch repair gene hMLH1 and cancer repression gene P53 were investigated on an ABI 310 genetic analyzer (Applied Biosystems, ABI), the result was proved by DNA sequencing. In addition, this method was used to type multiplex SNPs of Y chromosome [7] and Cytokine gene [8] in other published reports,

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but all above reports made use of the expensive commercial POP-4 matrix (ABI), which was composed of PDMA, used in the ABI capillary genetic analyzers. There are no reports about homemade LPA for SNaPshot analysis on the ABI capillary genetic analyzer, and no systematic comparison about the sieving capability of LPA and PDMA.

Oncogene ras product has a key role in controlling cell growth and differentiation through its intrinsic GTPase activity [9]. It also seems to interact with pathways that regulate programmed cell death [10] with other oncogenes, and may also play a part role in drug resistance mechanisms [11]. K-ras gene [12], one of three human ras genes recognized, is reported to mutate in about half of all patients with colorectal cancers [13]. And the hot spots of mutation are localized in codons 12 and 13 of Exon 1.

In this paper, we prepared an inexpensive homemade kit—LPA polymer and neutral coated capillary—and successfully applied it to SNaPshot analysis of K-ras gene on ABI 310 genetic analyzer. Results demonstrate that, under optimized conditions, homemade LPA has higher resolution and shorter analysis time than commercial POP-4 matrix. By using the home-made kit, four known SNPs of K-ras gene in 31 colorectal carcinomas were detected, our data indicate that mutations were present in 8(26%) of 31 patients investigated, and most mutations were localized in codon 12.

2. Materials and methods

2.1. Apparatus and materials

ABI 310 genetic analyzer and PE model 2700 PCR system were purchased from PE Applied Biosystems (ABI, Foster City, CA, USA). Bare fused-silica capillary with 50-μm internal diameter (I.D.) and 365-μm outer diameter (O.D.) were obtained from PE Applied Biosystems (Foster City, CA, USA) and Yongnian Optical Fiber Factory (Yongnian, Hebei, China).

γ-Methacryloxypropyltrimethoxysilane, acrylamide, ammonium peroxydisulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED) and Tris–boric–EDTA buffer (89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA, pH 8.3) were purchased from Sigma (St. Louis, MO, USA). PCR Amplification Kit and primers were obtained from TaKaRa Biotechnology (Dalian) Co., Ltd. SNaPshot Ready Reaction Mix (containing AmpliTaq DNA Polymerase FS, four labeled ddNTPs and reaction buffer), Exonuclease I(Exo I)and Shrimp Alkaline Phosphatase(SAP)were purchased from PE Applied Biosystems (Foster City, CA, USA).

2.2. Polymer synthesis and capillary coating

Short-chained LPA was synthesized in our laboratory. In a typical procedure, a solution of acrylamide (10 g) in deionized water (110 ml) was placed in a 300-ml airtight flask, immersed in a water bath kept at 50 °C, and degassed by bubbling N_2 for 30 min. Next, 3.5 ml 2-propanol, 0.625 ml 10% (w/v) APS and 0.625 ml 10% (v/v) TEMED were added in a consecutive fashion, and the resulting mixture was kept at 50 °C under N_2 flow for 1.5 h to ensure anaerobic conditions. The product was then loaded into a tubular cellulose ester membrane and dialyzed against deionized water for 3 days with daily water changes. Purified LPA was dissolved in 1×TBE buffer (89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA) at different concentrations containing 5 *M* urea and stored at 4 °C.

The inner surface of the capillary was modified by covalent bonding of hydrophilic polymer [14]. Firstly a new capillary was rinsed with 0.2 *M* NaOH, deionized water, and methanol respectively. Then the capillary was filled and left standing for 1 h with 1:1 3-methacryloxypropyltrimethoxysilan and methanol solution, and then for another hour with 3.4% acrylamide solution (10 ml 3.4% acrylamide solution containing 6 μl TEMED and 60 μl 10% APS). At last the capillary was rinsed with deionized water for 20 min. The detection window was formed by stripping 6 mm of polyimide coating off the capillary with a razor blade, and the effective length is 40 cm.

2.3. DNA extraction and PCR

The tissue samples used for analysis were obtained from the Secondary Affiliated Hospital of Dalian Medical University. All tumor tissues have been confirmed by pathology. Genomic DNA was extracted from frozen tissues by proteinase K digestion

and phenol–chloroform extraction using the method of Sambrook et al. [15] with minor modification.

Polymerase chain reaction (PCR) amplification of K-ras exon 1 was performed using 5′-ACTGAATATAAACCTGTGGTAGTTGGAGCT-3′ (forward) and 5′-CGTCCACAAAATGATTCTGAATTAGC-3′ (reverse) primers and the length of the product was 89 bp. The reaction mixture contained 10 m*M* Tris–HCl (pH 9.0), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 μ*M* each of dNTPs, 1 μ*M* each primer, 100–200 ng of extracted genomic DNA and 1.25 U Taq DNA polymerase in a total volume of 50 μl. PCR was performed on a Perkin-Elmer Model 2700 PCR system and the cycling parameters were 30 cycles under the following program: 40 s at 94 °C, 1 min at 50 °C, 2 min at 72 °C.

After PCR amplification, the resulting template is in solution, along with primers, dNTPs, enzyme and buffer components. To avoid participation in the subsequent primer-extension reaction, primers and unincorporated dNTPs must be removed with the following steps. Add 2 U Exo I and 5 U SAP to 15 μl PCR product, mix thoroughly and incubate at 37 °C for 1 h, followed by a 15-min incubation at 75 °C to inactivate the enzymes.

2.4. Multiplex single base extension reaction

Primers used for Multiplex single base extension reactions of K-ras gene were 5′-GCACTCTTGCCTAGGCCA-3′ (1, codon12-2, reverse); 5′-CGTCCACAAAATGATTCTGAATTAGC-3′ (2, codon22-2, reverse); 5′-ACTGAATATAAACTTGTGGTAGTTGGAGCT-3′ (3, codon12-1, forward); 5′ - ACTGAATATAAACTTGTGGTAGTTGGAGCTGGT-3′ (4, codon13-1, forward).

Uniplex SNaPshot reaction (3 μl PCR product, 2 μl primer, 2 μl SNaPshot Ready Reaction Mix, 3 μl ddH_2O) was performed as described by the manufacturer (ABI, CA) and the migration time of each uniplex SNaPshot reaction product was determined. Then multiplex single base extension reactions were performed in 12 μl solution containing 6 μl purified PCR product, 2 μl SNaPshot Ready Reaction Mix, 1 μl of each primer(0.5–2 pmol) and were carried out for 25 cycles under the following program: 10 s at 96 °C, 5 s at 50 °C, and 1 min at 60 °C.

After multiplex single base extension, removal of the 5′ phosphoryl groups by phosphatase treatment can alter the migration of the unincorporated ddNTPs and prohibit interference. So each reaction product was treated with 1 U SAP for 1 h at 37 °C, and followed by a 15-min incubation at 75 °C for SAP inactivation.

2.5. Capillary electrophoresis (CE) analysis

Homemade kit includes a 50 μm (I.D.)×365 μm (O.D.)×61 cm neutral coated capillary and 6–10% LPA (containing 5 *M* urea). Capillary electrophoresis analysis was performed on the ABI 310 genetic analyzer with homemade kit and POP-4 matrix. Subsequently, 5 μl purified extension product was diluted with 10 μl formamide, denatured at 95 °C for 5 min, chilled on ice and placed in the tray of the analyzer. CE analysis was automatically performed at 50 °C and 15 kV for 30 min. The buffer consisted of a 1×Tris–borate EDTA with 5 *M* urea. The data were collected by data collection software and analyzed by Gene-Scan 3.1 software.

3. Results and discussion

3.1. Multiplex single base extension reaction and primer design

In this method, a DNA oligomeric primer is designed that anneals one base 5′ of the locus that is to be typed. A DNA polymerase, in the presence of only ddNTP chain-terminating monomers, then appends a single DNA base to the 3′ end of the DNA primer. The identity of this appended base is then indicative of the genotype of the original template molecule. Using fluorescently labeled ddNTP in the reaction mix allows for facile determination of the appended base identity through fluorescence detection. Multiplexing single base extension to genotype multiplex SNPs in a single reaction can not only allow for decreased reagent consumption and lower cost of analysis but also requires the separation of the different reaction products, so multiple DNA primers that have different migration time must be designed.

It is reported,there is a linear relationship between

DNA size and migration time from approximately 75 to 500 bases in size under denatured condition [16], out of the range the effect of sequence diversity should not be ignored. In this experiment, the lengths of all SNaPshot reaction products are less than 40 bp, the sequence diversity can result in different mobility, even cause the overlap of the peaks. In order to avoid this overlap, the difference of adjacent primer in length had better be longer than 5–6 bp. Moreover, similar annealing temperature (melting temperature, T_m) of different fragments should be kept which allow for efficient nucleotide incorporation. In addition, the position of the extension primers is limited as primers have to be designed to bind immediately adjacent to the SNP, and the binding of extension primers on either the sense or antisense strand will lead to different bases being incorporated.

Multiplex SNaPshot reaction to genotype multiplex SNPs in a single reaction can not only decrease reagent consumption but also increase the throughput of detection. Through choosing a suitable ratio of template and corresponding primer, mixing all the primers and PCR products, Several SNPs can simultaneously be genotyped by any four-color or five-color fluorescent detection device. In this paper, we choose four known SNPs of K-ras gene, firstly type single SNP by uniplex SNaPshot reaction and determine the migration time of each SNaPshot reaction product, then perform multiplex SNaPshot reaction and simultaneously genotype four different SNPs.

3.2. Establishment of SNaPshot CE analytical method

To get an ideal resolution, the running conditions had to be optimized. Firstly, the effect of LPA concentration on SNaPshot analysis was investigated (Fig. 1). With the increase of gel concentration, resolutions of several peaks were improved. In order to get an efficient separation within a reasonable time, 8% LPA was chosen as an optimized gel concentration in the following experiment. Column temperature and urea usually help DNA samples to denature completely, so 50 °C was selected as the most convenient temperature in following experiments, and 5 *M* urea was added to all separation

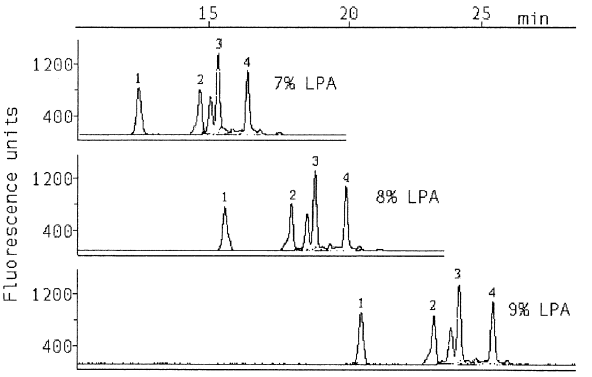

Fig. 1. Effect of LPA concentration on SNaPshot analysis of K-ras gene. Electrophoretic conditions: sieving medium, homemade LPA; DNA fragment, K-ras gene exon 1; buffer, 1×TBE (5 *M* urea); applied voltage, −15 kV; temperature, 50 °C; 1.codon 12-2; 2.codon 22-2; 3.codon 12-1; 4.codon 13-1.

matrices and buffer. In addition, separation voltage between 11 and 15 kV appeared to have little effect on SNaPshot analysis, 15 kV with a short analytical time was an optimized running voltage.

As we see, there are four peaks indicate four known SNPs of codon 12-2, codon 22-2, codon 12-1 and codon 13-1 in the electropherograms. In addition, there is a parasite peak between peak 2 and peak 3, which is not a homozygosity or heterozygosity mutation but a non-specific amplification product of primer 3, appearing in all tumor samples. In subsequent experiment we have found that this peak can be removed by changing either the initial PCR primer or SNaPshot reaction primer 3.

At a similar mode, the operational conditions for commercial POP-4 matrix was optimized, it was found that under optimized condition, homemade LPA exhibited higher resolution and shorter analysis time than commercial POP-4 matrix (Fig. 2).

It was suggested in some papers that the more hydrophobic character of PDMA is partly responsible for its lower performances in DNA sequencing [17]. With the same read length, the migration time of PDMA was approximately 20% longer than LPA [18]. Our results also demonstrate that for rapid and high-resolution analysis of SNaPshot, hydrophilic polymers such as LPA provide better performance than hydrophobic PDMA.

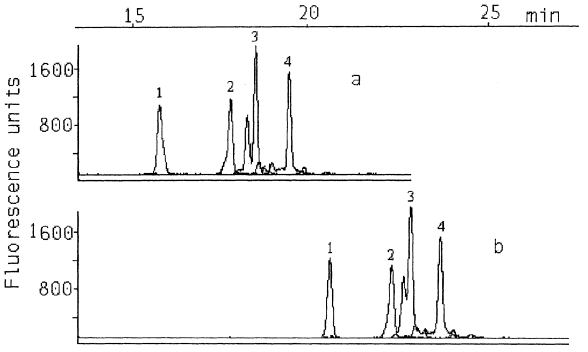

Fig. 2. SNaPshot electropherograms of K-ras gene with different matrix. Electrophoretic conditions: sieving medium, (a) Homemade 8% LPA matrix; (b) Commercial POP-4 matrix; DNA fragment, K-ras gene exon 1; buffer, 1×TBE (5 *M* urea); applied voltage, −15 kV; temperature, 50 °C; 1.codon 12-2; 2.codon 22-2; 3.codon 12-1; 4.codon 13-1.

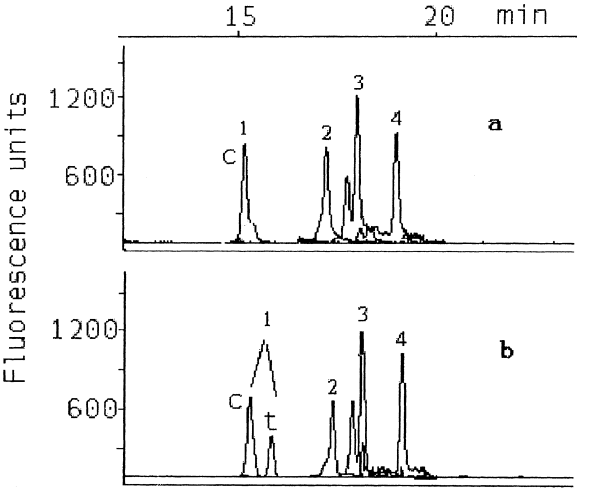

Fig. 3. SNaPshot electropherograms of wild-type and mutant-type samples. Electrophoretic conditions: sieving medium, homemade 8% LPA; DNA fragment. (a) K-ras gene exon 1 of wild-type sample. (b) K-ras gene exon 1 of mutant sample; buffer, 1×TBE (5 *M* urea); applied voltage, −15 kV; temperature, 50 °C; 1.codon 12-2; 2.codon 22-2; 3.codon 12-1; 4.codon 13-1.

3.3. Analysis of tumor samples

Thirty-one colon tumor samples were collected, four known SNPs of K-ras gene in these patients were detected using multiplex SNaPshot analysis. K-ras mutations were found in a total of 26%(8 of 31) of tumor samples. This finding is in good agreement with the frequency of 30% reported in a recent detection of K-ras mutation frequencies in colorectal cancer [19], and mutations were composed of several types of point mutations in the first and second positions of codon 12 and in the first position of codon 13. Fig. 3 shows electropherograms of the wild type and mutant (codon 12) sample of K-ras gene exon 1. The first peak is indicative of a ''C'' genotype in codon 12-2 of wild-type sample; but in the mutant sample, there are two peaks showing ''C'' and ''T'' genotype, which indicate a C→T heterozygosity mutation. Consistent with literature reports, the majority of K-ras mutations were base pair transitions, occurring predominantly in codon 12, with one case of mutation in codon 13, no mutations were found in codon 22 in this patient group. The detailed distribution of specific K-ras point mutations is shown in Table 1.

Although the real role of K-ras gene is not very clear, it has long been considered as an important initial event in the development of colorectal neoplasia [20,21], and associated with a lack of response to adjuvant chemotherapy [22]. The presence of most mutations in codon 12 is thought to be a critical step and plays an important role in human colorectal carcinogenesisas.

Similarly, this home-made kit can also be used to the CE-SSCP analysis and tandem CE-SSCP/heteroduplex analysis (HA) of P53 gene, the result will be presented in a separate article.

Table 1
Mutations of the K-ras gene exon 1

Case numbers	Mutation	Amino acid change
7	G→A	Gly12Asp
10	G→A	Gly12Asp
14	G→A	Gly12Asp
16	G→A	Gly12Asp
16	G→A	Gly12Ser
20	G→A	Gly12Ser
26	G→A	Gly12Ser
28	G→A	Gly12Ser
47	G→A	Gly12Ser
28	G→A	Gly13Ser

4. Conclusions

Detection of mutations in genes responsible for hereditary diseases (i.e. cancer) is important clinically. Here homemade LPA was firstly applied for SNaPshot analysis of K-ras gene on an ABI capillary genetic analyzer, its sieving capability is better than commercial POP-4 matrix, but the cost is lower. The application of multiplex SNP genotyping method can also significantly increase the throughput and decrease the cost of analysis. Detection results of K-ras gene in colorectal samples indicate that mutations were present in 8(26%) of 31 patients, and most mutations were localized in codon 12. The presence of these mutations is thought to be a critical step and plays an important role in human colorectal carcinogenesisas.

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