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Detection of rare point mutation via allele-specific amplification in emulsion PCR

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It is essential to analyze rare mutations in many fields of biomedical research. However, the detection of rare mutations is usually failed due to the interference of predominant wild-type DNA surrounded. Herein we describe a sensitive and facile method of detecting rare point mutation on the basis of allele-specific amplification in emulsion PCR. The identification and selective amplification of rare mutation are accomplished in one-pot reaction. The allele-specific primers coupled on magnetic beads allow the exclusive amplification and enrichment of the mutant amplicons. The productive beads bearing mutant amplicons are subsequently stained with the fluorescent dyes. Thus, the rare point mutations with a percentage as low as 0.1%, can be detected by fluorescent analysis. The relative percentages of mutation among different samples can be roughly accessed by counting the fraction of fluorescent positive beads through flow cytometry. [BMB Reports 2013; 46(5): 270-275]

INTRODUCTION

The detection of point mutation or single nucleotide polymorphism (SNP) is essential in many fields of biomedical research such as molecular diagnosis, personalized therapy, and drug development (1-3). In the last decades, the detection of rare mutation of cancer genes in the circulating free DNA (cfDNA) of patient's blood has attracted increasing attention for its potential in tumor diagnosis and personalized treatment (4-7). The mutations in the blood are contribute to the DNA release of apoptosis and necrosis of tumor cells because they bear the uncommon variations that consistent with that of DNA isolated from tumor cells. Detection of such mutations in the blood can provide wealth of information for tumor diagnosis, prognosis, and therapy. However, the detection of rare mutations in cfDNA has been proved very difficult (8-10). The percentage of mutation is usually less than 10% in the total cfDNA. In some case, it is even less than 0.1%. There are predominant wild-type alleles derived from the apoptosis of normal cells in the blood. And there is only one nucleotide variation in DNA sequence between wild-type DNA and point mutation. Thus, the rare mutations among large excess wild-type alleles are hard to be detected by conventional assays for gene variation. Take the gene sequencing technique, the golden standard for detecting gene variation first. The mutation with the percentage less than 20% is hardly to be identified by gene sequencing. Attempts to increase the percentage of rare mutation by polymerase chain reaction (PCR) often fail owing to the interference of wild-type DNA (11, 12). The predominant wild-type DNA tends to be amplified prior to the mutant alleles in PCR, which leads to the dilution of mutation. Similarly, the assays of gene variation based on DNA hybridization technique like southern blot cannot accurately discriminate the rare mutations among the predominant wild-type DNA alleles. The low specificity of DNA hybridization is apt to generate the false positive result. Other assays for gene sequence variation, such as the restriction fragment length polymorphism (RFLP), the single strand conformation polymorphism (SSCP) and the quantitative PCR (qPCR) based on TaqMan probe also fail to detect such rare mutation with the percentage lower than 1% due to their intrinsic problems in sensitivity, specificity, compliance and so on (11, 13-15).

In the last decade, microsphere-based suspension array technologies (namely microsphere arrays or liquid chip) like Luminex *xMAP*™ technique have attracted more and more attention for their advantages in high-throughput, sensitive and specific detection of nucleic acid (16, 17). Since the recognition of mutant sequence still depends on the conventional DNA hybridization in microsphere array, the application of microsphere array is limited for the detection of rare mutation. Recently, a sensitive method for detecting rare mutation, BEAMing technology, has been developed (10, 18, 19). Both wild-type and mutant alleles are amplified and the amplicons are bound to the magnetic beads in emulsion PCR. Then wild-type and mutant DNA amplicons are discriminated by the molecular beacons and subsequently labeled with two dis-

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tinguishing fluoresceins (18). The population of DNA fragments is transferred to the population of the beads stained with different fluorescence. Thus, the percentage of mutations can be roughly accessed by counting the fluorescent beads through flow cytometry assay. However, the whole detecting process is time-consuming because the processes of amplification and discrimination of mutations are performed separately. Moreover, the synthesis of molecular beacons is complex and expensive, which also limits the application of BEAMing technology in routine analysis of rare mutation in ordinary laboratory.

Herein, we developed a facile and sensitive method to detect the point mutation with a percentage as low as 0.1% among wild-type alleles. A point mutation contained in TP53 gene (674A>G) was used as the model for evaluation. The exclusive amplification and the discrimination of mutation were accomplished in one-pot reaction with the aid of allele-specific primers in emulsion PCR. The mutant and wild-type DNA templates were separated into single water-in-oil compartments to avoid the interference of predominant wild-type DNA on the amplification of rare mutation. The allele-specific primer (forward primer) coupled on magnetic beads allow the selective amplification of rare mutation in emulsion PCR. After amplification, the productive beads harboring thousands of mutant amplicons were separated from emulsion and then stained with streptavidin-R-phycoerythrin (SAPE). The fluorescent signal of mutations on the beads could be measured by fluorescent analysis.

RESULTS AND DISCUSSION

The whole process of detection was illustrated in Fig. 1. The exclusive amplification and the discrimination of rare mutation were simultaneously accomplished in emulsion PCR. The allele-specific primer (forward primer) was coupled to the surface (5′ attached) of micron-scale magnetic beads that were also involved in the emulsion PCR. The allele-specific primer allowed the exclusive amplification of mutant DNA owing to its unique structure. The reverse primer was modified with biotin at its 5′ end to facilitate the subsequent fluorescence staining with SAPE. The concentration of DNA template and bead were carefully controlled so that there was either one or zero template in most bead-containing compartments. Only in the productive compartments where both bead and mutant DNA molecule presented did the amplification take place. As a result, each productive bead would bear thousands of the copies corresponding to the amplification of a single mutant DNA molecule. The amount of mutant DNA molecules could be transferred to that of the productive beads due to their one-to-one correspondence in the compartments. After emulsion PCR, the beads were collected by magnetic separator and only the productive beads could be subsequently stained with SAPE owing to the reaction between biotin and streptavidin. Finally, the fluorescent signal of the beads was measured through fluorescent analysis to identify the rare mutation among large excess wild-type alleles.

The feasibility of exclusive amplification of point mutation was testified by the conventional PCR (Fig. 2A) and the emulsion PCR (Fig. 2B) respectively. The products of amplification were examined by agarose gel electrophoresis. The product derived from PCR reaction without adding DNA template was set as the negative control group (Negative). As shown in Fig. 2A, no significant band corresponding to the target sequence was yielded in the gel for both the negative control and the

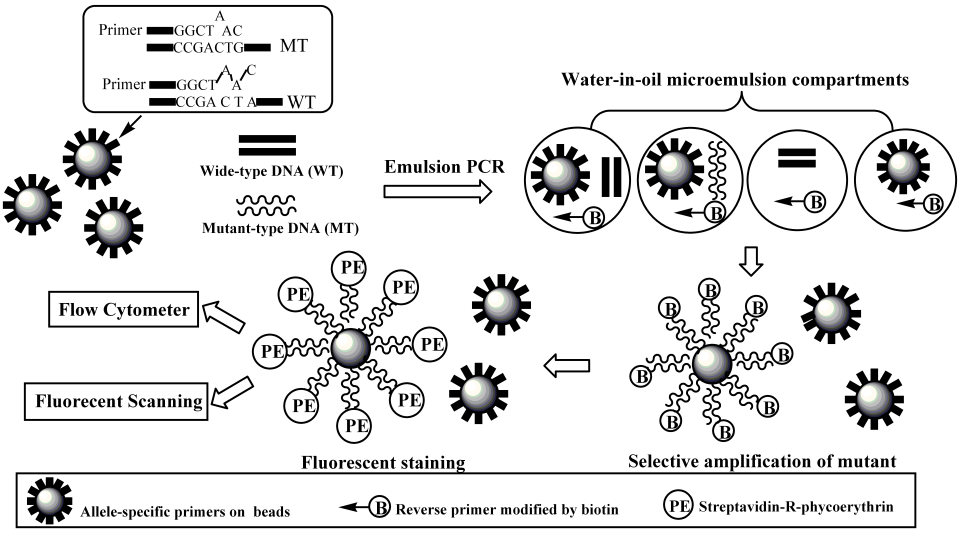

Fig. 1. Scheme of detecting the rare mutation via allele-specific amplification in emulsion PCR.

wild-type DNA assay, while a bright band of the target sequence presented for the mutation assay. Considering the difference between emulsion PCR and conventional PCR, we used the free allele-specific primers that did not couple with the beads to evaluate the specificity of amplification in emulsion PCR. As shown in Fig. 2B, only the mutant alleles significantly amplified through emulsion PCR, which was consistent with the result of the conventional PCR. The high specificity of amplification was attributed to the unique sequence of the allele-specific primer. As mentioned in the Material and Methods section, there were two mismatched nucleotides at 3′ end of the allele-specific primer for wild-type allele but only one mismatched nucleotide at the next near 3′ end of the primer for mutant allele. Beside the restricting effect of the mismatched nucleotide on the hybridization of allele-specific primer to target DNA, the structure variation between Taq DNA polymerase and the allelic primer was dominant for the amplification (20). The neighboring curving structure at the 3′ end of the allelic primer could not form a suitable substrate to combine Taq DNA polymerase for wild-type allele. As a result, the amplification of wild-type allele was limited while the rare mutation was successfully amplified.

After emulsion PCR, all of the beads were collected by magnetic separator and then stained with SAPE. The fluorescent intensities of the beads were measured by fluorescent scanner and the result was showed in Fig. 3. The blank beads (Beads) without involving in emulsion PCR were also stained with SAPE and its signal was measured. The beads that derived from the emulsion PCR without adding template DNA was set as the negative control (Negative). In order to evaluate the sensitivity of mutant detection of this assay, the mutant genomic DNA was diluted by wild-type genomic DNA to form a series of solutions with gradient percentages of mutation from 0.1% to 1%. The diluted solutions were used as the templates of pre-amplification and then the purified products were used as the templates in the subsequent emulsion PCR process. The resulting beads of emulsion PCR were represented by the corresponding percentages of mutations (%MT) in the original genomic DNA population. As shown in Fig. 3, the intensity of the beads of the negative control group was a little higher than that of the blank beads (5446 vs 4157). It might be attributed to the formation of a few cross dimers on the beads in the emulsion PCR because the cross dimers could also be stained with SAPE. The intensity of the beads of the pure wild-type DNA group (WT) was slightly higher than that of the beads from Negative group, but significantly less than that of the beads of the pure mutant DNA group (MT). It proved that the allele-specific primers had restricting effect on the amplification of wild-type DNA. The intensity of beads of 0.1% MT group was significantly higher than that of beads from wild-type DNA group (9733 vs 8255). It proved that the point mutation with a percentage as low as 0.1% could be detected by this assay. With the increase of the percentages of mutation in the original sample, the fluorescent intensity of the resulting beads gradually increased. But the fluorescent intensity of the beads did not increase in proportion to the rise of mutation percentages in original sample completely (Fig. 3 inset). The deviation of fluorescent intensity of the beads might be caused by the loss of the beads during the magnetic capture and washing processes.

Since the total fluorescent intensity of the beads was apt to be affected by the loss of the beads, it might be more reliable

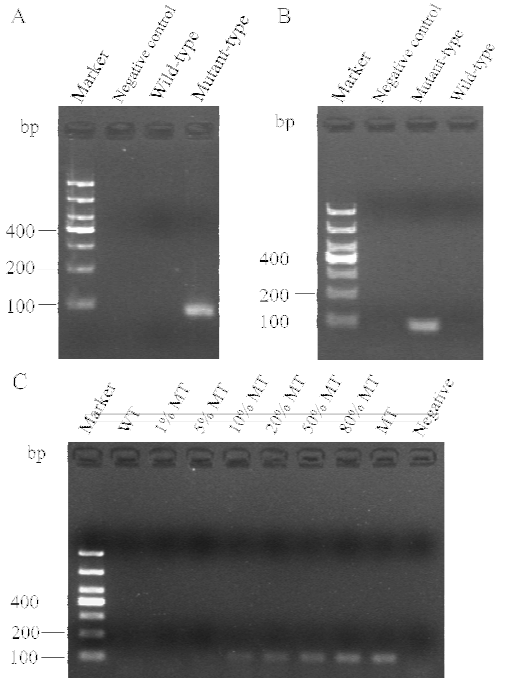

Fig. 2. The specificity of allele-specific amplification in conventional PCR (A) and emulsion PCR (B) were examined by agarose gel electrophoresis respectively. The sensitivity of mutation detection based on allele-specific PCR was also evaluated by agarose gel electrophoresis (C).

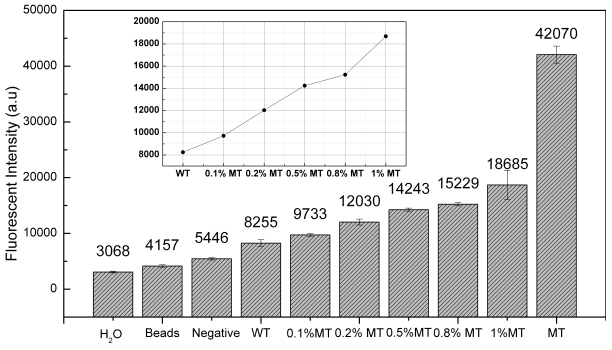

Fig. 3. Fluorescent intensity of the beads derived from emulsion PCR as a function of the percentage of mutations.

to measure the fraction of the fluorescent beads rather than the total fluorescent intensity in order to get the precise percentage of mutation. The fraction of the fluorescent-positive beads was counted by flow cytometry and the result was showed in Fig. 4. The fractions of fluorescent-positive beads increased with the rise of mutation percentages in original genomic DNA population. The fraction of fluorescent-positive beads of 0.1% MT was significantly higher than that of pure WT (2.45% vs 0.93%), which confirmed the high sensitivity of this assay for the rare mutation detection. For comparison, we evaluated the sensitivity of allele-specific PCR (ASP) based on agarose gel electrophoresis. The genomic DNA solutions with various percentages of mutant alleles (1-100% MT) were used as the DNA templates and the allele-specific primers as that involved in the emulsion PCR were used to exclusively amplify the mutant alleles in PCR. The products of PCR were examined by agarose gel electrophoresis and the result was showed in Fig. 2C. The product of PCR without adding the template DNA was set as the negative control (NC). With the decrease of percentage of mutant allele in the original DNA population, the target DNA sequence (88 bp) corresponding to the amplification of mutant allele decreased gradually. When the percentage of mutant allele was less than 5%, no remarkable target band was found in the gel. Thus, the point mutation with a percentage less than 5% in the original genomic DNA population could not be identified by ASP based on gel electrophoresis. On the contrary, the assay we developed could even detect the rare point mutation with the percentage as low as 0.1% in DNA population.

Comparing to the qualitative detection, the quantification of rare mutation was more complex in this assay. Theoretically, the amount of mutant alleles could be represented by the number of fluorescent-positive beads owing to their one-to-one corresponding relationship in the emulsion PCR. Thus, the percentage of mutant allele in the original DNA population could be transferred to the fraction of fluorescent positive beads. However, as shown in Fig. 4, the relationship between the fractions of fluorescent-positive beads and the mutation percentages was not completely in line as the theoretical expectation. The fraction of fluorescent positive beads was higher than the corresponding percentage of the mutant allele in the original DNA population. This deviation might be attributed to the formation of cross dimers and the false amplification of wild-type allele. As shown in Fig. 3, the fluorescent intensity of the beads of negative control (Negative) was slightly higher than that of the blank beads (Beads). Because no template DNA molecule was involved in the emulsion PCR, the fluorescent signal of Negative group must be attributed to the formation of the slight cross dimers. Since the beads harboring the cross dimers could also be stained by SAPE, which lead to the increase of the fluorescent intensity of beads. Moreover, the probability of false amplification of wild-type allele should not be ignored. The fluorescent intensity of WT group was slightly higher than that of Negative group (Fig. 3) and the fraction of fluorescent positive beads of WT group was also higher than that of Negative group (Fig. 4). Both results implied the existence of false amplification of wild-type alleles in emulsion PCR. These results seem to be inconsistent to the result of gel electrophoresis (Fig. 2). However, considering the complication of PCR, the restrict effect of allelic primer on the amplification of wild-type DNA is not absolute. It might be the reasonable speculation that a few products of false amplification

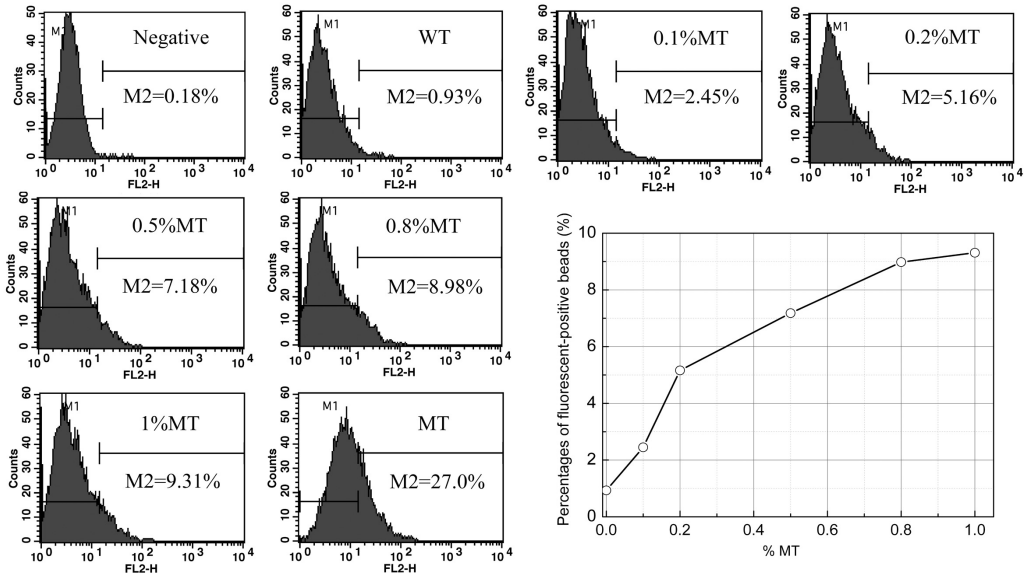

Fig. 4. Fraction of fluorescent-positive beads characterized by flow cytometry as a function of the percentage of mutations.

of predominant wild-type allele generated in PCR. Although these trace products of false amplification was not detected by gel electrophoresis owing to the low sensitivity of electrophoresis, they could be detected by the fluorescent analytical methods with high sensitivity like fluorescent scanning and flow cytometry. Both the situations (formation of cross dimer and false amplification of WT) give rise to the increase of fluorescent signal of beads. Therefore, it was still difficult to calculate the precise percentage of mutant allele in the original DNA population. However, the relative percentages of mutation were comparable for the DNA samples in the same batch by comparing the fraction of fluorescent-positive beads. This assay might be suitable for the prognosis of tumor patients by monitoring the variation of mutant alleles in the blood continuously.

MATERIALS AND METHODS

Materials

Wild-type and mutant genomes were purified from the cultured human liver hepatocellular carcinoma cell lines, HepG2 and HuH-7, respectively. The primers were synthesized by Sangon Biotech (Shanghai, China). Reagents for emulsion PCR were purchased from Applied Biosystems (SOLID^{TM} ePCR kit). Tosylactivated superparamagnetic beads of 1.08 μm in diameter (Myone^{TM} Tosylactivated) and Streptavidin R-phycoerythrin conjugate (SAPE) were purchased from Invitrogen. Hot-start DNA polymerase, PCR buffer, dNTP mix and DNA marker (DL1000) were purchased from Takara Biotechnology (Dalian, China). DNA gel extraction kit was purchased from Qiagen.

Pre-amplification

The wild-type and mutant genome DNA were extracted from the cell lines and then pre-amplified by conventional PCR to yield the amplicons of 368 bp that contained the mutant nucleotide (674A>G) of TP53 gene. The mutant genomic DNA was diluted by wild-type genomic DNA and then the dilute solutions with different percentages of mutation were used as the templates of pre-amplification. The DNA sequences of the primers were as follows: forward 5'-CAAGCAGTCACAGCAC AT-3′, reverse 5'-GGTCAAATAAGCAGCAGGAG-3′. PCR mixture consisted of 50 ng genomic DNA, 1 × PCR buffer, 0.1 mM of dNTP mix, 0.2 μM of primers, 2.5 units of Hot-start DNA polymerase and PCR grade water in a total volume of 50 μl. PCR was performed under the following thermal cycling: 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The products were examined by agarose gel electrophoresis and the target sequences were purified by DNA gel extraction. The purified DNA was quantified by ultraviolet (UV) spectrophotometry and then used as the templates of emulsion PCR.

Design of the primers for emulsion PCR

The DNA sequences of the primers were described as follows: Forward 5'-amino-C12-AAAAAAAAAACAGTTGCAAACCAGA CCTCAGGCGGCTAAC-3′ (allele-specific primer), Reverse 5′-Biotin-TTGCGTGTGGAGTATTTGGA-3′. The forward primer was allele-specific for the mutation, which was designed according to Amplification Refractory Mutation System (ARMS) (20-22). The allelic primer had at its 3′ end the nucleotide perfectly corresponding to the mutant allele but mismatched to the wild-type allele. An additional mismatch nucleotide for both alleles next near the 3′ end of the allelic primer was deliberately introduced to further improve the specificity of amplification for mutant allele. In order to reduce the steric hindrance between the allelic primer and the template DNA molecule in PCR, a spacer containing polyadenine sequence and C12 was added at 5′ end of the allelic primer. The reverse primer had at its 5′ end the nucleotide modified by biotin to facilitate the following fluorescent staining with SAPE.

Coupling the allelic primers to magnetic beads

The coupling was conducted according to the manufacturer's instructions. In brief, the beads (10 mg) were mixed properly with 500 μl of the allelic primers (5 μM) in the coating buffer (0.1 M borate buffer, pH 9.5). The mixture was incubated for 20 h at 37°C with slow tilt rotation. The amount of coupled primers was roughly estimated at 1×10^5 oligonucleotides per bead.

Emulsion PCR

The oil phase of emulsion PCR was prepared as instructed on ePCR kit. The aqueous solution in a total volume of 70 μl was composed of 0.1 ng template DNA (the products of pre-amplification), 1×PCR buffer, 40 μM of dNTP mix, 3 nM of free allelic primer, 0.3 μM of reverse primer, 1 unit of Hot-start DNA polymerase, and 1×10^8 beads coupled with the allelic primers.

The water-in-oil emulsion was prepared by adding 70 μl of the aqueous solution drop by drop to 115 μl of the oil phase in 2 min in the presence of vortex (1,500 rpm). Vortex continued for 10 min after the addition. The average diameter of the water-in-oil compartment was about 10 μm (see supplementary Fig. 1). The emulsion was transferred to PCR tubes with 60 μl per tube. Emulsion PCR was cycled under the following conditions: 94°C for 3 min; 94°C for 15 s, 58°C for 30 s, and 72°C for 75 s 35 times.

After emulsion PCR cycling, the emulsion was collected in a tube and then broken by the addition of 200 μl of butanol. After brief vortexing, the beads were pelleted by magnetic separator. The supernatant solution was discarded and the beads were suspended in 200 μl of Tm buffer (0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100, pH 8.0). After brief vortexing, the mixture was transferred to a new tube. The beads were pelleted and washed again with 200 μl of Tm buffer. Then the beads were resuspended in 100 μl of Tm buffer and stored at 4°C.

Fluorescence staining

The beads were pelleted by magnetic separator and then re-

suspended in 50 μl of Tm buffer containing 2 μg/ml of SAPE. Then, the mixture was incubated at 37°C for 20 minutes with slow tilt rotation. After incubation, the beads were pelleted by magnetic separator and the supernatant was discarded. The beads were washed by 100 μl of Tm buffer for 3 times and finally resuspended in 100 μl of Tm buffer and stored at 4°C.

Flow cytometry

The beads were diluted to the concentration of 1×10^6 beads per ml with Tm buffer. The detection and the count of florescent beads were performed with FACSCalibur (BD Biosciences).

Fluorescent scanning

The beads were suspended in Tm Buffer and then transferred to 96-well plate. The florescent scanning was performed with Infinite 200 Multimode Reader (Tecan, Switzerland).

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