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RESEARCH ARTICLE

Cloning of chimerical translocations as positive control for molecular genetic diagnosis of leukemia

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ABSTRACT

The diagnosis of leukemia-specific mRNAs by polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) require well-known positive standard controls. In general, the positive controls are obtained from cell lines and leukemia patients who have been diagnosed at the molecular level by RT-PCR. These are expensive and restricted sources for standard positive controls. Thus, there is a need for less expensive and reproducible standard positive controls in this area. We have cloned the t (9: 22) p190, t (9: 22) p210, t (4: 11), t (1: 19), t (15: 17), t (12; 21) breakpoint junctions of fusion genes into the plasmids. Cloned fusion genes are suitable for testing PCR experiments of the molecular genetic diagnosis of leukemia samples. We cloned and optimized fusion gene junctions as a standard positive control to check PCR efficiency and as a standard positive marker for diagnosis.

Key words: Fusion genes, cloning, reverse transcription-PCR

ÖZET

Lösemilerin moleküler genetik teşhisinde kullanılmak üzere tarnslokasyonların pozitif standart amaçlı olarak klonlanması

Lösemiye özgü kimerik mRNA'ların ters transkriptaz PCR ile saptanmasında standart Kontrollerin olması oldukça önemlidir. Lösemi hastalarından elde edilen kimerik genlere ait pozitif örnekler veya hücre hatlarından elde edilen standartlar yaygın olarak kullanılmaktadır. Gerek hasta örnekleri, gerekse hücre hatlarından elde edilen pozitif kontroller, kısıtlı, pahalı ve bir çok sorunlar oluşturmaktadır. Daha ucuz, tekrar üretilebilir stan-

dartların üretilmesi ihtiyaç haline gelmiştir. Biz p190, t (9: 22) p210, t (4: 11), t (1: 19), t (15: 17), t (12; 21) kimerik gen bölgelerini plasmidlere klonlayarak daha ucuz ve tekrar üretilebilen standart pozitif kontrolleri ürettik. Bu plasmidler, lösemilerin moleküler genetik teşhisinde standart pozitif marker olarak PCR ve RT-PCR sırasında kullanılabilir inancını taşımaktayız.

Anahtar kelimeler: Füzyon genler, klonlama, ters transkriptaz PCR

INTRODUCTION

Chromosomal translocation aberrations are frequently found in leukemia. These translocations are important for the diagnosis, prognosis, and treatment of diseases [1-6].

Chromosomal aberrations result in fusion of different genes, and the rearranged genes encode chimerical proteins [2,5]. The fusion transcripts can be detected by polymerase chain reaction (PCR), and reverse transcriptase (RT)-PCR [2,6].

The molecular genetic diagnosis of hematological cancer is still a challenge for the diagnostic genetic labs. The diagnosis of hematological cancer is based on gene expression analysis by RT-PCR. Total RNA is reverse transcribed by random hexamer, and the produced cDNA is amplified using specific primers [6,7]. Good positive controls for each translocation are important for the diagnosis of leukemia. In general, the positive controls used for diagnosis of leukemia come from leukemia cell lines [6] or leukemia patients who have been diagnosed at the molecular level by RT-PCR. Cheap, convenient and standardized positive controls can be helpful to improve molecular genetic diagnosis of leukemia. We have cloned the t(9:22) p190, t(9:22) p210, t(4:11), t(1:19), t(15:17), t(12; 21) chromosomal breakpoint junctions into plasmids to use as a standard positive control for the diagnosis of leukemia. In this paper, we report the cloned fusion gene junctions as a standard positive control to check PCR efficiency, and use standard positive marker for the diagnosis.

MATERIALS AND METHODS

Total RNA isolation and cDNA synthesis

The local ethics committee approved the protocol for this study, and a written informed consent was obtained for all procedures. Total RNA was isolated from 2 ml of fresh bone marrow using the total RNA purification kit according to the manufacturer's recommendations (Qiagen Co). Reverse transcription was done as follows: 5 ug of template total RNA and 0.2 ug random hexamer were added to 11 μl of nuclease free water, and incubated at 70°C for 5 min. 21 μl of reverse transcription mixture, 20 units of ribonuclease inhibitor, 1.0 mM final concentration dNTPs mix, and 200 units of RevertAid M-MuLV reverse transcriptase were added to template mixture [6,7]. The RT-PCR step was performed as follows: 60 min at 42°C, and the reaction stopped by heating at 98°C for 10 min.

cDNA samples were normalized to 0.2 ng/ml and diluted before use.

PCR

2 μl of cDNA was added to PCR mix and PCR was done as follows: one cycle at 95°C for 3 min; 30 cycles at 94°C for 30 sec, 66°C for 30 sec, and 72°C for 30 sec; and one final cycle at 72°C for 5 min. The PCR products were analyzed on agarose gel in order to determine the various fusion transcripts and PCR quality [6]. A cDNA spanning t(9:22) p210 chromosomal breakpoint junction, 450 bp, was amplified by forward BCR-b1-A and ABL-a3-B reverse primers (t(9:22) p210 BCR-b1-A 5'-GAAGTGTTTCAGAAGCTTCTCC-'3, ABLa3-B 5'-CCATTGTGATTATAGCCTA-'3) [6]. A cDNA t(9:22) p190, 550 bp long fragment was amplified by forward BCR-e1-A and reverse ABL-a3-B primers (t(9:22) p190 BCR-e1-A 5'-GACTGCAGCTCCAATGAGAAC-'3, ABL-a3-B 5'-TTCCCCATTGTGATTATAGCCTA-'3) [6]. A cDNA t(4:11) 559 bp was amplified by MLL-A forward and reverse AF4-B primers (t(4:11) MLL-A 5'-CCGCCTCAGCCACCTAC-'3, AF4-B 5'-TGTCACTGAGCTGAAGGTCG-'3) [6]. A cDNA from t(1:19), 373 bp long fragment was amplified by forward E2A-A and PBX1-B reverse primers (t(1:19)

E2A-A 5'-CACCAGCCTCATGCACAAC-'3 PBX1-B 5'-TCGCAGGAGATTCATCACG-'3) [6]. Three cDNA of t(15:17) chromosomal breakpoint junctions, 1329 bp, 819 bp and 376 bp long, were amplified by PMLA2 forward and RARA-B reverse primers (t(15:17) PMLA2 5-CTGCTGGAGGCTGTGGAC-'3, RARA-B 5'-GCTTGTAGATGCGGGGTAGA-'3) [6]. A t(12-21) cDNA, 298 bp, was amplified by TELA forward and AML1-B reverse primers (t(12-21) TEL-A 5'-TGCACCCTCTGATCCTGAAC-'3, AML1-B 5'-AACGCCTCGCTCATCTTGC-'3) [6].

Plasmid construction and sequencing

PCR fragments were recovered from the 2% agarose gel by cutting, and DNA was eluted from the sliced agarose gel by gel extract kit. All amplified fragments were cloned into the pGem-T easy vector by T-A cloning according to manufacturer's manual (Promega). Briefly, 50 ng of vector DNA and 1 μl of PCR product were incubated with 2x T4 DNA ligase buffer containing 1 ml (3u/μl) T4 DNA ligase at 4°C overnight. 2 μl of ligation reaction and 100 μl of DH5 alpha competent cells were heat-shock transformed and spread on agar plates containing 100 mg/ ml ampicillin. Plates were incubated overnight at 37°C. PCR tested and selected positive clones were confirmed with sequencing (Iontek Co, Turkey).

RESULTS AND DISCUSSION

The molecular genetic diagnosis of hematological cancer remains a challenge for the diagnostic genetics laboratories. Gene-expression analysis by RT-PCR and regular PCR are the main lab methods for the diagnosis. Standard positive controls are critical in the molecular genetic diagnosis of leukemia. In general, the positive controls that are used for diagnosis of leukemia come from leukemia cell lines or leukemia patients who have been diagnosed as positive at the molecular level by RT-PCR. Many factors may affect the quality and standardization of such positive controls. Also, cell lines are open to many changes at the molecular level and are expensive to maintain. It is difficult to standardize positive samples from leukemia patients, and there are restricted amounts of such samples. Cloning of a cost-effective approach can be helpful in overcoming such problems. Cloned fusion genes in leukemia can be used as positive controls for the molecular genetic diagnosis of leukemia.

These genes should be carefully selected so that they do not contain any sequences from the genomic DNA. We have cloned the corresponding fragments of the t (9:22) p190, t (9:22) p210, t (4:11), t (1:19), t(15:17), t (12; 21) chromosomal breakpoint junctions of fusion genes into the pGem-T-easy vector. The correct plasmids were selected from agar plates with PCR and confirmed by sequencing. We used 10pg, 100pg, and 1000pg plasmid dilutions to check PCR efficiency. The PCR was more efficient at 1000pg dilution at 30 cycles (Figure 1). We use 1000 pg plasmid dilutions for each translocation as positive control in our lab.

Plasmids are more stable, cheaper and easier to prepare compared to production of cell line processed cDNA as calibration material. These cloned fusion genes might be suitable for testing PCR experiments of the molecular genetic diagnosis of leukemia and can be helpful as a standard positive control to check PCR efficiency and as a standard positive marker for the molecular diagnosis.

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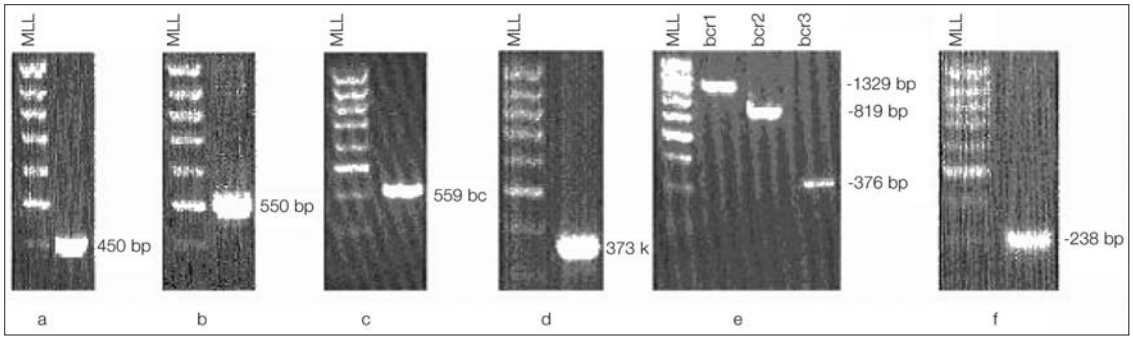

Figure 1. Ethidium bromide stained agarose gel showing PCR from cloned fusion fragments. a) M-BCR-ABL, b)m-BCR-ABL, c) MLL-AF4, d)E2A-PB:1, e) PML-PARA variants, and f) t(12,21)

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