PCR AND SOUTHERN BLOT FOR LYMPHOMA MALIGNA

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Abstrak

Analisis blot Southern tetap menjadi standar emas untuk mendeteksi T-sel reseptor antigen (TCR) penyusunan ulang gen, biasanya target gen-□ TCR karena penataan-kembali TCR-□ terlalu terbatas untuk membedakan andal monoklonal dari populasi T-sel poliklonal. Tetapi karena reaksi rantai polimerase (PCR) adalah cepat dan teknis yang kuat, secara bertahap menggantikan analisiş blot Southern sebagai metode pilihan untuk mendeteksi penyusunan ulang gen klonal TCR.

Kami retrospektif mempelajari 52 sampel jaringan segar-beku dari pasien klinis dicurigai keganasan T-sel. Sebuah penataan gen klonal TCR-□ terdeteksi pada 14 sampel dengan analisis DNA kurva leleh. Ketika pencairan DNA dibandingkan dengan metode standar emas blot Selatan atau denaturing gradien elektroforesis gel, itu mencapai sensitivitas sebesar 71% dan spesifisitas sebesar 94%. Kami juga membandingkan analisis kurva leleh dan polyacrylamide gel elektroforesis: analisa kurva leleh mencapai sensitivitas sebesar 100% dan spesifisitas sebesar 97%.

Kami menyimpulkan bahwa pemaparan analisis DNA kurva dalam sistem LightCycler memiliki potensi untuk penggunaan klinis sebagai metode, baru ultra-cepat untuk diagnosis awal penyusunan ulang gen klonal TCR-□.

Kata kunci: Southern blot, PCR, penyusunan ulang gen TCR, LightCycler system

PCR DAN BLOT SELATAN UNTUK LIMFOMA MALIGNA

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Abstract

Southern blot analysis remains the gold standard for detecting T-cell antigen receptor (TCR) gene rearrangements, it usually targets the TCR-□ gene because the rearrangements of TCR-□ are too limited to reliably distinguish monoclonal from polyclonal T-cell populations. But because the polymerase chain reaction (PCR) is rapid and technically robust, it has gradually replaced Southern blot analysis as the method of choice for detecting clonal TCR gene rearrangements.

We retrospectively studied 52 fresh-frozen tissue samples from patients clinically suspected of T-cell malignancy. A clonal TCR-□ gene rearrangement was detected in 14 samples by DNA melting curve analysis. When DNA melting was compared to the gold standard methods of Southern blot or denaturing gradient gel electrophoresis, it achieved a sensitivity equal to 71% and a specificity equal to 94%. We also compared melting curve analysis and polyacrylamide gel electrophoresis: melting curve analysis reached a sensitivity equal to 100% and a specificity equal to 97%.

We conclude that DNA melting curve analysis in the LightCycler system has potential for clinical use as a new, ultra-fast method for the initial diagnosis of clonal TCR-□ gene rearrangements.

Keywords: Southern blot, PCR, TCR gene rearrangements, LightCycler system

Introduction

The invention of polymerase chain reaction (PCR) by Kary Mullis in 1984 was considered as a revolution in science. Real-time PCR, hereafter abbreviated RT PCR, is becoming a common tool for detecting and quantifying expression profiles of selected genes (Deepak, 2007).

Southern blotting is still one of the standard techniques to determine the presence of a gene and its integrity. The method can be used to detect small mutations as well as large deletions, duplications and gene rearrangements. A Southern blot is a method routinely used in molecular biology for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization. The method is named after its inventor, the British biologist Edwin Southern in the 1970 (Southern, 1975).

PCR And SOUTHERN BLOT For Lymphoma Maligna

Various molecular methods have been developed to diagnose clonal T-cell receptor (TCR) gene rearrangements in clinical samples. Most polymerase chain reaction strategies for detecting clonal TCR gene rearrangements rely on either gel or capillary electrophoresis. However, a cumbersome manual transfer step separates amplification from analysis (

Xiao, 2005).

Recently, we developed a novel polymerase chain reaction assay using the LightCycler system to detect clonal immunoglobulin heavy chain gene rearrangement. In the current study, we extend this work to include the TCR. We report that clonal TCR-□ (TCR-□) gene rearrangements can be detected in less than 1 hour after preparing the DNA by measuring DNA melting immediately after amplification in a single closed capillary tube (Xiao, 2005).

We retrospectively studied 52 fresh-frozen tissue samples from patients clinically suspected of T-cell malignancy. A clonal TCR-□ gene rearrangement was detected in 14 samples by DNA melting curve analysis. When DNA melting was compared to the gold standard methods of Southern blot or denaturing gradient gel electrophoresis, it achieved a sensitivity equal to 71% and a specificity equal to 94%. We also compared melting curve analysis and polyacrylamide gel electrophoresis: melting curve analysis reached a sensitivity equal to 100% and a specificity equal to 97%. We conclude that DNA melting curve analysis in the LightCycler system has potential for clinical use as a new, ultra-fast method for the initial diagnosis of clonal TCR-□ gene rearrangements (Xiao, 2005).

Southern blot analysis remains the gold standard for detecting T-cell antigen receptor (TCR) gene rearrangements, it usually targets the TCR-□ gene because the rearrangements of TCR-□ are too limited to reliably distinguish monoclonal from polyclonal T-cell populations. But because the polymerase chain reaction (PCR) is rapid and technically robust, it has gradually replaced Southern blot analysis as the method of choice for detecting clonal TCR gene rearrangements (Xiao, 2005).

The TCR-□ gene has become a favorite target for PCR-based T-cell clonality assays for two reasons: it is rearranged in most □/□ and □/□ T cells and its structure, containing 14 V segments and 5 J segments, is relatively simple. In contrast, the TCR-□ locus is highly complex: It includes 64 to 67 V segments, 2 D segments, and 13 J segments. Thus, there have been few diagnostic PCR studies of TCR-□ gene arrangements compared to TCR-□ because of the necessity for many primers. But now a set of rigorously tested multiplex primers for TCR-□ are available (Xiao, 2005).

Traditional strategies for detecting TCR clonality using DNA-based PCR rely either on measuring the length of the amplicon or on detecting gel mobility variations owing to sequence-dependent conformational changes. Recently, we and others have shown that DNA melting curve analysis is a rapid and accurate method for detecting clonal immunoglobulin heavy chain (IGH) gene rearrangements. Melting curve analysis has also proven to be useful for studying clonal TCR-□ gene rearrangements. Because TCR-□ is structurally similar to IGH in terms of containing not only V and J but also D gene segments, we hypothesized that DNA melting curve analysis could detect clonal TCR-□ gene rearrangements as well. Therefore, we evaluated the diagnostic utility of combining TCR-□ PCR and melting curve analysis in a panel of T-cell malignancies and reactive lymphoid tissues (Xiao, 2005).

In the current report, we use LightCycler-PCR and DNA melting curve analysis to experimentally demonstrate that clonal TCR-□ gene rearrangements in T-cell lymphomas produce homoduplex PCR amplicons with a sharp − dF/dT peak. In contrast, nonclonal TCR-β gene rearrangements produce heteroduplexes in tonsil, B-cell lymphomas, and reactive lymphoid hyperplasia. To our knowledge, only one other LightCycler study has used DNA melting to analyze TCR gene rearrangements: clonal TCR-γ gene rearrangements were reported in a series of cutaneous T-cell lymphomas; the sensitivity equaled 59% by melting curve analysis and 72% by PAGE (Xiao, 2005).

The sensitivity of a TCR-β gene rearrangement PCR assay is dependent on both the primers and the analytic system. For example, PCR-PAGE assays yield a sensitivity of 44 to 76%. On the other hand, two-step PCR assays in combination with direct sequencing or seminested PCR in combination with GeneScan yield an enhanced sensitivity of 98 to 100%. However, all of these traditional methods require a cumbersome manual transfer step in between amplification and analysis

(Xiao, 2005).

The distribution of gene segments in either TCR-□ or TCR-□ genes are highly variable. For the TCR-□ gene, no single common sequence is sufficient to identify and amplify all of the possible rearrangements that occur in T-cell lymphomas. Therefore, multiple primer sets are needed. The same is true for the detection of TCR-□ gene rearrangements. Recently, standardized multiplex PCR primers have been tested in 32 diagnostic PCR laboratories in Europe (BIOMED-2 Concerted Action). In the TCR-□ PCR portion of this study, clonal TCR-□ gene rearrangements were detected by heteroduplex analysis in 86% of cases and by GeneScan analysis in 79% of cases of 29 Southern blot defined cases. A similar study in fresh-frozen tissues, using BIOMED-2 multiplex PCR primers, detected clonal TCR-□ gene rearrangements using heteroduplex and GeneScan analyses in 76% and 66% of cutaneous T-cell lymphomas, which was comparable to Southern blot analysis (68%). In the current report, we have used the BIOMED-2 TCR-□ primers for PCR amplification, but DNA melting for analysis. Overall, we achieved a sensitivity of 71%, which is equivalent to the other methodologies (Xiao, 2005).

Although we could routinely amplify TCR-□ using primer sets A and C, we were not successful using primer set B. Both primer sets A and B cover the identical 23 V□ gene segments. The major difference between primer sets A and B is in the coverage of the J region. Primer set A contains six J□1 primers and three J□2 primers: J□1.1 to 1.6, 2.2, 2.6, and 2.7. On the other hand, primer set B contains four J□2 primers and no J□1 primers: J□2.1, 2.3, 2.4, and 2.5. Primer set C contains two D□ primers and no V□ primers: D□1 and D□2. In addition, primer set C contains all 13 J□ indicated above. Therefore, the inability to amplify TCR-□ gene rearrangements using primer set B appears to be because of the J□2.1, 2.3, 2.4, and 2.5 DNA sequences. Thus, we will underestimate TCR-□ gene rearrangements that use these J□2 sequences (Xiao, 2005).

Real-time PCR using allele-specific oligonucleotides in the LightCycler can be used to quantify minimal residual disease in acute lymphoblastic leukemia at the 10^{-4} and 10^{-6} level of leukemia cells. Clearly, the TCR-β LightCycler-PCR method described here is not suitable for detecting minimal residual disease because the lower limits varied between 12.5% and 6.25% of clonal DNA. Nevertheless, the detection limit for TCR-β is of the same order of magnitude as other melting curve assays for IGH (12.5%) and for TCR-γ (10%) (Xiao, 2005).

Unfortunately, we could not detect TCR-β gene rearrangements in FFPE tissues using LightCycler and DNA melting curve analysis. In our previous study of IGH, DNA melting curve analysis did successfully detect gene rearrangements from FFPE tissues, but the size of those PCR amplicons was much smaller, ranging from 69 to 129 bp. In contrast, the BIOMED-2 TCR-β primer sets generate larger PCR products. Primer set A: 240 to 285 bp; primer set B: 240 to 285 bp; primer set C: 170 to 210 and 285 to 325 bp. Initially, we considered that the most likely reason why TCR-β clonality could not be detected in the LightCycler from FFPE tissues was because of the larger PCR products of TCR-β compared to IGH. However, after successfully reamplifying a 324-bp fragment of β-globin using conventional PCR soon after the LightCycler analysis, we conclude that DNA integrity by itself is not a sufficient explanation. We can only speculate that whereas the FFPE DNA could be amplified by conventional PCR, it may be suboptimal for producing relatively large amplicons under the LightCycler conditions. Also, DNA extracted from FFPE tissue tends to be severely fragmented and might contain PCR inhibitors that do not affect conventional PCR but could be detrimental in the LightCycler (Xiao, 2005).

We believe that DNA melting curve analysis for the detection of clonal TCR-β gene rearrangements has several unique advantages: monoclonal versus polyclonal T cells are distinguished based on fundamental DNA characteristic such as length, sequence, G:C content, and Watson-Crick base pairing, very fast temperature transition rates give rapid results (less than 1 hour after DNA preparation); precise temperature control produces accurate and reproducible Tms;

and combined PCR and DNA melting curve analysis in a closed system reduce cross-contamination risk. Using the same PCR products, melting curve analysis versus PAGE revealed sensitivity equal to 100% and specificity equal to 97%. Further, melting curve analysis compared to gold standard methods of Southern blot and DGGE revealed sensitivity equal to 71% and specificity equal to 94%. These results are within the typical range of most other methods for detecting PCR products. Because the sensitivity of detecting a T-cell clone diluted in tonsil DNA is between 6.25% and 12.5%, DNA melting curve analysis is clearly not suitable for assaying minimal residual disease in T-cell lymphomas. Nevertheless, we believe that this LightCycler DNA melting assay could play a role in rapidly evaluating clonal TCR-□ gene rearrangements in initial fresh or frozen tissue samples (Xiao, 2005).

Conclusion

Recently, we developed a novel polymerase chain reaction assay using the LightCycler system to detect clonal immunoglobulin heavy chain gene rearrangement. We conclude that DNA melting curve analysis in the LightCycler system has potential for clinical use as a new, ultra-fast method for the initial diagnosis of clonal TCR-□ gene rearrangements.

REFERENCES

Deepak SA, Kottapalli KR, Rakwal R, 2007. Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes, Current Genomics 8(4):234-251.

Heid CA, Stevens J, Livak KJ, Williams PM,1996. Real time quantitative PCR. *Genome Res.* **6**:986–994.

Innis MA, 1990. Academic Press. PCR Protocols: A Guide to Methods and Applications.

Shimizu H, and Burns J.C, 1995. in: PCR Strategies, Innis, M.A. et al. (eds.), Academic Press, San Diego, CA, 2.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel ellectrophoresis, J Mol Biol. 98:503-517.

Xiao Y, Dongsheng X, 2005. Rapid Detection of Clonal T-Cell Receptor-□ Gene Rearrangements in T-Cell Lymphomas Using The Light Cycler-Polymerase Chain Reaction with DNA Melting Curve Analysis. J. Mol Diagn 7:81-88.