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Detection of Common Deletional α-Thalassemia-2 determinants
常见的缺失型α地中海贫血-2的基因检测

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Abstract Using polymerase chain reaction to detect common deletional α-thalassemia-2. Specific primers selectively amplify the α2 and α1 genes. After electrophoresis of PCR products, then can make diagnosis for genotypes of the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions. This method is simple, fast, reliable and non-radioactive. It offers a new method for large screening and prenatal diagnosis of $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions in the area with high frequency of deletional α-thalassemia-2.

Key words deletional α-thalassemia-2, $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions, polymerase chain reaction (PCR)

摘要 应用聚合酶链反应技术检测缺失型α地中海贫血-2. 特异性引物选择性扩增α2和α1基因, PCR产物经琼脂糖凝胶电泳后即可对$-\alpha^{3.7}$和$-\alpha^{4.2}$缺失型作出基因诊断。此方法简便,快速和可靠。本研究为在缺失型α地中海贫血-2高发区进行$-\alpha^{3.7}$和$-\alpha^{4.2}$缺失型的筛查和产前诊断提供了一个新的方法。

关键词 缺失型α地中海贫血-2 $-\alpha^{3.7}$和$-\alpha^{4.2}$缺失型 聚合酶链反应 (PCR)
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The α-thalassemia (thal) is the most common genetic disorder which is due to an absence or diminished synthesis of α-globin chain of the hemoglobin[1]. The human α-globin gene cluster is located on chromosome 16p 13.3-ter and arranged in the order 5′-ζ2-ψζ1-ψα1-ψα2-α2-α1-θ1-3′. α-thalassemia frequently results from deletions involving one (-α) or both (--) α-globin genes. It is less frequency of resulting from nondelational mutations involving one or a few nucleotides within the structural gene ($\alpha^T\alpha$ or $\alpha\alpha^T$)[2].

The deletional α-thal-2 may involve one entire α-globin gene (either α2 or α1), part of α2-globin gene, or the 5′ end of α2, and 3′ end of α1. The numbers of deletions discovered thus far total to eight[3]. The most common α-thal-2 is the $-\alpha^{3.7}$ deletion, and followed by the $-\alpha^{4.2}$ deletion. Heteroduplex and DNA sequence analysis have shown that two α-globin genes are embedded within two highly homologous, 4kb duplication units. These regions are divided into homologous subsegments (X, Y and Z) by non-homologous elements (Ⅰ, Ⅱ and Ⅲ). Reciprocal recombination between Z segments, which are 3.7 kb apart, produces chromosomes with only one α-gene ($-\alpha^{3.7}$, rightward deletion) that causes α-thalassemia. Whereas, recombination between homologous X boxes, which are 4.2 kb apart, also gives rise to a α-thalassemia determinant ($-\alpha^{4.2}$)[4]. The high frequencies of the $-\alpha^{3.7}$ deletion are observed in Southern China, Southeast Asia, African and many other Mediterranean populations. The $-\alpha^{4.2}$ deletion also mainly presents in Southern China and Southeast Asia[3].

The molecular characterization of α-thalassemia-2 mentioned in previous reports were performed by restriction enzyme mapping of the Southern blot hybridization and some PCR based methods. In this study, we present a rapid, simple, accurate and non-radioactive PCR method for molecular diagnosis of $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions.

1 Materials and Methods

1.1 Patient selection

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43 cases of HbH disease were collected from the First Affiliated Hospital of Guangxi Medical University from July to October 1997. Hematological data were obtained by standard methods. DNA was extracted from peripheral blood by phenol and chloroform as described in reference [5]. Ten cases previously analyzed by Southern blot hybridization method were used as positive controls.

1.2 The primers and PCR procedure

The primers designed by E Baysal[6] were used with modification in PCR conditions. These specific primers were designed to allow the selective amplification of the α2 and α1 genes (Figure 1). The sequences are showed on table 1.

Primers A2proF, # 271 and # 273 were used to detect the presence of the $-\alpha^{3.7}$ genotype. Two separated reactions are run simultaneously for each DNA sample. One reaction contains primers A2proF+ # 271 which amplifies a segment of chromosome with deletion, while primers A2proF+ # 273 amplifies a comparable fragment of the normal gene. For testing $-\alpha^{4.2}$ genotype, primers D+ E amplify the fragment of the abnormal gene with $-\alpha^{4.2}$ deletion, while primers D+ F amplify that of the normal gene. The PCR reaction is run in one tube.

Table 1 The sequences of the primers

Primer	Sequences
A2proF	5′-CTTTCCCTACCCAGAGCCAGGTT-3′
# 271	5′-CCCATGCTGGCACGTTTCTGAGG-3′
# 273	5′-CCATTGTTGGCACATTCCGGGACA-3′
D	5′-CCTCCTCTCACTTGGCCCTGAG-3′
E	5′-CCCTGGGTGTCCAGGAGCAAGCC-3′
F	5′-GGCACATTCCGGGACAGAGAGA-3′

The PCR condition we used here is modified from original Baysal's one. Each of 50μL PCR reaction contains 0.5μg genomic DNA, 12.5 pmoles of the each primer, 67mmol Tris-HCl, pH 8.8, 16.6 mmol $(NH_4)_2SO_4$, 0.1 mg/mL BSA, 10 mmol β-mercaptoethanol, 4 mmol $MgCl_2$, 10% DMSO and 200μmol dNTPs. The PCR are carried out in the thermal cycler (Perkin Elmer Cetus, Normalk, CT). After denaturation at 99℃, added Taq polymerase, then a total of 32 cycles were performed under the following conditions: 95℃, 1 min; 63℃, 1 min; 72℃, 2.5 min with an additional 5 min extension at 72℃ in the final cycle. The PCR products were analyzed by electrophoresis on 1.5% agarose gel after ethidium bromide staining and visualized on Gel Doc 1000. (Bio-Rad, molecular analysis software).

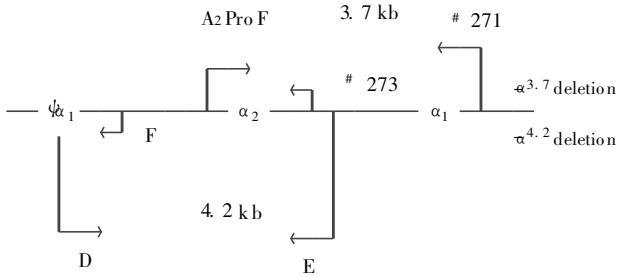

Fig. 1 The location of the primers

2 Results

The results are showed on Figure 2 and Figure 3. Figure 2 shows the results of the detection of the $-\alpha^{3.7}$ deletion: 1.8 kb band with primers A2proF+ # 271 indicates the presence of an amplification product from the $-\alpha^{3.7}$ chromosome, while a same size fragment with primers A2proF+ # 273 identifies the presence of a normal chromosome. Figure 3 shows the amplification of the $-\alpha^{4.2}$ genotype. 2.1 kb fragment with primers D+ E and 581bp fragment with D+ F indicate the chromosomes with $-\alpha^{4.2}$ deletion and normal gene respectively.

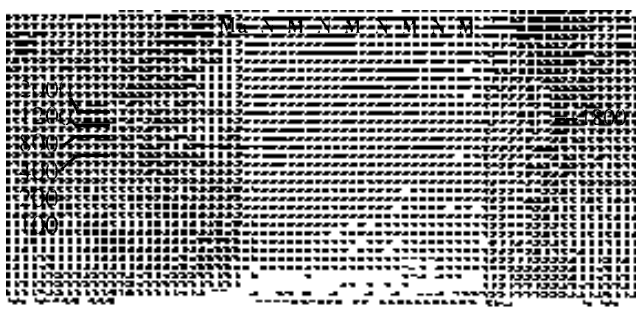

Fig. 2 PCR amplification product of $-\alpha^{3.7}$ deletion

Each DNA sample run in two separate tube for PCR reaction. N: normal, primers A2proF+ # 273, M: mutant, primers A2proF+ # 271. Lanes 2, 3 normal (αα/αα), lanes 4, 5 heterozygote ($-\alpha^{3.7}$/αα), lanes 6, 7 HbH disease ($-/-\alpha^{3.7}$), lanes 8, 9 homozygote ($-\alpha^{3.7}/-\alpha^{3.7}$). Ma: marker, DNA mass.

All the HbH cases here have an α-thal-1 allele of Southeast Asia type (data not shown), and an α-thal-2 allele of different types. Among them, 10 cases are with $-\alpha^{3.7}$ deletion and 7 cases with $-\alpha^{4.2}$ deletion. The remains had Hb Constant Spring (data not shown). These genotypes are confirmed by restriction enzyme mapping of Southern blot hybridization later.

Fig. 3 PCR amplification product of $-\alpha^{4.2}$ deletion
Three primers D+ E+ F are added together in a reaction. 2.1 kb band indicates $-\alpha^{4.2}$ deletion, while 581 bp indicates normal fragment. Lane1: HbH disease ($--/-\alpha^{4.2}$), lane2: heterozygote ($\alpha\alpha/-\alpha^{4.2}$), lane3: normal control ($\alpha\alpha/\alpha\alpha$). Ma: marker, DNA mass.

3 Discussion

α-thalassemia is mainly due to a part deletion of the α-globin gene cluster which has high GC content, and α1 and α2 gene are highly homologous, they differ only in IVS-2 and their 3′ non-coding region. It is quite difficult to use PCR-based method to detect deletional α-thal-2 genotype. Previous methods have entirely relied on DNA analysis by restriction enzyme mapping of Southern blot hybridization[7]. It is rather laborious and needs expensive restriction endonuclease and radioactive isotope.

In China, although the gene diagnosis of α-thal began in 1980′s, the only way for detecting deletional α-thal-2 is still the same as above up to date. Recently, Dode[8] has initially described a PCR-based method for the diagnosis of the $-\alpha^{3.7}$ genotype only. Ta-Chih Liu[9] used PCR followed by restriction enzyme digestion to detect $-\alpha^{4.2}$, $-\alpha^{3.7}$, $-\alpha^{2.7}$ and $-\alpha^{3.5}$ deletions. Lemuel[10] described a quantitative PCR technique, but it required fragment analysis by ACF DNA sequencer.

In this study, specific oligonucleotide selective amplification has been established for the detection of α-thal-2 deletions according to the method described by Baysal with modification. The sequences of primers used in this study are the same as that of the original method. Certain modification has been made to obtain optimal yield of PCR in primer concentration, initial denaturation temperature, annealing temperature and amplification cycles.

We used the concentration of each primer at 12.5 pmol/reaction and gained quite clear PCR product yield. For GC rich α-globin gene region requiring high denaturation temperature, incomplete denaturation allows the DNA strands to renature and reduce DNA template for polymerase reaction. When the initial denaturation temperature was changed to 99℃, amplification efficiency increased.

The temperature and the length for primer annealing depend on the base composition, length, and the concentration of amplification primers. Low temperature gives nonspecific product while high temperature enhances discrimination of incorrectly anneal primers. Comparing within different annealing temperatures of 55℃, 58℃, 63℃ and 65℃, we found that at 63℃, satisfactory PCR product was obtained.

The number of PCR cycles depends mainly on the starting concentration of target DNA. More cycles can increase the amount and complexity of nonspecific products and less cycles result in low product yield. We found that 25 cycles used by Baysal produced a low PCR product yield. When the cycles were increased to 32, the result was quite optimal.

The role of DMSO here is to increase the degree of strand separation of target DNA template by decreasing the stability of H-bands. It increased the efficiency of α-thal-2 amplification[11]. Although concentration of DMSO exceeding 10% is known to inhibit Taq polymerase activity by 50%, in this study the concentration of DMSO varying at 5%, 7.5% and 10%, we found that at 10% DMSO the amplification was the best.

This modified method for detecting deletional α-thal-2 can easily and efficiently identify $-\alpha^{3.7}$ and $-\alpha^{4.2}$ genotype. However, this method can not distinguish combined heterozygous α-thal-1/α-thal-2 (HbH, $--/-\alpha$) from homozygous α-thal-2 ($-\alpha/-\alpha$), and normal haplotype ($\alpha\alpha$) from Hb Constant Spring ($\alpha^{cs}\alpha$). But combining with PCR amplification of α-thal-1[12] and Hb Constant Spring[13] made it possible to resolve these problems.

In Guangxi province, the incidence of α-thal is 14.95%[14]. When delational α-thal-2 coinherits with deletional α-thal-1, the most common South-

east Asia Type, it becomes HbH (—/-α) disease with moderately hemolytic anemia. So it is quite important to do population screening and prenatal diagnosis. Our study develops a quite simple, low cost, rapid and reliable method. It is useful for large scale detection and prenatal diagnosis of the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions in the area where deletional α-thal-2 with high incidence.

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(上接第 287页 Continue from page 287)

争漆酶II型铜 (II) 部位产生的结果。

由于漆酶分子的底物种类较广, 专一性较差, 可以认为漆酶分子中无专用 "口袋" 来包络底物, 因此底物的氧化反应发生在漆酶分子的外围[8]。这表明II型铜 (II) 部位可能位于漆酶分子的浅表层处, 底物分子或阴离子通过酶分子表面的一道较浅可能也较窄的 "裂缝" 进入酶分子与II型铜 (II) 结合, 因而结合有可能是瞬时完成的。同时, 从 Cl^-、NO_3^- 和 SO_4^{2-} 离子对漆酶催化活性的抑制率依其离子半径的增大而下降的事实看, 阴离子对漆酶催化活性的抑制程度除了与它们和漆酶II型铜 (II) 的亲和力有关外, 可能还与它们的离子半径和离子形状有关。

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