PRIMERS FOR DIAGNOSING AVELLINO CORNEAL DYSTROPHY

TECHNICAL FIELD

The present invention relates to a real-time PCR primer pair and probe for diagnosing Avellino corneal dystrophy, and more particularly to such a real-time PCR primer pair and probe for diagnosing Avellino corneal dystrophy, which can accurately diagnose the presence or absence of a mutation in exon 4 of BIGH3 gene, which is responsible for Avellino corneal dystrophy.

BACKGROUND ART

Corneal dystrophy is an autosomal dominant hereditary disease, which begins with a blurry symptom in the center of cornea and gradually spreads and thus ends up vision loss as a patient gets older. It includes Avellino corneal dystrophy, Granular corneal dystrophy, lattice type I corneal dystrophy, Reis-bucklers corneal dystrophy, etc., and is caused by mutation of a gene coding ßIG-H3 protein.

Heterozygous patients suffering from Avellino corneal dystrophy appear to have severe loss of vision as getting older and homozygous patients appear to have complete loss of vision since 6 years old. Avellino corneal dystrophy is a newly named disease in 1988, divided from generally called Granular corneal dystrophy because it was found to have

discrete symptoms and genetic foundation. Also, it has been known to be the most common corneal dystrophy worldwide, 1/340 to 1/1000 of prevalence rate in Korea (the case of heterozygote) based on genetic analysis indicates that it is a common dystrophy (Holland, E.J. *et al.*, *Ophthalmology*, 99:1564, 1992; Kennedy, S.M. *et al.*, *Br. J. Ophthalmol.*, 80:489, 1996; Dolmetsch, A.M. *et al.*, *Can. J. Ophthalmol.*, 31:29, 1996; Afshari, N.A. *et al.*, *Arch. Ophthalmol.*, 119:16, 2001; Stewart, H.S. *Hum. Mutat.*, 14:126, 1999).

The present inventors has found that if a patient suffering from heterozygous Avellino corneal dystrophy has LASIK surgery, 2 years later, opacity of cornea starts to develop aggressively and eventually results in vision loss (Jun, R. M. *et al.*, *Opthalmology*, 111:463, 2004). Previously, eye surgery has been performed with an expectation that LASIK or Excimer Laser surgery would get rid of vision blurriness of a patient suffering from corneal dystrophy. Also, even in Korea, approximately 3 hundred thousand cases of LASIK surgery have been performed, which leads to the assumption that 300 people lost their vision, based on 1/1000 of minimum estimation of heterozygous patients suffering from Avellino corneal dystrophy. Patients who have undergone LASIK surgery are mainly in their 20's and 30's carrying out productive activities; therefore, their vision loss causes serious troubles in both society and economics.

In addition, after approval of LASIK surgery in year 2000 in USA, African American patients suffering from Avellino corneal dystrophy who underwent LASIK surgery have been found to lose eye sight, which infers that plenty of similar cases might be occurring throughout the world.

Therefore, although accurate diagnosis of Avellino corneal dystrophy is required to prevent the progression of Avellino corneal dystrophy by LASIK surgery, the diagnosis of Avellino corneal dystrophy is just conducted by microscopic observation of corneal opacity and thus often doctors miss latent symptoms of patients to perform LASIK surgery, which results in vision loss. Therefore, rapid and precise diagnosis of corneal dystrophy is desperately in need.

A DNA chip for detecting a mutation in BIGH3 gene, which is responsible for Avellino corneal dystrophy, was developed (Korean Patent Laid-Open Publication No. 10-2007-0076532). However, the diagnosis of Avellino corneal dystrophy using said DNA chip disadvantageously require several steps, including a step of amplifying DNA in a sample, a step of hybridizing the amplified DNA with the DNA chip, a step of washing the hybridized DNA chip, and a step of detecting a positive response.

Accordingly, the present inventors have made extensive efforts to develop a method capable of more efficiently diagnosing Avellino corneal dystrophy, and as a result, have

found that, if the diagnosis of Avellino corneal dystrophy is performed using a pair of primers having nucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 2 and probes having nucleotide sequences of SEQ ID NO: 13 and SEQ ID NO: 14 by a real-time PCR method, Avellino corneal dystrophy can be diagnosed in a more rapid and accurate manner than a conventional method, thereby completing the present invention.

DISCLOSURE OF INVENTION

A main object of the present invention is to provide a primer pair and probe for more efficiently and accurately diagnosing Avellino corneal dystrophy using a real-time PCR method.

To achieve the above object, the present invention provides a real-time PCR primer pair for diagnosing Avellino corneal dystrophy, which is represented by nucleotide sequences selected from the group consisting of SEQ ID NOs: 1 and 2, SEQ ID NOs: 3 and 4, SEQ ID NOs: 5 and 6, SEQ ID NOs: 7 and 8, SEQ ID NOs: 9 and 10, SEQ ID NOs: 11 and 12, SEQ ID NOs: 13 and 14, SEQ ID NOs: 15 and 16, SEQ ID NOs: 17 and 18, SEQ ID NOs: 19 and 20, SEQ ID NOs: 21 and 22, and SEQ ID NOs: 23 and 24.

The present invention also provides a real-time PCR probe for diagnosing Avellino corneal dystrophy, which is

represented by a nucleotide sequence selected from the group consisting of SEQ ID NO: 25 to SEQ ID NO: 42.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the results obtained from the design of real-time PCR primers and probes. In FIG. 1, "A" shows the results of real-time PCR carried out using optimal primers and probes, "B" and "C" show the results of real-time PCR carried out using primers different from those in "A".

FIG. 2 shows the results of real-time PCR carried out using real-time PCR primers according to the present invention in order to detect a gene mutation causing Avellino corneal dystrophy.

BEST MODE FOR CARRYING OUT THE INVENTION

In one aspect, the present invention is directed to a real-time PCR primer pair for diagnosing Avellino corneal dystrophy, which is represented by nucleotide sequences selected from the group consisting of SEQ ID NOs: 1 and 2, SEQ ID NOs: 3 and 4, SEQ ID NOs: 5 and 6, SEQ ID NOs: 7 and 8, SEQ ID NOs: 9 and 10, SEQ ID NOs: 11 and 12, SEQ ID NOs: 13 and 14, SEQ ID NOs: 15 and 16, SEQ ID NOs: 17 and 18, SEQ ID NOs: 19 and 20, SEQ ID NOs: 21 and 22, and SEQ ID NOs: 23 and 24.

Avellino corneal dystrophy is a disease caused by genetic abnormality in which the sequence CGC in exon 4 of BIGH3 gene is mutated to CAC so that arginine at residue of BIGH3 protein is mutated to histidine (R124H).

When a real-time PCR method is carried out using primers of the present invention, Avellino corneal dystrophy can be diagnosed in a more rapid and accurate manner than a conventional method that uses a DNA chip.

In the real-time PCR method, it is very difficult to establish temperature conditions, because experiments using primers and probes should be carried out in the same temperature conditions. Particularly, if only one mutation position like Avellino corneal dystrophy is to be detected, primers and probes should be used in temperature conditions in which they can bind. Also, the probes can bind in a very limited temperature range from 1 ℃ and 3 ℃ in a state in which only one nucleotide differs between the probe for detecting a normal gene and the probe for detecting a mutant gene.

Due to such conditions, it is important to find the same temperature conditions in which probes and primers can bind to a desired gene. Particularly, it is important to design a mutant probe and a normal probe such that the temperatures thereof can differ as much as possible within a limited range. In other words, the temperatures for primers and probes

should be well consistent with each other, the temperature conditions for the forward primer and the reverse primer should be well consistent with each other, and the difference between the temperatures for the mutant probe and the normal probe should be able to be maximized. FIG. 1A shows the results of using well-designed primers and probes, and FIGS. 1B and 1C shows the results of using primers different from those used in FIG. 1A while using the same probes as those used in FIG. 1A. As can be seen therein, the design of the primers and the probes has a significant effect on reading.

In the present invention, in order to construct optimal primers for diagnosing Avellino corneal dystrophy using a real-time PCR method, pairs of primers of SEQ ID NOs: 1 and 2, SEQ ID NOs: 3 and 4, SEQ ID NOs: 5 and 6, SEQ ID NOs: 7 and 8, SEQ ID NOs: 9 and 10, SEQ ID NOs: 11 and 12, SEQ ID NOs: 13 and 14, SEQ ID NOs: 15 and 16, SEQ ID NOs: 17 and 18, SEQ ID NOs: 19 and 20, SEQ ID NOs: 21 and 22 and SEQ ID NOs: 23 and 24 were designed, and real-time PCR was performed using each of the designed primer pairs. As a result, it was found that the use of the pair of primers of SEQ ID NOs: 1 and 2 showed the optimal results.

In another aspect, the present invention is directed to a real-time PCR probe for diagnosing Avellino corneal dystrophy, which is represented by a nucleotide sequence selected from the group consisting of SEQ ID NO: 25 to SEQ ID NO: 42.

In the present invention, in order to construct optimal probes for diagnosing Avellino corneal dystrophy using a real-time PCR method, probes of SEQ ID NOs: 25 to 42 were designed, and real-time PCR was performed each of the designed primers. As a result, it was found that the use of the probes of SEQ ID NOs: 13 and 14 showed the optimal results.

EXAMPLES

Hereinafter, the present invention will be described in further detail with reference to examples. It will be obvious to a person having ordinary skill in the art that these examples are illustrative purposes only and are not to be construed to limit the scope of the present invention. That is, the following steps will be described as one illustrative ones and do not limit the scope of the present invention.

Example 1: Construction of real-time PCR primers and MGB probes

In order to construct primers capable of amplifying a region comprising a mutation in exon 4 of BIGH3 gene, pairs of primers of SEQ ID NOs: 1 and 2, SEQ ID NOs: 3 and 4, SEQ ID NOs: 5 and 6, SEQ ID NOs: 7 and 8, SEQ ID NOs: 9 and 10, SEQ ID NOs: 11 and 12, SEQ ID NOs: 13 and 14, SEQ ID NOs: 15

and 16, SEQ ID NOs: 17 and 18, SEQ ID NOs: 19 and 20, SEQ ID NOs: 21 and 22 and SEQ ID NOs: 23 and 24 were designed using Primer Express 3.0 software (Applied Biosystems U.S.A).

ACD Fw primer : 5'-TCC ACC ACC ACT CAG CTG TA (SEQ ID NO: 1)

ACD Re primer : 5'-CCA TCT CAG GCC TCA GCT T (SEQ ID NO: 2) (60bp)

AV Fw primer : 5'-TGC AGC CCT ACC ACT CTC AA (SEQ ID NO: 3)

AV Re primer : 5'-AGG CCT CGT TGC TAG G (SEQ ID NO: 4) (150bp)

Real Fw primer : 5'-TAG TCT CTT ATT CTA ATA GA (SEQ ID NO: 5)

Real Re primer : 5'-GCT GCA GAC TCT GTG TTT AA (SEQ ID NO: 6) (860bp)

ACD Fw2 primer : 5'-CCA TCC CTC CTT CTG TCT TCT G (SEQ ID NO: 7)

ACD Re2 primer : 5'-CGG GCC CCT CCA TCT C (SEQ ID NO: 8) (140bp)

ACD Fw3 primer : 5'-CAG AGA AGG GAG GGT GTG GTT (SEQ ID NO: 9)

ACD Re3 primer : 5'-GGG CGA AGA TGG TGA AGC T (SEQ ID NO: 10) (190bp)

ACD Fw4 primer : 5'-TCC TCG TCC TCT CCA CCT GTA (SEQ ID NO: 11)

ACD Re4 primer: 5'-AGC TGG CAA GGA GGC CC (SEQ ID NO: 12)

ACD Fw5 primer : 5'-TTT GGG CTT TCC CAC ATG C (SEQ ID NO: 13)

ACD Re5 primer : 5'-GGC AGA CGG AGG TCA TCT CA (SEQ ID NO: 14)

ACD Fw6 primer : 5'-GTA GTA CCG TGC TCT CTG (SEQ ID NO: 15)

ACD Re6 primer : 5'-AGT TCC CCA TAA GAA TCC CCC SEQ ID NO: 16)

ACD Fw7 primer : 5'-GGC TGG ACC CCC AGA GG (SEQ ID NO: 17)

ACD Re7 primer : 5'-ACC CCT CGG GGA AGT AAG G (SEQ ID NO: 18)

ACD Fw8 primer : 5'-AAC CTT TAC GAG ACC CTG GGA (SEQ ID NO: 19)

ACD Re8 primer : 5'-GAC TCC CAT CCA TCA TGC CC (SEQ ID NO: 20)

ACD Fw9 primer : 5'-AGT CGT TGG ATC CAC CAC CA (SEQ ID NO: 21)

ACD Re9 primer : 5'-GAC GTC ATT TCC TAC TGT TTC AGG (SEQ ID NO: 22)

ACD Fw10 primer : 5'-CCC CCC AGA AAC AGC CTG (SEQ ID NO: 23)

ACD Re10 primer : 5'-TTC TAA GGG GTT AAG GAG AAA GCT T (SEQ ID NO: 24)

In order to detect a guanine-to-adenine mutation in exon 4 of BIGH3 gene, probes of SEQ ID NOs: 25 to 42 were constructed.

The probe binding to a normal gene fragment having no mutation was labeled with VIC, and the probe binding to a gene fragment having a mutation was labeled with FAM, and a minor groove binder (MGB) was attached to the probe so as to facilitate binding to a complementary gene fragment.

Normal probe 1 : VIC-CAC GGA CCG CAC GGA-NFQ (SEQ ID NO: 25)(15bp)

Mutant probe 1 : FAM-CAC GGA CCA CAC GGA-NFQ (SEQ ID NO: 26)

Normal probe 2 : VIC-ACA CGG ACC **G**CA CG-NFQ (SEQ ID NO: 27)

Mutant probe 2 : FAM-ACA CGG ACC **A**CA CG-NFQ (SEQ ID NO: 28)(14bp)

Normal probe 3 : VIC-TAC ACG GAC C**G**C A-NFQ (SEQ ID NO: 29)

Mutant probe 3 : FAM-TAC ACG GAC C**A**C A-NFQ (SEQ ID NO: 30)(13bp)

Normal probe 4 : VIC-CTG TAC ACG GAC C**G**C ACG-NFQ (SEQ ID NO: 31)

Mutant probe 4 : FAM-CTG TAC ACG GAC C**A**C ACG-NFQ (SEQ ID NO: 32)(18bp)

Normal probe 5 : VIC-CTG TAC ACG GAC C**G**C ACG GAG-NFQ (SEQ ID NO: 33)

Mutant probe 5 : FAM-CTG TAC ACG GAC C**A**C ACG GAG-NFQ (SEQ ID NO: 34)(21bp)

Normal probe 6 : VIC-GCT GTA CAC GGA CC**G** CAC GGA GAA-NFQ (SEQ ID NO: 35)

Mutant probe 6 : FAM-GCT GTA CAC GGA CC**A** CAC GGA GAA-NFQ (SEQ ID NO: 36)

Normal probe 7 : VIC-ACC GCA CGG AGA AGC-NFQ (SEQ ID NO: 37)

Mutant probe 7 : FAM-ACC ACA CGG AGA AGC-NFQ (SEQ ID NO: 38)

Normal probe 8 : VIC-ACC GCA CGG AGA AGC TGA GGC-NFQ (SEQ ID NO: 39)

Mutant probe 8 : FAM-ACC ACA CGG AGA AGC TGA GGC-NFQ (SEQ ID NO: 40)

Normal probe 8 : VIC-ACC GCA CGG AGA AGC TGA GGC CTG-NFQ (SEQ ID NO: 41)

Mutant probe 8 : FAM-ACC ACA CGG AGA AGC TGA GGC CTG-NFQ (SEQ ID NO: 42)

Example 2: Diagnosis of Avellino corneal dystrophy using real-time PCR

Samples were taken from the blood, hair root and oral epithelial cells of test subjects, and DNA was isolated from the samples. The isolation and purification of DNA were performed using a partial modification of the phenol/chloroform extraction method (Miller, SA *et al.*, *Nucl. Acids Res.* 16:1215, 1988), and the isolated DNA was dissolved in a suitable amount of TE buffer (10mM Tris-Cl, 1mM EDTA,

pH7.4) and confirmed by electrophoresis on 1% agarose gel and used as template DNA in PCR.

The PCR reactions were performed using the primers (SEQ ID NOs: 1 to 12) for amplifying the fragment containing the mutation region, and the probes (SEQ ID NOs: 13 to 24), constructed in Example 1.

25 ㎕ of a master mix containing 10 pmol of each primer and 5 pmol of each probe was prepared and used in the PCR reaction.

The real-time PCR reaction was performed in the following conditions: 36 cycles each consisting of 10 min at 95 ℃, 15 sec at 92 ℃ and 1 min at 60 ℃, followed by a reaction for 5 min at 60 ℃.

After each cycle, fluorescence was measured. The sample positive to the VIC dye was diagnosed as having the normal gene, and the sample positive to the FAM gene was diagnosed as having the mutant gene.

As a result, it could be seen that the use of the primer pair of SEQ ID NOs: 1 and 2 and the probes of SEQ ID NOs: 25 and 26 showed the most accurate and effective results (FIG. 2).

Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope

of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

Industrial Applicability

The use of the primer pair and probe according to the present invention can diagnose Avellino corneal dystrophy in a more rapid and accurate manner than a conventional method that uses a DNA chip or PCR.

THE CLAIMS

What is Claimed is:

1. A real-time PCR primer pair for diagnosing Avellino corneal dystrophy, which is represented by nucleotide sequences selected from the group consisting of SEQ ID NOs: 1 and 2, SEQ ID NOs: 3 and 4, SEQ ID NOs: 5 and 6, SEQ ID NOs: 7 and 8, SEQ ID NOs: 9 and 10, SEQ ID NOs: 11 and 12, SEQ ID NOs: 13 and 14, SEQ ID NOs: 15 and 16, SEQ ID NOs: 17 and 18, SEQ ID NOs: 19 and 20, SEQ ID NOs: 21 and 22, and SEQ ID NOs: 23 and 24.

2. A real-time PCR probe for diagnosing Avellino corneal dystrophy, which is represented by a nucleotide sequence selected from the group consisting of SEQ ID NO: 25 to SEQ ID NO: 42.

3. The real-time PCR probe for diagnosing Avellino corneal dystrophy of claim 2, wherein the real-time PCR probe is labeled with VIC or FAM.