

DNA CHIP FOR DIAGNOSIS OF CORNEAL DYSTROPHY

TECHNICAL FIELD

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The present invention relates to oligonucleotides for diagnosis of corneal dystrophy. More particularly, the present invention relates to oligonucleotides for detecting mutation of BIGH3 gene for diagnosis of corneal dystrophy including Avellino corneal dystrophy, which must be precisely diagnosed before vision correction surgery, and a DNA chip for diagnosis of corneal dystrophy, which has the oligonucleotides fixed thereon.

BACKGROUND ART

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Corneal dystrophy is a hereditary, autosomal dominant 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 β IG-H3.

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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. And it has known to be the most common corneal dystrophy worldwide, 1/340~1/1000 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,

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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
5 Avellino corneal dystrophy has LASIK surgery, 2 years later, opacity of cornea
starts to develop aggressively (Jun, R.M. *et al.*, *Ophthalmology*, 111:463, 2004) and
eventually results in vision loss. 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. And, even in Korea,
10 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
15 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
20 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
25 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.

Accordingly, the present inventors have made extensive efforts to develop a DNA
30 chip for swiftly and accurately detecting mutations of BIGH3 gene that causes

corneal dystrophy, and as a result, generated a probe comprising a specific mutation region of BIGH3 gene and manufactured a DNA chip on which the probe is fixed, and confirmed that corneal dystrophy can be effectively diagnosed using the DNA chip, thereby completing the present invention.

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SUMMARY OF INVENTION

The main object of the present invention is to provide oligonucleotides comprising
10 a specific mutation region of BIGH3 gene that causes corneal dystrophy.

Another object of the present invention is to provide a DNA chip for diagnosis of corneal dystrophy, on which said oligonucleotides are fixed.

15 To achieve the above object, the present invention provides oligonucleotides for diagnosis of corneal dystrophy, which essentially comprises one or more nucleotide sequence selected from the group consisting of SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID
20 NO: 35, SEQ ID NO: 37, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 50, SEQ ID NO: 53 and SEQ ID NO: 59.

In the present invention, the oligonucleotide essentially comprising the nucleotide sequence of SEQ ID NO: 50 is preferably set forth in SEQ ID NO: 12, and the
25 oligonucleotide essentially comprising the nucleotide sequence of SEQ ID NO: 53 is preferably set forth in SEQ ID NO: 13.

In the present invention, the oligonucleotide essentially comprising the nucleotide sequence of SEQ ID NO: 59 is preferably set forth in SEQ ID NO: 15, and the
30 oligonucleotide essentially comprising the nucleotide sequence of SEQ ID NO: 35

is preferably set forth in SEQ ID NO: 65.

In the present invention, the length of the oligonucleotides is 13 to 17 bp.

- 5 The present invention also provides a DNA chip for diagnosis of corneal dystrophy, on which the oligonucleotides are fixed.

In the present invention, the DNA chip for diagnosis of corneal dystrophy preferably additionally immobilizes oligonucleotides essentially comprising one or
10 more nucleotide sequences selected from the group consisting of SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, and SEQ ID NO: 56, thereon.

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In the present invention, the oligonucleotide essentially comprising the nucleotide sequence of SEQ ID NO: 47 is preferably set forth in SEQ ID NO: 11, the oligonucleotide essentially comprising the nucleotide sequence of SEQ ID NO: 56 is preferably set forth in SEQ ID NO: 14, and the oligonucleotide essentially
20 comprising the nucleotide sequence of SEQ ID NO: 34 is preferably set forth in SEQ ID NO: 62.

In the present invention, the DNA chip for diagnosis of corneal dystrophy preferably has all oligonucleotides, essentially comprising the nucleotide sequence
25 of the group consisting of SEQ ID NO: 16 to SEQ ID NO: 47, SEQ ID NO: 50, SEQ ID NO: 53, SEQ ID NO: 56 and SEQ ID NO: 59, fixed thereon.

The present invention also provides a DNA chip for diagnosis of corneal dystrophy, which has all oligonucleotides of the group consisting of SEQ ID NO: 11 to SEQ
30 ID NO: 15, SEQ ID NO: 62 and SEQ ID NO: 65 fixed thereon.

The present invention also provides a pair of primers selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2; SEQ ID NO: 3 and SEQ ID NO: 4; SEQ ID NO: 5 and SEQ ID NO: 6; SEQ ID NO: 7 and SEQ ID NO: 8; and SEQ
5 ID NO: 9 and SEQ ID NO: 10.

Other features and embodiments of the present invention will be more fully apparent from the following detailed description and appended claims.

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BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows spots on a DNA chip to measure detection efficiency of DNA chip depending on spotting solutions.

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FIG. 2 shows hybridization results after applying exon 4 PCR product from normal individuals to the DNA chip of FIG. 1.

FIG. 3 shows hybridization results after applying exon 4 PCR product from
20 patients with heterozygous Avellino corneal dystrophy to the DNA chip of FIG.1.

FIG. 4 shows hybridization results after applying exon 4 PCR product from patients with homozygous Avellino corneal dystrophy to the DNA chip of FIG.1.

25 FIG. 5 shows hybridization results after applying exon 4 PCR product from patients with heterozygous Reis-bucklers corneal dystrophy (CD1) to the DNA chip of FIG.1.

FIG. 6 illustrates spot arrangement of a DNA chip designed depending on the
30 concentration of probes.

FIG. 7 shows the anticipated result of hybridization according to each patient when the DNA chip of FIG. 6 was used.

- 5 FIG. 8 shows hybridization results depending on hybridization time of each primer set when the DNA chip of FIG. 6 was used.

FIG. 9 displays graphs of hybridization results of the DNA chip depending on the length of probes.

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FIG. 10 illustrates spot arrangement of the DNA chip to estimate hybridization efficiency of DNA chip depending on probe length.

- FIG. 11 shows hybridization results of the DNA chip depending on the length of
15 probes.

FIG. 12 illustrates spot arrangement of the DNA chip manufactured with 50 μ M of 15mer probe using 3X SSC spotting solution.

- 20 FIG. 13 shows hybridization results of the DNA chip of FIG. 12.

DETAILED DESCRIPTION OF THE INVENTION, AND PREFERRED EMBODIMENTS

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- The present inventors have paid attention to the fact that BIGH3 gene mutation causes corneal dystrophy having the symptom of corneal opacity and constructed a probe for diagnosing each corneal dystrophy, based on point mutations in BIGH3 gene shown in patients of various corneal dystrophies such as Avellino corneal
30 dystrophy, Lattice corneal dystrophy, Granular corneal dystrophy, Reis-bucklers

corneal dystrophy.

The probes constructed in the present invention comprise nucleotide sequences of SEQ ID NO: 11 to SEQ ID NO: 67.

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In order to manufacture a DNA chip capable of effectively detecting corneal dystrophy using the probe, optimal spotting solution, optimal time for immobilization, and the optimal length of probes were examined. Consequently, best result was generated by using 3X SSC as a spotting solution for 6 hours of hybridization time with 15mer probe for detecting exon 4 mutation or with 17mer probe for detecting exon 12 mutation in patients with corneal dystrophy, respectively.

The following definitions serve to illustrate the terms and expressions used in the different embodiments of the present invention as set out below.

An "isolated" nucleic acid molecule is one separated from other nucleic acid molecules that are present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules separated from the chromosome with which the genomic DNA is naturally associated.

The term "probe" or "nucleic acid probe" refers to single-stranded and sequence specific oligonucleotides, the sequence of which is sufficiently complementary to hybridize to a target nucleotide sequence to be detected

By "composition" is meant that a probe complementary to prokaryotic or eukaryotic genetic sequences may be a purified nucleic acid or in combination with other probes. In addition, the probe may be in a dry state in combination with salt or buffer, and may be a precipitate in an alcohol solution or in an aqueous solution.

The term "target" refers to nucleic acid molecules derived from a biological sample, which has a nucleotide sequence complementary to any one of the nucleic acid probes of the present invention. The target nucleic acid can be single- or double-
5 stranded DNA (optionally obtained after amplification) or RNA, and contain a sequence at least partially complementary to at least one oligonucleotide probe.

The phrase "a biological sample" refers to a specimen such as clinical samples (e.g., pus, sputum, blood, urine, etc.), environmental samples, bacterial colonies,
10 contaminated or pure culture products, purified nucleic acid, etc., in which the target sequence of interest is confined.

By "oligonucleotide" is meant a nucleotide polymer generally about 10 to about 100 nucleotides in length, but the length may be longer than 100 or shorter than 10
15 nucleotides.

By "nucleotide" is meant a subunit of a nucleic acid consisting of a phosphate group, a 5-carbon sugar and a base containing nitrogen. In RNA, the 5-carbon sugar is ribose. In DNA, it is 2-deoxyribose. For a 5-nucleotide, the sugar contains
20 a hydroxyl group (-OH) at the second carbon. The term also includes analogs of such subunits the same as a methoxy group at the second carbon of ribose.

The term "homologous" is synonymous with identical, meaning that polynucleic acids e.g., 90% homologous have 90% identical base pairs in the same position,
25 when sequences are aligned.

"Hybridization" involves annealing of a complementary sequence to its target nucleic acid (a sequence to be detected). The ability of two polymers of nucleic acids containing complementary sequences to recognize each other and anneal
30 through base pairing interaction is a well-known phenomenon.

The term "primer" refers to a single-stranded oligonucleotide sequence of DNA providing an initiation point for synthesis and elongation of a product that is complementary to a strand of nucleic acid to be copied. The length and the
5 sequence of a primer are recommended as much as they allow priming the synthesis and elongation of products. Preferably, the primer is about 5-50 nucleotides long. Specific length and sequence will depend on the complexity of target DNAs or RNAs, as well as on ambient conditions for primer use such as temperature and ionic strength.

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The term "label" as used herein refers to any atom or molecule that can be used to produce a detectable (preferably quantifiable) signal and attached to a nucleic acid.

Labels may provide detectable signals of fluorescence, radioactivity, colorimetry,
15 gravimetry, X-ray diffraction or absorption, magnetism, and the like.

By "hybrid" is meant the complex formed between two single stranded nucleic acid sequences by Watson-Crick base pairing or non-canonical base pairing between the complementary bases.

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The phrase "probe specificity" refers to characteristic of a probe, describing its ability to distinguish between a target and a non-target sequence. In this regard, the term "specific" means that a nucleotide sequence will hybridize to a defined target sequence and will substantially not or minimally hybridize to a non- target
25 sequence. Probe specificity is dependent on sequence and assay conditions.

The phrase "a standard strain" includes a microorganism commercially or readily available in the art.

Identification of Probes

To produce nucleic acid probes that can detect various prokaryotic or eukaryotic genetic sequences, each detection probe needs to be specific to a target sequence.

- 5 For this, screening of probe candidates specific to each target gene or organism is preceded.

Probe candidates are selected in the region of a gene embracing their target sequences. Specificity of probe candidates is first examined by BLAST search,
10 comparing homology of each nucleotide sequence, confirmed by hybridization using the "target" *in vitro*. And probes that only anneal to target genes among candidates are finally determined for gene identification.

Additionally, sensitivity of the probes for gene identification, selected by the
15 procedure above, is examined by clinical trials using a variety of biological samples.

The probe of the present invention are 11~17mer oligonucleotides, and preferably 70%, 80%, 90% or more than 95% homologous and exactly complementary to their target sequences to be detected. The length of the inventive probe for detecting and
20 identifying each "target" may be or more than 50 nucleotides. The nucleotides used in the present invention may include ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine or nucleotides containing analogue groups, but should not alter their hybridization characteristics.

25 Use of Probes

The probes of the present invention can be used for all the well-recognized hybridization techniques investigating presence or absence of a target nucleic acid in a biological sample for diagnostic purposes, such as a point-spotting technique
30 on a filter called "Dot-blot" (Maniatis *et al.*, *Molecular Cloning*, Cold Spring

Harbor, 1982), Southern Blot (Southern, E. M., *J. Mol. Biol.* 98, 503(1975)), or Northern Blot.

The probes of the invention can also be used in sandwich hybridization system that
5 enhances specificity of a nucleic acid probe-based assay. The principle and the
procedure of sandwich hybridization in a nucleic acid probe-based assay have been
already described (Dunn and Hassel, *Cell*, 12: 23-36; 1977; Ranki *et al.*, *Gene*, 21:
77-85; 1983). The sandwich hybridization technique uses a capture probe and/or a
10 detection probe. Those probes may hybridize with two different regions of their
target nucleic acid, and at least one of the probes (generally the detection probe)
may hybridize with a target region specific to a target species. It is understood that
the capture probe and the detection probe must have at least partly distinct
nucleotide sequences.

15 Although a direct hybridization assay has favorable kinetics, sandwich
hybridization is advantageous with respect to a high signal-to-noise ratio. Moreover,
sandwich hybridization can improve specificity of a nucleic acid probe-based assay.
The incubation and subsequent washing steps that constitute the key steps of the
sandwich hybridization process are each carried out at a constant temperature
20 between about 20°C and 65°C. It is known that nucleic acid hybrids have a
dissociation temperature which depends on the number of hybridized bases (the
temperature tends to rise in proportion to the size of the hybrid), and which also
depends on the nature of hybridized bases and their adjacent bases. The
hybridization temperature used in sandwich hybridization technique should be
25 chosen below the half-dissociation temperature relating to a given probe and its
complementary target sequence. And the temperature may be determined by simple
routine experiments.

The probes of the present invention can also be used in a competition hybridization
30 protocol. In competition hybridization, a target molecule competes for hybrid

formation with a specific probe and its complement. The more targets exist, the less amount of probe-complement hybrids becomes. A positive signal indicating existence of a specific target tends to decline in hybridization reaction as compared with a system without a target. In a particular embodiment, a specific
5 oligonucleotide probe, conveniently labeled, is hybridized with its target molecule. Next, the mixture is transferred to a microtiter dish well, in which complementary oligonucleotides and the specific probe are fixed, and allowed to keep all hybridized. After washing, the hybrids of the complementary oligonucleotides and the probes are measured preferably quantitatively based on used label.

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In addition, the probes of the present invention can be used in reversed hybridization (*Proc. Natl. Acad. Sci. USA*, 86: 6230-6234, 1989). For the method, a target sequence can first be enzymatically amplified by PCR using 5'-biotinylated primers. In a second step, the amplified products are detected upon hybridization
15 with specific oligonucleotides immobilized on a solid support. Reversed hybridization may also be carried out without amplification steps. In that particular case, prior to hybridization, the nucleic acids present in a biological sample have to be labeled or modified, specifically or non-specifically by addition of certain dyes or chemical reaction.

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The nucleic acid probes of the present invention may be included in a kit that can apply to rapidly determine presence or absence of pathogenic species of interest. The kit includes all components necessary for assay determining presence of a target gene. In the universal concept, the kit includes a stable reagent containing
25 labeled probes, a hybridization solution in either dry or liquid form for hybridizing target and probe polynucleotide, a solution for washing and removing undesirable and unbound polynucleotide, a substrate for detecting labeled duplex, and optionally an instrument for detecting the label.

30 One specific embodiment of the present invention embraces a kit that utilizes the

concept of sandwich assay. This kit includes a first component for collecting samples from patients including a scraping device or paper points, vials for containment, and buffers for dispersing and dissolving the sample. A second component includes a medium in either dry or liquid form for hybridizing target
5 and probe polynucleotide and a medium for washing out to remove undesirable and unbound polynucleotide. A third component includes a solid support, on which or to which nucleic acid probe(s) is conjugated, which is unlabeled and complementary to part of target polynucleotide. In the case of multiple target analysis, more than one capture probe, each specific to its own ribosomal RNA, is
10 applied to different discrete regions of dipstick.

A fourth component contains a labeled probe that is complementary to different regions from the same rRNA strand hybridizing to the immobilized and unlabeled nucleic acid probe of the third component. The probe components described herein
15 include combination of probes in dry form such as lyophilized nucleic acid or in precipitated form such as alcohol-precipitated nucleic acid, or in a buffered solution. The label may be any of the labels described above.

For example, probes can be biotinylated using conventional ways, and presence of
20 a biotinylated probe can be detected by adding an avidin-conjugated enzyme such as horseradish peroxidase, and then supplying a substrate of peroxidase that can be monitored visually or by a colorimeter or a spectrophotometer. This labeling method and other enzyme-conjugating labels have advantages of being economical, highly sensitive, and relatively safe, compared with radioactive labeling methods.
25 Kits of assorted components include various reagents for detection by labeled probes, an instruction, containers for mixing and reacting positive and negative controls, etc.

DNA chip

The probes of the present invention are also used in a DNA chip. In a preferred embodiment, the present invention provides a DNA chip that nucleic acid probes
5 are immobilized on its solid support. DNA chip, a narrow support that is mounted with various fragments of nucleotides with high density, is applied to identify DNA information by hybridizing unknown DNA extracted from unknown samples with immobilized DNA thereof. Examples of solid supports for holding probe oligonucleotides include inorganic materials such as glass and silicon, and
10 polymeric materials such as acryl, polyethylene terephthalate (PET), polystyrene, polycarbonate and polypropylene. The surface of solid substrates may be flat or highly porous. The probes are immobilized by covalent bond of either 3'-end or 5'-end on the substrate. The immobilization can be achieved by conventional techniques, for example, using electrostatic force, fixing amine group-attached
15 oligomers on aldehyde-coated slide, or spotting on an amine coated slides, L-lysine-coated slides or nitrocellulose-coated slides. In one embodiment of the present invention, a base with an amine group was incorporated into 3' position of the probe during its synthesis, which makes it easy to bind covalently onto aldehyde-coated glass slides.

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Immobilization and arrangement of various probes onto solid substrates are carried out by pin microarray, inkjet, photolithography, electric array, etc. In an embodiment of the invention, probes are separately dissolved in a buffer solution, and the resulting solution is spotted onto the substrate by using a microarrayer
25 prepared by a known method (Yoon *et al.*, *J. Microbiol.Biotechnol.*, 10(1), 21-26, 2000).

The principle of microarrayer is that minutely constructed pins pick probe DNA from a reservoir and transfer to one site on a support appointed by a computer. For
30 fixing the probes transferred by a microarrayer, immobilization reaction proceeds at

least for one hour under humid condition of from 45% to 65%, preferably, from 50% to 55%, and it was left to stand at least for 6 hours to induce reaction between an amine group at 3' end of the probe and an aldehyde group coated onto the glass slide.

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For detecting cells derived from living organisms or a living organism itself, the cells occasionally need partial and/or total lysis by a chemical and/or a mechanical procedure for easy access to their RNA and/or DNA, and then they are brought in contact with one or several probes of the present invention. The contact may be carried out on an appropriate support such as nitrocellulose, cellulose or a nylon filter in a solution, or in a liquid medium, which may take place under a suboptimal, an optimal or a restrictive condition. Such conditions include temperature, concentration of reactants, presence of substances lowering an optimal temperature of nucleic acid pairing (e.g., formamide, dimethylsulfoxide and urea), and presence of substances apparently lowering reaction volume and/or accelerating hybrid formation (e.g., dextran sulfate, polyethyleneglycol or phenol).

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Preparation of Probes

Traditional cloning methods may produce large quantities of nucleic acid probes by either gene manipulation of the desired sequence as described in Maniatis, *T., et al. Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1982, or by chemical synthesis using a commercially available DNA synthesizer.

The probes of the present invention can be prepared to be single-stranded or double-stranded by conventional methods. A representative example of preparing a single-stranded probe is to synthesize probes comprising a desired number of nucleotides after dimethoxytrityl (DMT) off method by an automated DNA synthesizer and stripping protection.

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One end of the probes, while synthesized, is labeled with a fluorescent dye (fluorescein isothiocyanate, FITC) to confirm presence or absence of nucleic acids of interest. Alternatively, A DNA probe complementary to a single-stranded DNA template is prepared by annealing primers to its template DNA and polymerizing
5 with Klenow enzyme and fluorescent-labeled dNTP. The probes, thus exhibit high sensitivity and specificity owing to the fluorescent dye.

For a preparation of a double-stranded probe, genomic DNA or plasmid DNA is digested with specific restriction enzymes to obtain probes comprising desired
10 regions of a gene or a nucleotide fragment. Random priming method is one way of polymerizing fluorescent-labeled probes with various lengths by six random hexamers. Alternatively, Probes can be synthesized by attaching ^{32}P to 5'-end of DNA with T4 polynucleotide kinase and also synthesized by generating nicks in double-stranded DNA molecules with DNase I, polymerizing DNA with DNA
15 polymerase I and fluorescent-labeled dNTP. The synthetic double-stranded probes are denatured to make it single-stranded, which is then used for hybridization.

The probes of the present invention may be advantageously labeled. Any conventional label can be utilized. The probes can be labeled by means of
20 radioactive tracers such as ^{32}P , ^{35}S , ^{125}I , ^3H and ^{14}C . The radioactive labeling can be carried out according to any conventional method such as terminal labeling at the 3' or 5' end using a radiolabeled nucleotide, polynucleotide kinase, terminal transferase, or ligase.

25 Another method for radioactive labeling is chemical iodination of probes of the present invention attaching several ^{125}I on the probes. If one of the probes of the present invention is radioactive, detection of ionizing ray is generally conducted by autoradiography, liquid scintillation, gamma counting or any other conventional method.

The probes of the present invention can be labeled by means of non-radioactive residues featuring: immunological properties (e.g., antigens or haptens); specific affinity for some reagents (e.g., ligands); detectable properties by enzymatic reaction (e.g., enzymes, co-enzymes, enzyme substrates or intermediates taking
5 part in an enzymatic reaction); and physical properties including fluorescence, emission or absorption of light at a certain wavelength. Antibodies can be used to specifically detect a hybrid of probes and targets. Non-radioactive labels can be provided when the probe of the present invention is chemically synthesized. Adenosine, guanosine, cytidine, thymidine and uracyl residues are easily coupled
10 with other chemical residues, enabling to detect the probe, or hybrids of probes and their complementary DNA or RNA fragments.

Target

15 Nucleic acids are extracted from a sample to provide nucleic acid substrates for diagnosis of diseases with biological samples. The nucleic acids may be extracted from a variety of clinical samples using standard techniques or commercially available kits. For example, kits for isolating RNA or DNA from tissue samples are available from Qiagen, Inc. (Chatsworth, CA, USA) and Stratagene (La Jolla, CA,
20 USA). For example, QIAamp Blood extraction kit can isolate DNA from blood, bone marrow, body fluids or cell suspensions. QIAamp tissue extraction kit can purify DNA from tissues such as muscles and organs.

If double-stranded, target DNA is recommended to denature prior to proceeding
25 detection. Denaturation of double-stranded DNA can be conducted by chemical, mechanical or enzymatic method heating up to a proper temperature over 80°C.

Target DNA hybridizing to a probe is usually prepared by two kinds of methods. A first method is Southern blot or Northern blot in which genomic DNA or plasmid
30 DNA is digested with appropriate restriction enzymes, and digested DNA

fragments are separated and purified by using agarose gel electrophoresis. A second method is amplification of desired DNA regions by PCR.

Examples of PCR include: typical PCR using same amounts of forward and reverse
5 primers; Asymmetric PCR using different amount of primers and resulting in
double-stranded and single-stranded bands; Multiplex PCR using various primer
pairs and amplifying different regions simultaneously; Ligase chain reaction (LCR)
using specific 4 primers and ligase and measuring fluorescence intensity by ELIA
(Enzyme Linked Immunosorbent Assay); besides, Hot Start PCR, Nest-PCR, DOP-
10 PCR (Denaturated oligonucleotide primer PCR), RT-PCR (Reverse Transcription
PCR), Semi-quantitative RT-PCR, Real time PCR, RACE (Rapid Amplification of
cDNA Ends), Competitive PCR, STR (Short Tandem Repeats), SSCP (Single
Strand Conformation Polymorphism), DDRT-PCR (Differential Display Reverse
Transcriptase), etc.

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In one preferred embodiment of the present invention, asymmetric PCR is
performed to polymerize a gene fragment using DNA extracted from a specimen as
a template. The gene fragment is polymerized by one reaction of PCR using
different amount of forward and reverse primers in a ratio of 1: 5.

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In a preferred embodiment of the PCR, 5 ul of 10X PCR buffer solution (100 mM
Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂), 4 ul of dNTP mixture (dATP,
dGTP, dCTP, dTTP, each 2.5 mM), 0.5 ul of 10 pmole forward primer, 2.5 ul of 10
pmole reverse primer, 1 ul of 1/10 diluted template DNA (100 ng) and 0.5 ul of Taq
25 polymerase (5 unit/ul, Takara Shuzo Co. , Shiga, Japan) are mixed and distilled
water is added to the cocktail to be up to 50 ul. The PCR is performed by following
steps: First denaturation at 94°C for 7 min once, 10 cycles of second denaturation
at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min;
30 cycles of third denaturation at 94°C for 1 min, annealing at 52°C for 1 min and
30 extension at 72°C for 1 min, and then 72°C for 5 min only at the last extension.

The PCR products are confirmed by agarose gel electrophoresis.

Hybridization and Washing

- 5 A particular hybridization technique is not essential to the present invention. Hybridization techniques are generally described in *Nucleic Acid Hybridization: A Practical Approach*, Ed. Hames, B. D. and Higgins, S. J. , IRL Press, 1987; Gall and Pardue (1969), *Proc. Natl. Acad. Sci., U.S.A.*, 63: 378-383, and John, Burnsteil and Jones (1969) *Nature*, 223: 582-587.

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The hybridization conditions are determined by the "stringency" that is to say the strictness of the operating conditions. The more stringent a condition is, the more specific probes and targets hybridize. Stringency indicates especially the base composition of a probe/target duplex as well as the degree of mismatch between
15 nucleic acids. Stringency can likewise reflect functional parameters of hybridization reaction, such as concentration and type of ionic species present in a hybridization solution, nature and concentration of denaturing agents and/or hybridization temperature. Stringency of a hybridization condition depends especially on probes used. All data about the conditions are well known, and an
20 appropriate condition of individual hybridization may be established by routine experiments. With regard to the length of probes, temperature of hybridization reaction generally lies in approximately within the range of about 20°C and 65°C, in particular, between 35°C and 65°C in about 0.8 to 1M of a saline solution.

- 25 Nucleic acid hybridization of labeled oligonucleotide probes and nucleic acid targets can be enhanced by the use of "unlabeled Helper Probes" as disclosed in U. S. Pat. 5,030,557. Helper probes are oligonucleotides that bind to another region of nucleic acid, not where the assay probe is targeting. The helping interaction of additional probes accelerates the binding of assay probes, forming new secondary
30 and tertiary structures on the targeted region of single stranded nucleic acids.

It will be appreciated by those skilled in the art that factors affecting thermal stability of probe/target hybrids can also affect probe specificity. Thus, the melting profile, including melting temperature (T_m) of probe/target hybrids needs to be
5 determined. The preferred method is described in U.S. Pat. 5,283,174.

T_m of probe/target hybrids may be determined by a hybridization protection assay as follows. With the excess amount of targets, probe/target hybrids are formed in a lithium succinate buffered solution containing lithium lauryl sulfate. Each aliquot
10 of the "preformed" hybrid is diluted in hybridization buffer and incubated for five minutes at various temperatures, gradually increasing 2 - 5°C from below anticipated T_m (typically 55°C). The solutions are then diluted with a mildly alkaline borate buffer and incubated at lower temperature (for example, 50°C) for ten minutes. Under this condition, while the acridinium ester attached to single-
15 stranded probes is hydrolyzed, the acridinium ester attached to hybridized probes is relatively protected. This is referred to as a hybridization protection assay (HPA). The amount of chemiluminescence's remnants is proportional to the amount of hybrids, and is measured in a luminometer after adding hydrogen peroxide and alkali in order. The data is plotted by percentage of maximum signal (usually from
20 the lowest temperature) versus temperature.

T_m is defined by a point reaching at 50% of maximum signal. As an alternative, T_m of a probe/target hybrid may also be determined by isotopic methods well known to those skilled in the art. It should be noted that T_m of a given hybrid may
25 vary depending on the concentration of salts, detergents and other solutes existing in hybridization solution, which affect relative stability of the hybrid during thermal denaturation (*Molecular Cloning: A Laboratory Manual* Sambrook *et al.*, eds. Cold Spring Harbor Lab Publ., 9.51 (2nd ed., 1989)).

30 Hybridization conditions can be monitored by several parameters, e.g.,

hybridization temperature, nature and concentration of components in the media, and temperature at which hybrids are washed. Hybridization and washing temperature are limited by maximum temperature corresponding to probe's nucleic acid composition, composition and length, and the maximum temperature of probes
5 for hybridization or wash described herein is about 30°C to 60°C. At higher temperature, the duplexing competes with the dissociation or denaturation of probe/target hybrid. A preferred hybridization medium contains about 3X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), about 25 mM of phosphate buffer pH 7.1, and 20% deionized formamide, 0.02% Ficoll, 0.02% bovine serum
10 albumin, 0.02% polyvinylpyrrolidone and about 0.1 mg/ml sheared denatured salmon sperm DNA. A preferred wash medium contains about 3X SSC, 25 mM phosphate buffer pH 7.1 and 20% deionized formamide. However, when modifications are introduced either in the probes or in the media, the temperatures, suitable for hybridization to bring out required specificity, should change based on
15 known relationships as described in the following reference: B. D. HAMES and S. J. HIGGINS, (eds.). *Nucleic acid hybridization. A practical approach*, IRL Press, Oxford, U.K., 1985.

In this respect, it should also be noted that, generally because DNA:DNA hybrids
20 are less stable than RNA:DNA or RNA:RNA hybrids, their conditions of hybridization need to be carefully adjusted to achieve specific detection depending on hybrid features.

In a preferred embodiment of the present invention, amplified target fragments of a
25 gene is added in a hybridization buffer solution (6X SSPE (0.15M NaCl, 5mM $C_6H_5Na_3O_7$, pH 7.0), 20% (v/v) formamide), applied onto a glass slide holding probes, and then incubated at 30°C for 6 hours, so that said probes can complementarily hybridize with said targets. The glass slide is washed sequentially with 3X SSPE, 2X SSPE and 1X SSPE for 5 min.

Hybrids can be quantified by labeling targets with a fluorescence chemical or a radioactive isotope in accordance to conventional methods. Label incorporation may be carried out by using labeled primers or labeled nucleotides during polymerization and amplification.

5

Examples

The present invention will hereinafter be described in further detail by examples. It will however be obvious to a person skilled in the art that these examples are
10 given for illustrative purpose only, and the scope of the present invention is not limited to or by the examples.

All the following experiments in Examples were approved by IRB (Institutional Review Board) in Severance hospital in Korea and conducted by prior agreement
15 of every attendee in accordance with the declaration of Helsinki.

Example 1: Determining mutation types of BIGH3 protein

In order to construct diagnostic probes for detecting genetic mutation of BIGH3,
20 one of the causes of ophthalmologic diseases including Avellino corneal dystrophy, its mutation sites were determined to produce probes.

The mutation sites of BIGH3 protein were identified, ensuring their amino acid and nucleotide sequences by GenBank and OMIM (Online Medelian Inheritance in
25 Man), databases of nucleotide sequences of NCBI (National Center for Biotechnology Information), and allele information of each disease was comprehended. The types of mutation to be searched were determined to test efficacy of DNA chip. Especially among hot spots for BIGH3 gene mutation, mutation regions causing Avellino dystrophy, Lattcie type I dystrophy and Reis-
30 bucklers I were selected (Table 1).

Table 1. Ophthalmologic diseases caused by genetic mutation of BIGH3

Phenotype	Mutated Amino acid	Sequence change	Exon
Avellino dystrophy	R124H	CGC → CAC	4
Granular dystrophy	R555W	CGG → TGG	12
Lattice dystrophy	R124C	CGC → TGC	4
Lattice dystrophy	L518P	CTG → CCG	12
Lattice dystrophy	L527R	CTG → CGG	12
Lattice dystrophy	H626R	CAT → CGT	14
Lattice dystrophy	N622H	AAC → CAC	14
Lattice dystrophy	N544S	AAT → AGT	12
Lattice type IIIA	P501T	CCA → ACA	11
Lattice type IIIA	A546T	GCC → ACC	12
Reis-bucklers I (Reis-bucklers(CDB1))	R124L	CGC → CTC	4
Reis-bucklers	F540 deletion	TTT → ---	12
Reis-bucklers (CDB2)	R555Q	CGG → CAG	12
Reis-bucklers	G623D	GGC → GAT	14

Example 2: Obtaining Search regions by PCR

5

In order to search all mutations shown in Table 1, five pairs of primers encompassing exon 4, exon 11, exon 12 and exon 14 were designed. Among them, two pairs of primers were used to amplify the mutation region of exon 4. One of the pairs (Primer 1 and Primer 2) was concluded suitable for experiments of diagnostic DNA chip, and the other was designed for direct DNA sequence analysis (Primer 3 and Primer 4). In addition, the DNA probes and their complementary primers (reverse primers) to be searched were labeled with a fluorescent chemical on their 5'-hydroxyl group. By Cy5 and Cy3 that were bound to primer 2 and primer 4, respectively, those primer pairs were hence effectively distinguished.

15

Table 2. Primers for amplifying genetic regions encompassing mutation sites

Primer #	SEQ ID NO.	Sequence (5' -> 3')
----------	------------	---------------------

1	1	agc cct acc act ctc aa
2	2	cag gcc tcg ttg cta ggg
3	3	ccc cag agg cca tcc ctc ct
4	4	ccg ggc aga cgg agg tca tc
5	5	ctc gtg gga gta taa cca gt
6	6	tgg gca gaa gct cca ccc gg
7	7	cat tcc agt ggc ctg gac tct act atc
8	8	ggg gcc ctg agg gat cac tac tt
9	9	ctg ttc agt aaa cac ttg ct
10	10	ctc tcc acc aac tgc cac at

Gene fragments were generated by Asymmetric PCR using 10-100 ng of DNA extracted from blood as a template, considering PCR reaction volume to be total 50 μ l. The fragments were polymerized by one PCR reaction using different amount of forward and reverse primers in a ratio of 1: 5 to 1:10. The first denaturation was carried out at 98°C for 5 min. just once, and then main 35 cycles of PCR were performed by secondly denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. PCR was terminated by maintaining 72°C for 7 min once for the last extension.

10

Example 3: Manufacturing probes for diagnosis of BIGH3 gene mutation

In order to search mutations of corneal dystrophy as shown in Table 1, probes were designed to embrace 5- 8 more nucleotide sequences expanding from both sides of a hotspot, more preferably 7 more nucleotides. Probes for normal counterparts were designed to embrace the same sequences as the above but normal instead of mutated nucleotide sequences at the center.

15

Table 3. Probes constructed for diagnosing mutation of ophthalmologic diseases

SEQ ID NO.	Genotype	Normal/ Mutation	Probe sequence	Exon
11	Normal	R124	acg gac cgc acg gag	4
12	Avellino dystrophy	R124H	acg gac cac acg gag	4

13	Reis-bucklers(CDB1)	R124L	acg gac ctc acg gag	4
14	Normal	R124	cac gga ccg cac gga	4
15	Lattice type I	R124C	cac gga ctg cac gga	4
16	Normal	P501	gac ccc ccc aat ggg	11
17	Lattice typellIA	P501T	gac ccc cac aat ggg	11
18	Normal	L518	agc atg ctg gta gct	12
19	Lattice dystrophy Pro	L518P	agc atg ccg gta gct	12
20	Normal	L527	gca gga ctg acg gag	12
21	Lattice dystrophy Arg	L527R	gca gga cgg acg gag	12
22	Normal	F540	aca gtc ttt gct ccc	12
23	Reis-bucklers	F540 deletion	ac aca gtc gct ccc ac	12
24	Normal	N544	ccc aca aat gaa gcc	12
25	Lattice dystrophy ser1	N544S	ccc aca agt gaa gcc	12
26	Normal	N544	ccc aca aac gaa gcc	12
27	Lattice dystrophy ser2	N544S	ccc aca agc gaa gcc	12
28	Lattice dystrophy ser3	N544S	ccc aca tet gaa gcc	12
29	Lattice dystrophy ser4	N544S	ccc aca tcc gaa gcc	12
30	Lattice dystrophy ser5	N544S	ccc aca tca gaa gcc	12
31	Lattice dystrophy ser6	N544S	ccc aca tcg gaa gcc	12
32	Normal	A546	aaa tga agc ctgc cga	12
33	Lattice typellIA thr	A546T	aaa tga aac ctgc cga	12
34	Normal	R555	aag aga acg gag cag	12
35	Granular dystrophy	R555W	aag aga atg gag cag	12
36	Normal	R555	aga gaa cgg agc aga	12
37	Reis-bucklers(CDB2)	R555Q	aga gaa cag agc aga	12
38	Normal	N622	ggc cac aaa tgg cgt	14
39	Normal	N622	ggc cac aaa cgg cgt	14
40	Lattice dystrophy his	N622H	ggc cac aca cgg cgt	14
41	Normal	G623	aca aat ggc gtg gtc	14
42	Reis-bucklers-1	G623D	aca aat gat gtg gtc	14
43	Normal	G623	caa acg gcg tgg tcc	14
44	Reis-bucklers-2	G623D	caa acg atg tgg tcc	14
45	Normal	H626	gtg gtc cat gtc atc	14
46	14Lattice dystrophy	H626R	gtg gtc cgt gtc atc	14

Example 4: Manufacturing DNA chip for diagnosing corneal dystrophy

Each probe selected in Example 3 was synthesized to apply to DNA chip.

Mononucleotides (Proligo Biochemie GmbH Hambrug Co. GE) were injected into Automatic synthesizer (ExpediteTM 8900, PE Biosystems Co. USA) with information of nucleotide sequences and input scales, and were polymerized to be 0.05 μ M of pure nucleic acid probes. The synthesis of resulted probes was confirmed by electrophoresis.

In order to immobilize DNA probes on a solid support, amine-aldehyde covalent bonds were used. The 3' end of synthetic oligonucleotide probes was modified to insert amine residues using an amino linker column (Cruachem, Glasgow, Scotland) for immobilization, and dissolved in spotting buffer (3X SSC; 0.45 M NaCl; 15 mM $C_6H_5Na_3O_7$, pH 7.0). The resulting solution was spotted on the aldehyde-coated glass slides (CEL Associates, Inc. Huston, USA) using microarrayer, and then the slides were kept under more than 55% humid condition to allow amine and aldehyde to bind for more than 1 hour, followed by immobilizing DNA probes for 6 hours. Position markers and each probes were fixed at the concentration of 2-5 μ M and 10-100 μ M, respectively. To evaluate efficiency of immobilization, the glass slides were dyed with SYBRO green II (Molecular Probes, Inc., Leiden, Netherlands).

Example 5: Diagnosis of corneal dystrophy using DNA chip for diagnosing thereof

To confirm the specificity and sensitivity of probes, hybridization was performed by applying the PCR products prepared in Example 2 to the DNA chip capturing probes that were generated in Example 4. After hybridizing with genomic DNA extracted from blood of patients or normal individuals, whether a probe produced a positive hybridization signal and crossreactivity (specificity) with other probes were tested.

DNA chip was hydrated with a water vapor and then soaked in 70% ethanol to

remove any probes that were not immobilized on the glass of DNA chip. The DNA chip was transferred to a blocking solution (1.3 g of NaBH₄, 375 ml of PBS, 125 ml of 100% ethanol) and shaken for 5 minutes in order to prevent fluorescent chemicals from attaching aldehyde groups on the surface of a glass, which might
5 result in boosting total intensity of fluorescence and cause the eclipse of specific and positive signals. The DNA chip was then washed with 0.2% SDS for 5 min. and ensued by twice or three times with sterile water for 1 min. each. The liquid remains on the glass surface of DNA chip was eliminated by centrifugation (at 1,000 rpm for 2 min).

10

10-20 μ l of asymmetric PCR products was added with hybridization buffer [6X SSPE (0.9M NaCl, 10 mM NaH₂PO₄-H₂O, 1 mM EDTA, pH 7.4) or 20X SSPE (3M NaCl, 0.2M NaH₂PO₄-H₂O, 0.02M EDTA, pH 7.4)], 20% (v/v) formamide (Sigma Co., St. Louis USA) to be total 200 μ l. The resulting mixture was applied
15 on a glass slide onto which probes were immobilized and covered with a probe-clip press-seal incubation chamber (Sigma Co., St. Louis, MO).

The hybridization reaction was continued for 4-6 hours in shaking incubator at 30°C, preventing dehydration of ambient air around glass by placing wet cotton
20 tissues in the chamber during hybridization to induce complementary binding. After the completion of hybridization, the slides were washed subsequently with 3X SPE (0.45 M NaCl, 15 mM C₆H₅Na₃O₇, pH 7.0), 2X SSPE (0.3 M NaCl, 10 mM C₆H₅Na₃O₇, pH 7.0) and then 1X SSPE (0.15 M NaCl, 10 mM C₆H₅Na₃O₇, pH 7.0) for 5 min. each. The liquid remains on the glass surface of DNA chip was
25 eliminated by centrifugation (at 1,000 rpm for 2 min).

The hybrids were detected by DNA chip scanner (CCD camera based scanner, ArrayWoRx, Applied Precision LLC, USA), with exposure time of 0.2 ~ 0.5 sec. Fluorescence intensity was measured at 595 nm for Cy3, 685 nm for Cy5 and 530
30 nm for FITC and the detected signals were interpreted by ImaGene 6.0 software

(Biodiscovery Inc., USA).

Example 6: Detection efficiency of DNA chip depending on spotting buffer

- 5 The detection efficiency of DNA chip was assessed depending on spotting buffers, in order to manufacture efficient DNA chip that can selectively detect point mutation of one or two nucleotides for diagnosing corneal dystrophy.

DNA chip was manufactured with different spotting solutions, but in which 0.1 M probes produced in Example 4 are contained. Probes on 2 blocks in the left among 10 4 blocks as shown in FIG. 1 were spotted with a commercial buffer (Telechem, USA), one block in the upper left was spotted with 3X SSC, and the other block in the lower right, with 50% DMSO solution. Yellow regions represent probes detecting normal individuals, and pink and bluish green represent probes detecting 15 Avellino dystrophy and Reis-bucklers CD1, respectively.

FIG. 2 ~ FIG. 5 show the result of hybridization using DNA chip designed as described above and exon 4 PCR products. Cy5 (Red) fluorescence stands for the result by applying PCR product that was amplified with primer 1 and primer 2, and 20 Cy3 (green) fluorescence does the result with primer 3 and primer 4. A box located in the right stands for a comparable result, such as FIG. 2 for normal individuals, FIG. 3 for heterozygous Avellino dystrophy, FIG. 4 for homozygous Avellino dystrophy and FIG. 5 for heterozygous Reis-Bucklers CD1.

- 25 As shown in FIG. 2 ~ FIG. 5, spotting with 3X SSC produced most precise and clear signals, hence, in the following examples, all hybridization was performed with 3X SSC.

Example 7: Detection efficiency of DNA chip depending on probe
30 **concentration and hybridization time**

The detection efficiency of DNA chip was assessed depending on probe concentration and hybridization time, in order to manufacture efficient DNA chip that can selectively detect point mutation of one or two nucleotides for diagnosing
5 corneal dystrophy.

DNA chip was manufactured by spotting 10 -100 μ M probes produced in Example 4 with 3X SSC. As shown in FIG. 6, one DNA chip was manufactured to contain two blocks. FIG. 7 shows the anticipated result of hybridization depending on
10 patients.

FIG. 8 shows the hybridization result using the DNA chip above and primer sets depending on hybridization time. Red spots are the hybridization result of PCR product amplified with primer 1 and primer 2 and labeled with Cy5. Hybridization
15 time and applied patients were enlisted on each drawing.

As a result of PCR product of exon 4 region amplified with primer 1 and 2, the optimal condition for hybridization turned out that probe concentration was 30- 50 μ M of probes and hybridization time was recommended for 2-6 hours, more
20 preferably 4-6 hours and most preferably 6 hours.

Example 8: Detection efficiency of DNA chip depending on probe length

The detection efficiency of DNA chip was assessed depending on probe length, in
25 order to manufacture efficient DNA chip that can selectively detects point mutation of one or two nucleotides for diagnosing Avellino corneal dystrophy, Reis-bucklers I corneal dystrophy, Lattice type I corneal dystrophy and Granular corneal dystrophy.

30 Based on the mutation sites enumerated in Table 3, variety of probe length such as

11mer, 12mer, 15mer and 17mer were synthesized, based on the probe sequences of primer 11 to primer 15, and primer 34 and primer 35.

Table 4. Probe sequences for determining optimal probe length.

Probe	Length	Sequence	SEQ ID NO.	
not ACD	11	ggaccgcacgg	47	Normal/Hetero
	13	cggaccgcacgga	48	
	15	acggaccgcacggag	11	
	17	cacggaccgcacggaga	49	
ACD	11	ggaccacacgg	50	Avellino
	13	cggaccacacgga	51	
	15	acggaccacacggag	12	
	17	cacggaccacacggaga	52	
RBCD	11	ggacctcacgg	53	Reis-Bucklers I
	13	cggacctcacgga	54	
	15	acggacctcacggag	13	
	17	cacggacctcacggaga	55	
not LCD I	11	cggaccgcacg	56	Normal/Hetero
	13	acggaccgcacgg	57	
	15	cacggaccgcacgga	14	
	17	acacggaccgcacggag	58	
LCD I	11	cggactgcacg	59	Lattice type I
	13	acggactgcacgg	60	
	15	cacggactgcacgga	15	
	17	acacggactgcacggag	61	
not GCD	15	aagagaacggagcag	34	Normal/Hetero
	17	caagagaacggagcaga	62	
	16	caagagaacggagcag	63	
	16	aagagaacggagcaga	64	
GCD	17	caagagaatggagcaga	65	Granular type
	16	caagagaatggagcag	66	
	16	aagagaatggagcaga	67	

5

DNA chip was manufactured with 3X SSC spotting solution and 50 μ M probes produced in Example 4.

The ratio (W/M) of mutation signals (M) to normal signals (W) was defined by the ratio of spot signals (W) of probes detecting normal sequences to spot signals (M) of probes detecting dystrophy. And the ratio was optimized and interpreted. As shown in FIG. 9, Avellino corneal dystrophy was detected most successfully by 13mer probe as well as quite efficiently by 15mer probe. Reis-bucklers I corneal

dystrophy and Lattice type I corneal dystrophy were detected most efficiently by 15mer probe. Therefore, the detection of mutation regions of exon 4 for Avellino corneal dystrophy, Reis-bucklers I corneal dystrophy and Lattice type I corneal dystrophy was conducted with 15mer probe. In Table 4, the optimal length of probes was emphasized by bold fonts for diagnostic DNA chip of corneal dystrophy.

The detection of Granular corneal dystrophy, containing point mutations in exon 12 was performed most effectively with 17mer probe using DNA chip in FIG. 10 (FIG. 11).

Example 9: Hybridization of DNA chip for diagnostic application

Based on the results of Example 6, Example 7 and Example 8, the detection efficiency of DNA chip was assessed depending on probe concentration and hybridization time, in order to manufacture efficient DNA chip that can selectively detect point mutations of one or two nucleotides for diagnosing corneal dystrophy.

Eventually, DNA chip was manufactured with 3X SSC spotting solution and 50 μ M of 15mer probes (FIG. 12). FIG. 13 shows the anticipated results of hybridization depending on patients.

FIG. 13 shows the result of hybridization using DNA chip manufactured as described above and PCR products of patients, amplified with primer 1 and primer 2. Subjects were enlisted in each drawing. The application result of DNA chip of the present invention to patients in diagnosis confirmed possibility of precise diagnosis for all patients, and proved superiority of specificity as well as sensitivity.

By the result of hybridization, DNA chip was manufactured, which is applicable to diagnosis of ophthalmologic diseases with 15mer probes detecting for Avellino

corneal dystrophy, Reis-bucklers I corneal dystrophy and Lattice type I corneal dystrophy, and 17mer probes detecting for Granular corneal dystrophy. The procedure, that 30-50 μ M probes in 3X SSC was spotted and hybridized at 30°C for 6 hours, was found to generate optimal diagnostic result. And the result delivered a fact that hybridization can be performed at 15-20°C higher than T_m corresponding to probe length. Therefore, DNA chip of the present invention can be processed by controlling hybridization temperature that is determined by probe length fixed on the chip, and utilized for diagnosis of corneal dystrophy including Avellino corneal dystrophy caused by BIGH3 mutation.

10

Using the optimized DNA chip for diagnosis of corneal dystrophy manufactured above, blood samples from 98 patients were subjected to diagnosis of corneal dystrophy. As a result, as shown in Table 5, 27 patients turned out to be normal, 10 patients to be homo Avellino corneal dystrophy, 57 patients to be hetero Avellino corneal dystrophy, 1 patient to be hetero Lattice type I corneal dystrophy, 1 patient to be hetero Reis-Bucklers type I corneal dystrophy, and 2 patients to be Granular corneal dystrophy.

15

Table 5. Diagnosis result of clinical samples

Illness	Mutation	Mutation site	Mutation type	Exon	Pateint.No.
Avellino	R124H	CGC→CAC	heterozygous	Exon 4	57
Avellino	R124H	CGC→CAC	homozygous	Exon 4	10
Lattice type I	R124C	CGC→TGC	heterozygous	Exon 4	1
Reis-Bucklers I	R124L	CGC→CTC	heterozygous	Exon 4	1
Granular type	R555W	CGG→TGG		Exon 12	2
normal	None		normal		27
Total					98

20

Said diagnosis result was confirmed by DNA sequence analysis of clinical samples, which proved 100% of accuracy.

INDUSTRIAL APPLICABILITY

As described above in detail, the present invention has an effect of providing oligonucleotides containing a mutation region of BIGH3 gene that causes corneal
5 dystrophy, and a DNA chip for diagnosis of corneal dystrophy, which has the oligonucleotides fixed thereon. According to the present invention, conventional microscopic diagnosis of corneal dystrophy can be replaced with a precise genetic method, which prevents a patient with corneal dystrophy from losing eyesight by eyesight correction surgery after erroneous diagnosis.

10

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 solely 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
15 the appended claims and equivalents thereof

THE CLAIMS

What is claimed is:

- 5 1. Oligonucleotides for diagnosis of corneal dystrophy, which essentially comprises one or more nucleotide sequences selected from the group consisting of SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 40, SEQ ID NO:
10 42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO: 50, SEQ ID NO:53 and SEQ ID NO: 59.
2. The oligonucleotide for diagnosis of corneal dystrophy according to claim 1, wherein said oligonucleotide essentially comprising the nucleotide sequence of
15 SEQ ID NO: 50 is set forth in SEQ ID NO: 12.
3. The oligonucleotide for diagnosis of corneal dystrophy according to claim 1, wherein said oligonucleotide essentially comprising the nucleotide sequence of
20 SEQ ID NO: 53 is set forth in SEQ ID NO: 13.
4. The oligonucleotide for diagnosis of corneal dystrophy according to claim 1, wherein said oligonucleotide essentially comprising the nucleotide sequence of
SEQ ID NO: 59 is set forth in SEQ ID NO: 15.
- 25 5. The oligonucleotide for diagnosis of corneal dystrophy according to claim 1, wherein said oligonucleotide essentially comprising the nucleotide sequence of
SEQ ID NO: 35 is set forth in SEQ ID NO: 65.
6. The oligonucleotide for diagnosis of corneal dystrophy according to claim 1,
30 wherein the length of the oligonucleotide is 13 to 17 bp.

7. A DNA chip for diagnosis of corneal dystrophy, wherein the oligonucleotide of any one claim among claims 1 to 6 is fixed on the substrate thereof.

5 8. The DNA chip for diagnosis of corneal dystrophy according to claim 7, which additionally immobilizes oligonucleotides, essentially comprising one or more nucleotide sequences selected from the group consisting of SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO:
10 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, and SEQ ID NO: 56, thereon.

9. The DNA chip for diagnosis of corneal dystrophy according to claim 8, wherein said oligonucleotide essentially comprising the nucleotide sequence of SEQ ID
15 NO: 47 is set forth in SEQ ID NO: 11.

10. The DNA chip for diagnosis of corneal dystrophy according to claim 8, wherein said oligonucleotide essentially comprising the nucleotide sequence of SEQ ID NO: 56 is set forth in SEQ ID NO: 14.

20

11. The DNA chip for diagnosis of corneal dystrophy according to claim 8, wherein said oligonucleotide essentially comprising the nucleotide sequence of SEQ ID NO: 34 is set forth in SEQ ID NO: 62.

25 12. The DNA chip for diagnosis of corneal dystrophy according to claim 7, which immobilizes all oligonucleotides, essentially comprising the nucleotide sequence of the group consisting of SEQ ID NO: 16 to SEQ ID NO: 47, SEQ ID NO: 50, SEQ ID NO: 53, SEQ ID NO: 56 and SEQ ID NO: 59, thereon.

30 13. The DNA chip for diagnosis of corneal dystrophy, wherein all oligonucleotides

of the group consisting of SEQ ID NO: 11 to SEQ ID NO:15, SEQ ID NO: 62 and SEQ ID NO: 65 are fixed thereon..

14. A pair of primers selected from the group consisting of SEQ ID NO: 1 and SEQ
5 ID NO: 2; SEQ ID NO: 3 and SEQ ID NO: 4; SEQ ID NO: 5 and SEQ ID NO: 6;
SEQ ID NO: 7 and SEQ ID NO: 8; and SEQ ID NO: 9 and SEQ ID NO: 10.