Method and/or kit for determining response to muscarinic receptor antagonist treatment

Field of the invention

The present invention relates to nucleic acid modifications in the muscarinic receptor genes for determining the response of a subject to muscarinic receptor antagonist treatment for eye conditions. The invention also relates to a method and/or kits for detecting the nucleic acid modifications for determining the response of the subject.

Background of the invention

The eye is a complex organ responsible for vision. In the normal eye, light rays are focused on the retina. However, in eye conditions such as myopia or nearsightedness, there is a refractive defect in the eye such that the image is focused in front of the retina. As a result, near objects can be clearly seen but distant objects are blurred. Myopia is especially prevalent among Asians and has been reported to be as high as 70-90% in Asian countries. In Singapore, 20% of children are myopic at 7 years, with the prevalence exceeding 70% upon completing college education. Most commonly, treatment of myopia include the use of corrective lenses (such as glasses or contact lenses) or refractive eye surgery (such as LASIK).

In addition, muscarinic receptor antagonists, in particular atropine, have been used to retard the progression of myopia in human subjects (Chua *et al.*, 2006). Typically, the atropine is formulated in eyedrops, which is administered topically. As muscarinic receptor antagonist therapy is a proven method of retarding myopic progression, many subjects will be keen to consider this method of treatment. However, not all myopic subjects respond positively to atropine administration. In responsive subjects, administration of atropine results in the retardation of the progression of myopia. However, the myopia of

non-responsive subjects continue to deteriorate despite the administration of atropine.

The mechanism of action of the muscarinic receptor antagonists in the retardation of myopia is not well understood. The reason why some subjects respond to the therapy and some do not remains unclear. The action of the muscarinic receptor antagonists in myopia treatment may involve the muscarinic receptors, of which five subtypes have been identified, m1-m5 (Klett *et al.*, 1999). Although the muscarinic receptor antagonist pirenzepine may be relatively selective for the m1 receptor (Tan *et al.*, 2005) it is uncertain if other muscarinic receptor antagonists interact with any, some or all of the muscarinic receptors to bring about retardation of myopia progression.

Muscarinic receptor antagonist therapy for myopia may involve years of instillation of eyedrops, with possible short term complications of mydriasis, glare, loss of accommodation. In addition, there is the possibility that the subject may not be able to take part in outdoor activities. Further, long term potential complications include cataracts and light induced maculopathy. Many myopic subjects are affected by the condition at a young age and it takes great discipline to persevere with the treatment regime of administering the eyedrops over many years.

It would therefore be desirable to have a method of determining if a myopic subject is likely to respond to muscarinic receptor antagonists therapy before beginning the therapy.

Summary of the invention

According to a first aspect, the present invention provides a method for determining the response of a subject to at least one muscarinic receptor antagonist treatment for at least one eye condition comprising determining, from a sample isolated from the subject, the genotype(s) of the subject for at least one nucleic acid modification of at least one muscarinic receptor gene and/or its transcriptional and/or translational product, wherein the genotype(s) of the subject for the nucleic acid modification(s) is correlated to the probability of the subject responding to the treatment.

In particular, the eye condition to be treated is myopia. The muscarinic receptor antagonist may be selected from the group consisting of atropine, benztropine, darifenacin, dexetimide, dicyclomine, dimenhydrinate, diphenyhydramine, flavoxate, glycopyrrolate, homatropine, hyoscyamine, ipatropium, orphenadrine, oxybutyrin, pirenzepine, procyclidine hydrochloride, propiomazine, scopolamine, solifenacin, tiotropum, tolterodine and trihexyphenidyl. The list of muscarinic receptor antagonist provided is not intended to be exhaustive and accordingly it is understood that other muscarinic receptor antagonists not listed may be used for the invention.

In addition, any of the muscarinic receptor genes, m1, m2, m3, m4 or m5 are contemplated in the method of the invention. Preferably, the muscarinic receptor gene is the m1 muscarinic receptor gene or a sequence at least 85 % homologous to SEQ ID NO: 1 or a fragment thereof. SEQ ID NO: 1 is derived from the sequence having a GenBank accession no. NM_000738 but has the single nucleotide polymorphism at position 1353 indicated as n, where n is C or T.

The method of determining the nucleic acid modification may include but is not limited to hybridization, primer extension, ligation, invasive cleavage, sequencing, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), multiplex ligation-dependent probe amplification (MLPA), micro PCR systems, microfluidic chip systems, allele specific amplification, snapshot mini-sequencing, strand displacement amplification, transcriptional mediated amplification, nucleic acid sequence-based amplification and/or helicase dependent amplification.

The nucleic acid modification referred to in the method of the invention may be at least a single nucleotide polymorphism (SNP), a double nucleotide polymorphism (DNP), a nucleotide deletion, a nucleotide addition, a nucleic acidamplification, a rearrangement of the muscarinic receptor gene and/or its transcriptional and/or translational product, and/or alternative splicing of the transcriptional and/or translational product. Further, the nucleic acid modification may be in one or more alleles.

In addition, the nucleic modification may be in a coding and/or non-coding region of the muscarinic receptor gene.

According to another aspect, the present invention provides a probe for determining the response of a subject to at least one muscarinic receptor antagonist treatment for at least one eye condition, wherein the probe is for determining from a sample isolated from the subject the presence of at least one nucleic acid modification of at least one muscarinic receptor gene and/or its transcriptional and/or translational product.

In particular, the probe hybridises and/or is complementary to at least one muscarinic receptor gene and/or its transcriptional and/or translational product comprising at least one nucleic acid modification or fragment thereof.

According to another aspect, the invention relates to kits for determining the response of a subject to at least one muscarinic receptor antagonist treatment for at least one eye condition. The kit may comprise the probe(s) according to the invention.

Brief description of the figures

Figure 1 illustrates two methods to determine the genotype at rs2067480; (A) illustrates determination by restriction fragment length polymorphism and (B) illustrates determination by sequencing. The sequences in Figure 1(B) are represented by SEQ ID NOs: 3 and 7.

Definitions

"Determine" or "determining" means to find out or come to a decision about by investigation, reasoning, or calculation, or to settle or decide from alternatives or possibilities; "determine" or "determining" encompasses the meaning of "predicting" or "assessing".

A non-coding region of a gene would include but is not limited to areas that exert and influence the gene, such as promoters, gene activator and repressor binding sites and the like.

"Nucleic acid modification" includes, but it is not limited to, a single nucleotide polymorphism (SNP), a double nucleotide polymorphism (DNP), a nucleotide deletion, a nucleotide addition, a nucleic acid amplification, a rearrangement of the muscarinic receptor gene and/or its transcriptional and/or translational product, and/or alternative splicing of the transcriptional and/or translational product.

A single polynucleotide polymorphism (SNP) refers to a DNA and/or RNA sequence variation occurring when a single nucleotide in an organism's DNA

sequence differs between members of the species (or between paired chromosomes in the organism).

A double polynucleotide polymorphism (DNP) refers to two single polynucleotide polymorphisms, and includes the circumstances when the two SNPs are positioned next to each other, separated by other nucleotides, on different strands of the same nucleic acid molecules, or on different nucleic acid molecules.

"Determining the genotype of a subject with respect to a nucleic acid modification" refers to determining the genetic constitution or makeup of the subject for that nucleic acid modification and includes determining the absence or presence of the nucleic acid modification and; where the nucleic acid modification comprises several alternatives or variations, determining the alternative or variation in the subject.

"Correlation of a genotype of a nucleic acid modification to the probability of a subject to respond to treatment" refers to a correlation or link between the nucleic acid modification and the likelihood of responding to the treatment or not. For example, this includes the SNP rs542269, wherein a C at the position 52 of SEQ ID NO: 2 indicates that a subject is likely to respond to muscarinic receptor antagonist treatment. In another example, a DNP genotype comprising (i) for the rs2067480 position, a C at position 1353 of SEQ ID NO: 1 and for the rs542269 position a TT at position 52 of SEQ ID NO: 2 indicates that a subject is likely to not respond to muscarinic receptor antagonist treatment and (ii) any other DNP genotype than in (i) indicates that a subject is likely to respond to muscarinic receptor antagonist treatment.

"CI" refers to confidence interval.

A probe refers to a DNA or RNA molecule which is complementary and/or hybridises to a target DNA or RNA sequence and may be used to locate and/or

identify the DNA or RNA sequence. Probes may usually be labelled by standard methods, for example, radioactively or with fluorescent markers. For example, probes may be used to detect differences in DNA or RNA sequences, including single nucleotide polymorphism(s).

A primer refers to an oligonucleotide to which deoxyribonucleotides may be added by a DNA polymerase. A single primer may be used to amplify a DNA or RNA region, for example, for sequencing.

A primer pair usually comprises a first primer complementary to one strand of a DNA or RNA molecule and a second primer complementary to a second strand of a DNA or RNA molecule, with both primers flanking a target DNA or RNA region, to be amplified by a DNA polymerase.

Detailed description of the invention

The invention relates to a method for determining the response of a subject to at least one muscarinic receptor antagonist treatment for at least one eye condition comprising determining, from a sample isolated from the subject, the genotype of the subject for at least one nucleic acid modification of at least one muscarinic receptor gene and/or its transcriptional and/or translational product, wherein the genotype(s) of the subject for the nucleic acid modification(s) is correlated to the probability of the subject responding to the treatment.

According to one particular aspect, the invention provides a method for determining the response of a subject to at least one muscarinic receptor antagnonist treatment for at least one eye condition comprising determining, from a sample isolated from the subject, the presence of at least one nucleic acid modification of the m1 muscarinic receptor gene or a sequence at least

85% homologous to SEQ ID NO: 1 or a fragment thereof. The m1 muscarinic receptor gene (chrm1) resides on chromosome 11 and consists of 1 large exon which contains the entire coding region of the M1 muscarinic receptor (M1 receptor), a member of the G-protein coupled receptor (GPCR) superfamily (Lucas *et al.*, 2001). Several single nucleotide polymorphisms (SNPs) of the m1 receptor gene have been identified (Lucas and Sadee., 2001)

In particular, two polymorphisms in the m1 muscarinic receptor gene have been identified by the present inventors as having a link to the ability of a subject to respond to muscarinic receptor antagonist treatment for myopia. The two polymorphisms are the rs2067480 and rs542269 polymorphisms, both of which have been previously described (Maeda *et al.*, 2006).

The rs2067480 polymorphism is in a coding region of the m1 muscarinic receptor gene and is at position 1353 of SEQ ID NO: 1 (Lucas and Sadee, 2001). On the chromosome 11 contig sequence (Genbank accession number NT_033903), this polymorphism is at position 7983015. The polymorphism at rs2067480 can be either a C or a T. Accordingly, when both alleles are considered, the polymorphism at rs2067480 can be CC, TC or TT.

The rs542269 polymorphism is in a non-coding terminal region and is at position 7991332 of the chromosome 11 contig sequence (Genbank accession number NT_033903).

This position is indicated in capital and in bold in the portion of the chromosome 11 contig reproduced below where the position is indicated by a C. The numbers on the left above the first row indicate the position of the first base on the chromosome 11 contig NT_033903. This position relates to position 52 of SEQ ID NO: 2, indicated by an "n" where "n" is C or T.

7991281

tggatccagg ccgtctgcct ccgagaccaa cctcttgaac cctactcctg cCcttgaggg

gttgcggggg tttgccagcc tccttgccca cctcagctag cttactcaag cccaactttg

aatcagttaa cgatgagcaa tcggtccttc tttcagaatc tggcctcctt tgtgtgcgcg

The polymorphism at rs542269 can be either a C or a T. Accordingly, when both alleles are considered, the polymorphism at rs542269 can be CC, TC or TT.

When both alleles are considered for both rs2067480 and rs542269, the genotype can be CCCC, CCCT, CCTT, CTCC, CTCT, CTTT, TTCC, TTTC and TTTT.

Interestingly, the rs2067480 polymorphism does not result in an amino acid change in the corresponding polypeptide at ser451 (Lucas and Sadee, 2001). In addition, the rs542269 polymorphism is in a non-coding region. These polymorphisms may alter mRNA stability which in turn may affect cellular functions involved in the response to treatment and myopia progression.

According to one embodiment, the invention involves determining the genotype of a myopic subject at rs2067480 from a sample isolated from the subject.

In another embodiment, the invention involves determining the genotype of a myopic subject at rs542269 from a sample isolated from the subject.

In yet another embodiment, the invention involves determining the genotype of a myopic subject at both rs2067480 and rs542269 from a sample isolated from the subject.

In one embodiment of the invention, a C at rs542269 indicates that a subject is likely to respond to muscarinic receptor antagonist treatment (a positive result).

Further, in one embodiment of the invention, CC or TC at rs542269 position for both alleles indicates that a subject is likely to respond to muscarinic receptor antagonist treatment (a positive result).

In another embodiment of the invention,

(i) a DNP genotype comprising C at rs2067480 and a TT at rs542269 indicates that a subject is likely to not respond to muscarinic receptor antagonist treatment (negative result), and

(ii) any DNP genotype other than that in (i) indicates that a subject is likely to respond to muscarinic receptor antagonist treatment (positive result).

In another embodiment of the invention,

(i) a DNP genotype comprising CC or TC at rs2067480 and a TT at rs542269 indicates that a subject is likely to not respond to muscarinic receptor antagonist treatment (negative result), and

(ii) any DNP genotype other than that in (i) indicates that a subject is likely to respond to muscarinic receptor antagonist treatment (positive result).

Determining the genotype at rs2067480 and/or rs542269 may be carried out by restriction fragment length polymorphism (RFLP) or sequencing. However other methods of ascertaining SNP genotypes which are known in the art may be used. For example, methods of ascertaining SNP genotypes which are readily scalable to handle many samples may be utilised. These techniques include but are not limited to modifications of micro PCR systems, microfluidic chip systems, allele specific amplification, multiplex ligation-dependent probe amplification, snapshot mini-sequencing or the like. Other methods include

competing methods of DNA amplification that are not based on PCR such as strand displacement amplification (Becton Dickinson, Franklin Lakes, NJ), transcription mediated amplification (Gen-Probe Inc., San Diego, CA), nucleic acid sequence-based amplification (New England BioLabs, Beverley, MA).

The sample may be isolated from any body fluid and/or material. For, example, the sample may be isolated from the buccal mucosal, lacrimal gland secretions, tears, conjunctival tissue, cornea cells, blood and/or saliva. The isolated sample may be used directly. Alternatively, DNA may be extracted from the isolated sample.

The present invention enables a method as well as the construction of a commercial assay or kit to determine the response to muscarinic receptor antagonist treatment for myopia. In clinical scenarios where the desire to commence treatment is tempered by a concern over the side effects of the treatment, a quick and relatively non-invasive genetic assay may be performed.

Accordingly, the present invention provides a kit for performing the method of the invention. The kits may be used for determining the response of a subject to at least one muscarinic receptor antagonist for at least one eye condition. Accordingly, the kit may comprise at least one probe according to the invention.

The probe may be used for determining the response of a subject to at least one muscarinic receptor antagonist for at least one eye condition. In particular, the probe may be complementary to at least one muscarinic receptor gene and/or its transcriptional and/or translational product comprising at least one nucleic acid modification or fragment thereof. The probe may be used for detecting at least one SNP, in particular, the SNP at position 1353 of SEQ ID NO: 1 (rs2067480) and/or the SNP at position 52 of SEQ ID NO: 2 (rs542269).

The kit of the invention may further comprise at least one means to detect the presence of the labelled probe.

In addition, according to another aspect, the kit according to the invention may detect the SNP at rs2067480, comprising at least one primer comprising SEQ ID NO: 3 and/or 4, at least one polymerase, and/or at least one restriction enzyme.According to another aspect, the kit according to the invention may detect the SNP at rs542269, comprising at least one primer comprising SEQ ID NO: and/or 6, at least one polymerase, and/or at lest one restriction enzyme.

Having now generally described the invention, the same will be more readily understood through reference to the following embodiments and examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

Examples

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russel, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2001).

Example 1 - Determination of genotype

The methods used in determining the genotype of the SNPs was either restriction fragment length polymorphism (RFLP) or sequencing. The different SNPs were determined separately in different reactions.

DNA Extraction

Cells from the buccal mucosal were collected on a sterile cytobrush by vigorously brushing the inner cheek with the cytobrush. The brush was stored at -80 °C until prior to DNA extraction. For DNA extraction, the cytobrush was thawed and DNA extraction was performed using the QIAmp DNA Micro Kit

(Qiagen Inc., Valencia CA, USA) according to the manufacturer's instructions. The extracted DNA was used for subsequent analysis for the SNPs.

SNP at rs2067480.

The methods for determining the genotype of the SNP at rs2067480 either by RFLP (Figure 1A) or by sequencing (Figure 1B)

RFLP

DNA from the sample isolated from the subject was used in the RFLP method. In this example, the DNA was isolated from the buccal scrape samples as described above and used for the RFLP.

As illustrated in Figure 1A, the first step in the RFLP method involved amplifying a region of the m1 muscarinic receptor gene from the DNA of the subject using the following primers, 5' CCACCTTCTGCAAGGACTGT 3' (rs2067480 forward primer, SEQ ID NO: 3) and 5' CTGGGAATAGCGAAGTCTGG 3' (rs2067480 reverse primer, SEQ ID NO: 4) by PCR. The restriction enzyme used to digest the PCR amplicon was *Nla*IV. The digestion was performed for a minimum period of at least 8 hours at 37 °C. Alternatively, the digestion was performed overnight.

The digestion products were visualised by agarose gel electrophoresis. Figure 1A shows schematic diagrams of the agarose gel electrophoresis indicating the sizes of the fragments obtained after *Nla*IV digestion when the SNP at rs2067480 is T or C.

If the genotype is TT, three DNA fragments of sizes 31, 65 and 307 bp are observed. If the genotype is CC, four fragments of sizes 31, 65, 119 and 118 bp are observed. In the event that the genotype is heterozygous, where both C and T are present, five bands of sizes 31, 65, 119, 118 and 307 bp are observed.

Sequencing

In the sequencing method illustrated in Figure 1B, only the forward primer of SEQ ID NO: 3 was used in the sequencing. The sequencing was by the fluorescent sequencing methods, where the dNTPs are tagged with fluorescent dyes. As illustrated by Figure 1B, sequencing identified if the genotype was a TT or a CC. In the event of heterozygosity, the peaks corresponding to both C and T will be observed at that position.

Accordingly, the primers of SEQ ID NO: 3 and/or 4 may be included in a kit, as well as a polymerase, and/or any suitable restriction enzyme. In particular, the enzyme included in the kit is *Nla*IV. Optionally buffers for the PCR and restriction digest may also be included in the kit. The kit may be used to detect the SNP at rs2067480 for determining the response of the subject to muscarinic receptor antagonist treatment.

SNP at rs542269

Detection of the SNP at rs542269 is similar to the method for rs2067480 as described above, except that the primers used for PCR amplification are 5' TTTGCAAAAGGCCTAACCTG 3' (rs542269 forward primer, SEQ ID NO: 5) and 5' CCTCTTCCCACAGCACTGTTA (rs542269 reverse primer, SEQ ID NO: 6). The restriction enzyme used for the digestion was *BslI*, which has the recognition site CCNNNNN^NNGG (SEQ ID NO: 8), cutting at the position ^, producing sticky ends.

The restriction digestion was performed at 37 °C for 8 hours. Alternatively, the digestion may be performed overnight. On electrophoresis, three fragments (58, 71 and 177 bp) were visualised when the genotype was CC, two fragments (71 and 235 bp) were visualised when the genotype was TT. In the event of heterozygosity at rs542269, four fragments (58, 71, 177 and 235 bp) were observed.

Further, the primer having SEQ ID NO: 5 may also be used for sequencing to determine the SNP at rs542269.

Accordingly, the primers of SEQ ID NO: 5 and/or 6 may be included in a kit, as well as a polymerase and/or any suitable restriction enzyme. In particular the enzyme included in the kit is *Bsl*I. Optionally, buffers for the PCR and restriction digest may be included in the kit. The kit may be used to detect the SNP at rs542269 for determining the response of the subject to muscarinic receptor antagonist treatment.

Example 2 - Association of SNPs with response to atropine treatment

105 subjects were treated with atropine according to the treatment regime described in Chua *et al.*, 2006. The subjects were selected according to the eligibility criteria described in Chua *et al.*, 2006 and the study was approved by the Singapore Eye Research Institute Review Board. Subjects who responded to atropine treatment were defined as those with progression of sphericial equivalent, SE (cycloplegic autrorefraction) of less than 0.5D Diopters) in a period of two years. Cycloplegic autorefraction was measured according to the method described in Chua *et* al., 2006. Treatment was deemed to be ineffective when the myopia increased by more than 0.5 D. Worsening of spherical equivalent (SE) by 0.5 D or more was considered as undesirable and constitutes the non-responsiveness. Worsening of SE by less than 0.5 D indicated that the myopia was stable and constitutes satisfactory response to atropine.

DNA extraction was undertaken from buccal scrape samples according to the method described in Example 1. A total of five SNPs in the m1 gene were investigated and these were the rs2067477, rs2067478, rs2075748, rs542269 and rs2067480. From the five SNPs studied, the present inventors have

surprisingly identified an association between two SNPs of the m1 receptor gene and the response to therapeutic treatment of myopia with muscarinic receptor antagonists. The method of determining the polymorphisms was by restriction fragment length polymorphisms (RFLP) according to the method described in Example 2. The procedure took approximately two days, with a minimum 8 hour digest period.

Accordingly, this association was used in a test to determine the response of a myopic subject to treatment with muscarinic receptor antagonists.

The two polymorphism were:

1. rs2067480: polymorphisms of CC, TC, TT.

2. rs542269: polymorphisms of CC, TC and TT.

Table 1. Observed frequency of subjects with various genotypes and response to atropine treatment.

Genotype at rs2067480 (first 2 alphabets) and rs542269 (third and fourth alphabet)	No of subjects: SE worse by at least 0.5 D (Non-responders)	No of subjects: SE worse by less than 0.5 D (Responders)	Total no of subjects
CCTT	25	39	64
CTTT	4	10	14
TTTT	0	1	1
CCCC	0	1	1
CCCT	4	16	20
CTCT	0	2	2
NNNC[1]	0	3	3
Total	33	72	105

[1] This notation "NNNC" indicates any genotype at rs2067480, and TC or CC at rs542269.

Table 1 illustrates that a test criteria using the outcome(s) in these SNPs could predict drug response.

The rows indicate the genotypes at the 2 positions (loci): the first two letters represent the genotype at rs2067480, and the third and fourth letters represent the genotype at rs542269, respectively. The genotype at each locus is specified by 2 letters or bases, one for each chromosome 11. The cells in the table with numbers indicate the number of subjects with the specified genotypes for that row that fulfilled the drug response requirement or otherwise. The totals for each row are specified on the extreme right column. The last row represents the total number of responders and non-responders.

For the genotype NNNC, insufficient material in the isolated samples of 3 subjects prevented the determination of the genotype at rs542269 and one allele at rs2067480. However, Table 1 showed that a criterion may encompass the use of CC and CT in the rs542269 position as a positive test regardless of the SNP at rs2067480. Alternatively, a C in the rs542269 position is indicative of a positive test regardless of the SNP at rs2067480.

By collapsing the genotypes (rows in Table 1 above), the final test contingency table can be expressed as shown in Table 2. The first two rows in Table 1 were merged to give the genotypes representing a negative test in Table 2, and the last five rows in Table 1 were merged to give the positive test genotypes.

Table 2 Two by two table showing test result and effectiveness of treatment.

	NON-RESPONDERS: intervention not effective SE worsened by at least 0.5D over 2 years	RESPONDERS: intervention effective SE worsened by less than 0.5D (or any improved) over 2 years	Total
NEGATIVE TEST (rs2067480: CC or TC) and rs542269 TT	29	49	78 (74%)
POSITIVE TEST Any genotype not covered above	4	23	27 (26%)
	33 (31%)	72 (69%)	105

From Table 2, it can be seen that a DNP genotype comprising a C at rs2067480 and a TT at rs542269 indicated that a subject was likely to not respond to treatment (negative test) and any other DNP genotype indicated that a subject was likely to respond to treatment (positive test). Put in another way, a DNP genotype comprising a CC or TC at rs2067480 and a TT at rs542269 indicated a negative test and any other DNP genotype indicated a positive test.

If the test outcome is positive – expected to be 26% (95% CI 18-35) there is a 85% chance (calculated from 23/27 x 100%) that the subject is a responder to treatment and treatment may be justified on the basis of the likelihood of efficacy. If the test is negative, there is still a chance that the subject may still respond to treatment. In this instance, the decision to start treatment or otherwise for myopia could be made based on clinical grounds including the age of the subject, the history of progression over the last year and/or the family history of myopia.

Statistics

Statistics were calculated according to standard formulae.

Fisher's exact p value 0.0331
Odds ratio 3.40 (95%CI: 1.07-10.82) Sensitivity 32%(95%CI: 22-44)
Specificity 88%(95%CI: 71-96)
Positive predictive value 85%(95%CI: 65-95)
Negative predictive value 37%(95%CI: 27-49)

Note that the percentages on the extreme right column of Table 2 indicate the proportion of subjects with negative and positive test results, not the proportion of responders. These percentages showed that more subjects obtained a negative test result compared to a positive test result in our trial (these percentages may be different in a different target screening population). The last row in Table 2 indicates the percentages of responders and non-responders to treatment. These showed more subjects responding to treatment rather than not responding.

Interpretation of statistics

When the odds ratio is above the value of one, it indicates that the test result (genotype combination) is significantly associated with the drug response (myopia progression). The Fisher's exact probability test was used to test for statistical significance because one of the cells has a number of less than 7. The p value of 0.0331 indicates statistical significance at the level of alpha=0.05. A high specificity and moderate sensitivity of the test has been achieved. In any diagnostic test, the threshold for considering a result to be positive or negative may be varied so that the sensitivity and/or specificity will change. However, the change in the sensitivity and/or specificity is reciprocal to one another. In other words, it is difficult to have a perfect test of very high

sensitivity and/or specificity at the same time. The ideal combination of sensitivity and/or specificity in a test is often decided according to the clinical scenario, or, in the case where the threshold is a continuous variable (like body mass index or blood glucose), the characteristics of a receiver operating curve. The relatively high positive predictive value implies that when the test result is positive, the chances of responding to the treatment is high, whereas a modest negative predictive value implies that a negative test may not be very predictive of treatment failure.

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Claims

1. A method for determining the response of a subject to at least one muscarinic receptor antagonist treatment for at least one eye condition comprising determining, from a sample isolated from the subject, the genotype of the subject for at least one nucleic acid modification of at least one muscarinic receptor gene and/or its transcriptional and/or translational product, wherein the genotype(s) of the subject for the nucleic acid modification(s) is correlated to the probability of the subject responding to the treatment.

2. The method according to claim 1, wherein the eye condition is myopia.

3. The method according to claim 1 or 2, wherein the musacarinic antagonist is selected from the group consisting of atropine, benztropine, darifenacin, dexetimide, dicyclomine, dimenhydrinate, diphenyhydramine, flavoxate, glycopyrrolate, homatropine, hyoscyamine, ipatropium, orphenadrine, oxybutyrin, pirenzepine, procyclidine hydrochloride, propiomazine, scopolamine, solifenacin, tiotropum, tolterodine and trihexyphenidyl.

4. The method according to any one of the preceding claims, wherein the sample is isolated from the buccal mucosal, lacrimal gland secretions, tears, conjunctival tissue, cornea cells, blood and/or saliva.

5. The method according to any one of the preceding claims, wherein the nucleic acid modification is determined by hybridization, primer extension, ligation, invasive cleavage, sequencing, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), sequencing, multiplex ligation-dependent probe amplification (MLPA), micro PCR systems, microfluidic chip systems, allele specific amplification, snapshot mini-

sequencing, strand displacement amplification, transcriptional mediated amplification, nucleic acid sequence-based amplification and/or helicase dependent amplification.

6. The method according to any one of the preceding claims, wherein the nucleic acid modification comprises at least a single nucleotide polymorphism (SNP), a double nucleotide polymorphism (DNP), a nucleotide deletion, a nucleotide addition, a nucleic acid amplification, a rearrangement of the muscarinic receptor gene and/or its transcriptional and/or translational product, and/or alternative splicing of the transcriptional and/or translational product.

7. The method according to any one of the preceding claims, wherein the nucleic acid modification is in one or more alleles.

8. The method according to any one of the preceding claims, wherein the nucleic acid modification is in a coding region of the muscarinic receptor gene.

9. The method according to any one of the preceding claims, wherein the nucleic acid modification is in a non-coding region of the muscarinic receptor gene.

10. The method according to any one of the preceding claims, wherein the muscarinic receptor gene is selected from the group consisting of the m1, m2, m3, m4 and m5 muscarinic receptor genes.

11. The method according to any one of the preceding claims, wherein the muscarinic receptor gene is m1 muscarinic receptor gene.

12. The method according to any one of the preceding claims, wherein the muscarinic receptor gene comprises a sequence at least 85 % homologous to SEQ ID NO: 1 or a fragment thereof.

13. The method according to any one of the preceding claims, wherein the nucleic acid modification comprises at least one SNP at position 1353 of SEQ ID NO: 1 (rs2067480) and/or one SNP at position 52 of SEQ ID NO: 2 (rs542269).

14. The method according to any one of the preceding claims, wherein the nucleic acid modification comprises a C at rs542269, thereby indicating that a subject is likely to respond to muscarinic receptor antagonist treatment.

15. The method according to any one of the preceding claims 13, wherein the nucleic acid modification comprises a CC or TC at rs542269 for both alleles, thereby indicating that a subject is likely to respond to muscarinic receptor antagonist treatment.

16. The method according to any one of the preceding claims, wherein the muscarinic receptor antagonist is atropine.

17. The method according to any one of the preceding claims, wherein the nucleic acid modification comprises at least one DNP comprising a SNP at position 1353 of SEQ ID NO: 1 (rs2067480) and a SNP at position 52 of SEQ ID NO: 2 (rs542269).

18. The method according to claim 17, wherein

(i) a DNP genotype comprising a C at rs2067480 and a TT at rs542269 indicates that a subject is likely to not respond to muscarinic receptor antagonist treatment and

(ii) any DNP genotype other than that in (i) indicates that a subject is likely to respond to muscarinic receptor antagonist treatment.

19. The method according to claim 17 or 18, wherein

(i) a CC or a TC at rs2067480 and a TT at rs542269 indicates that a subject is likely to not respond to muscarinic receptor antagonist treatment and

(ii) any DNP genotype other than that in (i) indicates that a subject is likely to respond to muscarinic receptor antagonist treatment.

20. The method according to any one of claims 13 to 19, wherein the method of determining the SNP is by restriction fragment length polymorphism (RFLP) and/or sequencing.

21. The method according to claim 20, wherein the RFLP for determining the SNP at rs2067480 comprises the steps of:

(i) amplifying a region of the m1 muscarinic receptor gene using at least one primer comprising SEQ ID NO: 3 or a fragment thereof and/or at least one primer SEQ ID NO: 4 or a fragment thereof to produce at least one amplicon;

(ii) digesting the amplicon with at least one restriction enzyme;

(iii) determining the size of the fragment(s) obtained after the enzyme digestion; and

(iv) determining the SNP genotype.

22. The method according to claim 21, wherein the restriction enzyme comprises *Nla*IV.

23. The method according to claim 20, wherein sequencing involved sequencing a region of the m1 muscarinic receptor gene with at least one primer comprising SEQ ID NO: 3 or a fragment thereof to determine the genotype of the SNP at rs2067480.

24. The method according to claim 20, wherein the RFLP for determining the SNP at rs542269 comprises the steps of:
(i) amplifying a region of the m1 muscarinic receptor gene using at least one primer comprising SEQ ID NO: 5 and/or at least one primer comprising SEQ ID NO: 6 to produce at least one amplicon;
(ii) digesting the amplicon with at least one restriction enzyme;
(iii) determining the size of the fragments obtained after the enzyme digestion; and
(iv) determining the SNP genotype.

25. The method according to claim 24, wherein the restriction enzyme comprises *Bsl*I.

26. A probe for determining the response of a subject to at least one muscarinic receptor antagonist treatment for at least one eye condition, wherein the probe is for determining from a sample isolated from the subject the genotype of at least one nucleic acid modification of at least one muscarinic receptor gene and/or its transcriptional and/or translational product.

27. A probe according to claim 26, wherein the probe hybridises and/or is complementary to at least one muscarinic receptor gene and/or its transcriptional and/or translational product comprising at least one nucleic acid modification or fragment thereof.

28. The probe according to claim 26 or 27, wherein the eye condition is myopia.

29. The probe according to any one of claims 26 to 28, wherein the the musacarinic antagonist is selected from the group consisting of atropine, benztropine, darifenacin, dexetimide, dicyclomine, dimenhydrinate, diphenyhydramine, flavoxate, glycopyrrolate, homatropine, hyoscyamine, ipatropium, orphenadrine, oxybutyrin, pirenzepine, procyclidine hydrochloride, propiomazine, scopolamine, solifenacin, tiotropum, tolterodine and trihexyphenidyl.

30. The probe according to any one of claims 26 to 29, wherein the nucleic acid modification comprises at least a single nucleotide polymorphism (SNP), a double nucleotide polymorphism (DNP), a nucleotide deletion, a nucleotide addition, a nucleic acid amplification, a rearrangement of the muscarinic receptor gene and/or its transcriptional and/or translational product, and/or alternative splicing of the transcriptional and/or translational product.

31. The probe according to any one of claims 26 to 30, wherein the nucleic acid modification is in one or more alleles.

32. The probe according to any one of claims 26 to 31, wherein the nucleic acid modification is in a coding region of the muscarinic receptor gene.

33. The probe according to any one of claims 26 to 32, wherein the nucleic acid modification is in a non-coding region of the muscarinic receptor gene.

34. The probe according to any one of claims 26 to 33 wherein the muscarinic receptor gene is selected from the group consisting of the m1, m2, m3, m4 and m5 muscarinic receptor genes.

35. The probe according to any one of claims 26 to 34, wherein the muscarinic receptor gene comprises the m1 muscarinic receptor gene.

36. The probe according to claim 35, wherein the probe is for detecting at least one SNP.

37. The probe according to claim 35, wherein the SNP comprises an SNP at position 1353 of SEQ ID NO: 1 (rs2067480) and/or an SNP at position 52 of SEQ ID NO: 2 (rs542269).

38. The probe according to any one of claims 26 to 37, wherein the probe comprises at least one sequence of the m1 muscarinic receptor gene including the nucleotide at position 1353 of SEQ ID NO: 1.

39. The probe according to claim 38, comprising at least 10 contiguous nucleotide sequence of the m1 muscarinic receptor gene including the nucleotide at position 1353 of SEQ ID NO: 1.

40. The probe according to any one of claims 26 to 37, wherein the probe comprises a sequence of the m1 muscarinic receptor including the nucleotide at position 52 of SEQ ID NO: 2

41. A probe according to claim 40, comprising at least 10 contiguous nucleotide sequence of the m1 muscarinic receptor gene including the nucleotide at position 52 of SEQ ID NO: 2

42. The probe according to any one of claims 26 to 41, wherein the probe is labelled.

43. The probe according to claim 42, wherein the probe is labelled with a radioactive tag, a fluorescent tag, a chemiluminescence tag or any other tag which enables the probe to be detected.

44. A kit for performing the method according any one of claims 1 to 25 comprising at least one means to determine the nucleic acid modification.

45. A kit for determining the response of a subject to at least one muscarinic receptor antagonist treatment for at least one eye condition, comprising at least one probe according to any one of claims 26 to 43.

46. A kit according to claim 45, further comprising at least one means to detect the presence of the labelled probe.

47. A kit for determining the response of a subject to at least one muscarinic receptor antagonist treatment for at least one eye condition by for the SNP at rs2067480, wherein the kit comprises at least one primer comprising SEQ ID NO:: 3 and/or 4, at least one polymerase, and/or at least one restriction enzyme.

48. A kit for determining the response of a subject to at least one muscarinic receptor antagonist treatment at least one eye condition for the SNP at rs542269, wherein the kit comprises at least one primer comprising SEQ ID NO: 5 and/or 6, at least one polymerase, and/or at least one restriction enzyme.

49. A kit according to any one of claims 44 to 48, wherein eye condition is myopia.

50. A kit according to any one of claims 44 to 50, wherein the muscarinic receptor antagonist is selected from the group consisting of atropine, benztropine, darifenacin, dexetimide, dicyclomine, dimenhydrinate, diphenyhydramine, flavoxate, glycopyrrolate, homatropine, hyoscyamine, ipatropium, orphenadrine, oxybutyrin, pirenzepine, procyclidine hydrochloride, propiomazine, scopolamine, solifenacin, tiotropum, tolterodine and trihexyphenidyl.