

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
27 September 2007 (27.09.2007)

PCT

(10) International Publication Number
WO 2007/107785 A2

(51) International Patent Classification:

C12Q 1/68 (2006.01)

(21) International Application Number:

PCT/GB2007/050111

(22) International Filing Date: 8 March 2007 (08.03.2007)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

0605544.6

20 March 2006 (20.03.2006)

GB

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

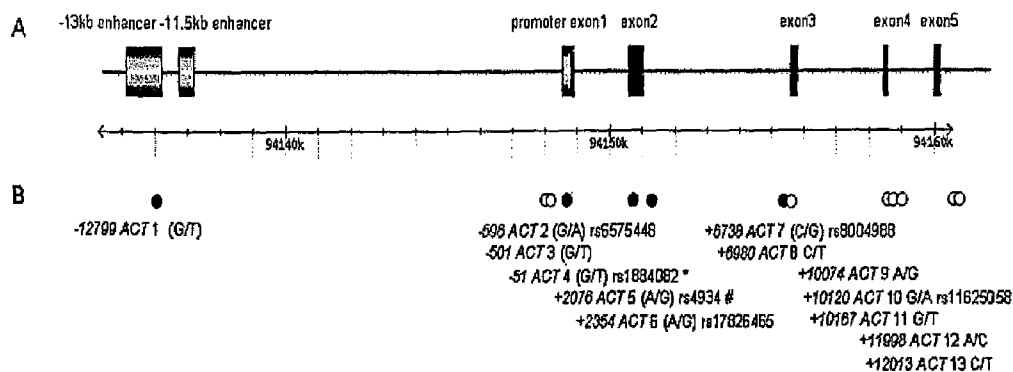
— of inventorship (Rule 4.17(iv))

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: GENETIC MARKER



(57) Abstract: The invention provides a method of identifying the existence of, or susceptibility to, neurodegenerative disorder in a subject. The method comprising: (i) obtaining a sample from a subject; and (ii) detecting, in the sample, the presence of a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (ACT) gene, wherein the polymorphism is associated with neurodegenerative disorder. The invention also provides a kit for carrying out the method, methods of treatment, and medical uses of nucleic acids harbouring polymorphisms in the ACT gene and of peptides encoded thereby.

GENETIC MARKER

The present invention relates to genetic markers, and particularly to genetic markers for use in methods for detecting the susceptibility to, or the existence of, neurodegenerative disorders. Particularly, although not exclusively, the markers are associated with Alzheimer's disease, and in particular, early-onset Alzheimer's disease. The method extends to kits for carrying out the method, and also to methods of treatment of neurodegenerative disorders, such as Alzheimer's disease. The invention also provides medical uses of nucleic acids harbouring such genetic markers and also peptides encoded thereby.

Alzheimer's disease is a neurodegenerative disorder characterised by the formation of senile plaques and neurofibrillary tangles in the brain. Senile plaques result from the extracellular deposition of β -amyloid protein ($A\beta$). Intracellular $A\beta_{1-42}$ is proteolytically derived from amyloid precursor protein (APP) by β - and γ -secretases, and induces neuronal cell death, resulting in severe cognitive impairment. Known mutations in *APP* and in the presenilin genes, *PSEN1* and *PSEN2*, cause familial early-onset Alzheimer's disease, accounting for around 5% of Alzheimer's cases. Remaining cases constitute "sporadic Alzheimer's" for which genetic associations, other than the apolipoprotein (*APOE*; MIM 107741) $\epsilon 4$ allele risk and the ACE indel polymorphism, are currently not fully established. These cases include both early-onset Alzheimer's disease (EOAD) and late-onset Alzheimer's disease (LOAD) defined by age-at-onset less than or greater than 65 years respectively.

Since alpha-1-antichymotrypsin has been detected in abundance in senile plaques, the *ACT* gene (*ACT*, *SERPINA3*; MIM 107280), located on chromosome 14q32.1, has become an Alzheimer's disease candidate. The protein, ACT, is an acute-phase reactant that increases up to four-fold during inflammation in response to cytokines. Whilst ACT is synthesised mainly in the liver, it is also produced in the brain by activated astrocytes, which surround the senile plaque.

The exact role of ACT in senile plaques pathology is undetermined. Some studies report that ACT promotes rapid disaggregation of $A\beta$ fibrils and forms part of the

inflammatory response to A β aggregation. However, there is increasing biochemical and pathological evidence that ACT may act as a molecular chaperone, both increasing the neurotoxicity of the A β peptide and promoting amyloid filament formation. *In vitro* ACT forms a complex with the putative neurotoxic A β ₁₋₄₂ in a dose-dependent manner, and this has recently been shown to act synergistically to alter gene expression in astrocytes. Transgenic mouse models confirm that ACT directly inhibits A β degradation and promotes A β deposition in brain.

- Supporting genetic evidence in humans has been difficult to obtain and is controversial. Following the initial observation that a common variant (Ala) in the *ACT* signal sequence (rs4934, herein termed *ACT* 5) was associated with increased risk of Alzheimer's disease, a number of studies refuted this, whilst others were supportive. Conflictingly, the Thr (A) allele of this single nucleotide polymorphism (SNP) has also been implicated in EOAD reducing the amount of mature glycosylated ACT secreted by transfected rat glioma cells. The Thr allele is in strong linkage disequilibrium (D' 0.97) with the T-allele of the previously reported *ACT* promoter SNP (rs1884082, here termed *ACT* 4), which is associated with a 22% mean higher serum ACT concentration, increased functional activity (~30%) *in vitro* and a more rapid cognitive decline.

- In order to resolve this apparent conflict regarding *ACT* SNPs and Alzheimer's, and to verify earlier observations implicating the *ACT* gene in EOAD and cognitive decline, the inventors used a high density SNP mapping approach combined with haplotype analysis in a large case-control association study (N=1,087). Since Alzheimer's disease can often present with variable phenotypes, the inventors appreciate that even a large sample size such as this may not achieve adequate statistical power for a case-control haplotype association study. Therefore, considering the most severe cases only, those with EOAD, was thought to improve statistical power by increasing the phenotypic homogeneity, but at the expense of a reduction in sample size. For this reason, analyses of the correlation of *ACT* haplotypes with pathological and cognitive indices, as described herein is considered to be a more sensitive test of genetic effects.

A genetic marker refers to a sequence in the genome that is known to vary among individuals, ie a polymorphic region which is used in genetic analyses. Genetic testing (also referred to as genetic screening, genotyping or molecular diagnostics) may be defined as the testing of a nucleic acid molecule of an individual or patient in an analytical capacity in order to determine whether the patient carries a genetic marker, polymorphic alleles or mutations causing, or associated with, a certain disease state or linked to a mutation or allele causing, or associated with, that disease. The number of diseases in which genetic testing and other biological methods are informative is continually increasing. The early detection of a pre-disposition to a genetic disease presents the best opportunity for medical intervention. In addition, genetic markers associated with an increased risk of severe clinical course of a particular disease may be screened. In addition, early genetic diagnosis and screening for markers associated with disease severity, complications or rate of progression may improve the prognosis for a patient through supervision and early intervention before the clinically detectable disorder occurs. In cases where patients with similar symptoms are treated with variable success, sophisticated genetic testing can differentiate individual patients with subtle or undetectable differences and can lead to more suitable individual treatments. It is even possible that early intervention may eventually involve methods such as gene therapy. It will therefore be appreciated that there is an ongoing need to provide new and improved genetic markers which are associated with neurodegenerative disorders, such as Alzheimer's disease, and which may be used in diagnostic methods and kits, and in therapeutic methods to treat such disorders.

Therefore, it is one of the aims of embodiments of the present invention to address the problems outlined above and to provide a method by which physicians can assess the risk to a patient of suffering from Alzheimer's disease and which could thereby act as an early indication for increased monitoring and proactive clinical management. It is a further aim of the invention to provide a kit by which physicians can detect the susceptibility of a patient to developing Alzheimer's disease. The ability to identify high-risk individuals by such a detection system

would allow physicians to focus preventative measures on those individuals who may gain the greatest benefit, ie. those patients with higher risk of developing Alzheimer's disease, and would provide strong incentives for those at higher risk to comply with such approaches so as to slow down or prevent progression of the disease to developing late onset Alzheimer's disease.

In order to identify new genetic markers associated with Alzheimer's disease, the inventors used a high-density *ACT* single nucleotide polymorphism (SNP) map, constructed haplotypes and explored correlations with phenotype. SNPs were identified by sequencing, and were then used to construct haplotypes in 668 AD patients and 419 controls, and a case-control association study was performed. Five SNPs, comprising five common haplotypes, represented 93% of *ACT* gene variation. Upon detailed analysis of their data, the inventors were surprised to find an SNP in intron 2 of the *ACT* gene, which showed association with Alzheimer's disease, and in particular, with later onset and more rapid cognitive decline ($P=0.04$). The inventors have therefore, fortuitously, identified a novel genetic marker in the *ACT* gene that is clearly associated with the development of neurodegenerative disorders.

Hence, according to a first aspect of the invention, there is provided a method of identifying the existence of, or susceptibility to, neurodegenerative disorder in a subject, the method comprising:-

- (i) obtaining a sample from a subject; and
- (ii) detecting, in the sample, the presence of a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) gene, wherein the polymorphism is associated with neurodegenerative disorder.

The method according to the first aspect of the invention is useful for enabling a clinician to make decisions with regards to the best course of treatment for a subject who is pre-disposed to a neurodegenerative disorder. It is preferred that the method of the first aspect of the invention is useful for enabling a clinician to decide how to treat a subject who is suffering from neurodegenerative disorder. In addition, the method of the first aspect is useful for monitoring the efficacy of a

putative treatment for neurodegenerative disorder. It will therefore be appreciated that the discovery of the novel genetic marker in intron 2 of the *ACT* gene for neurodegenerative disorders *per se* will have significant utility in the diagnosis, prognosis and treatment of such diseases.

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The inventors were particularly surprised to find that the genetic marker they have found was not part of the coding sequence of the *ACT* gene, but was in fact located in a non-coding intron. Hence, the marker is a single nucleotide polymorphism (SNP) having no known function.

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The term "polymorphism" refers to the co-existence within a population, of more than one form of a gene or portion thereof (eg. an allelic variant), at a frequency that is too high to be explained by recurrent mutation alone. A portion of a gene of which there are at least two different forms, ie. two different nucleotide sequences, is referred to as a "polymorphic region of a gene". A specific genetic sequence at a polymorphic region of a gene is known as an allele. The sequence variants contributing to the polymorphism according to the invention may be single or multiple base changes, including without limitation insertions, deletions, or substitutions, or may be a variable number of sequence repeats.

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The term "susceptibility", when used in relation to neurodegenerative disorder or any similar phrase such as "propensity" or "pre-disposition", means that certain alleles have been discovered to be associated with, or predictive of, progression of neurodegenerative disorder. The alleles are thus over-represented in frequency or carriage rate in individuals who are at risk of developing neurodegenerative disorder compared to individuals who are not susceptible to neurodegenerative disorder. Hence, the term "a subjects susceptibility to neurodegenerative disorder" refers to a statistically higher frequency or rate of progression of neurodegenerative disorder in an individual carrying a particular polymorphic allele, or genotype (ie. allelic or polymorphism pattern) in comparison to the frequency or rate of progression in a member of the population that does not carry the particular polymorphic allele, or genotype (allelic or polymorphism pattern).

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By the term “neurodegenerative disorder”, we mean a condition characterized by a degeneration of nervous tissue or nerve cells, ie neurons.

It is preferred that the methods according to the invention may be used to detect a diseased characterized by a decline in cognitive function. The skilled technician will understand that there are numerous neurodegenerative disorders that may be diagnosed using the method according to the invention. For example, preferred neurodegenerative disorders which may be identified according to the invention include Parkinson’s disease; Lewis body dementia; Fronto Temporal degeneration (FTD); Picks disease; as well as normal aging, which results in gradual decline of cognitive function or performance.

A preferred condition which may be diagnosed is Alzheimer’s disease. Preferably, the method according to the invention is useful for detecting the susceptibility to, or the existence of, early-onset Alzheimer’s disease. Preferably, the invention may be used to identify sporadic early-onset Alzheimer’s disease, as opposed to familial early-onset Alzheimer’s disease.

By the term “early-onset Alzheimer’s disease”, we mean disease with an age-of-onset less than 65 years with no evidence of a known familial mutation.

The *ACT* gene is located on chromosome 14q32.1 (Baker C *et al.*, 2007, “Review on Serpin A3 (aka alpha-1-antichymotrypsin)” - Frontiers in Bioscience). The coding DNA sequence and amino acid sequence of *ACT* are known and are readily accessible at www.ncbi.nlm.nih.gov (Accession Number: AL049839, AAA51543, respectively).

The polymorphism which is detected in the method of the first aspect is located in intron 2 of the *ACT* gene at position 64231 with respect to contig sequence AL049839. The inventors have found that intron 2 of the *ACT* gene has a single nucleotide Cytosine/Guanine (C/G) polymorphism located at nucleotide position 6738 of the gene. When referring to polymorphisms herein, usual nomenclature is adhered to with the first allele (Cytosine) denoting the wild-type allele and the

second allele (Guanine) denoting the mutant allele. This means that at this nucleotide position, a subject may have either a guanine allele (mutant) or a cytosine allele (wild-type). This polymorphism has been designated as rs8004988, and is referred to herein as *ACT 7*. As shown in Figure 5e, the data shows that subjects possessing the cytosine allele in the *ACT 7* polymorphism show a statistically significant propensity to show a decline in cognitive function.

The inventors have found a strong correlation between the novel genetic marker in intron 2 of the *ACT* gene with the onset of Alzheimer's disease. Surprisingly, the marker suggests pre-disposition to the disease condition at least eight years before actual disease onset. Referring to Figure 5e, the inventors were astounded to find that homozygotes for a G (mutant) allele of the genetic marker in intron 2 of the *ACT* gene (*ACT 7* GG) show pre-disposition to the disease at least 8 years before homozygotes for a C (wild-type) allele or heterozygotes. This was particularly surprising as differences in pre-disposition to disease conditions are generally altered by only a matter of months at most. Hence, this 8 year alteration in pick-up rate by diagnosing for the G allele in *ACT 7* was most unexpected. It will be appreciated that the maximum life expectancy for most Alzheimer's patients from initial onset is no more than ten years. Accordingly, by diagnosing pre-disposition to the disease at least eight years early, it is possible to determine which patients require treatment well before treatment is actually required. The method according to the first aspect therefore provides a powerful tool for early diagnosis of the disease condition, and enables effective treatment thereof.

Hence, preferably, the polymorphism which is detected comprises the G allele at marker rs8004988 in intron 2 of the *ACT* gene.

Preferably, the method of the invention comprises determining whether the subject is homozygous or heterozygous for alleles at the genetic polymorphism of intron 2 of the *ACT* gene, or other regions genetically linked thereto. For example, the method comprises determining GG, CG or CC subjects. It will be appreciated from the foregoing that GG subject are more pre-disposed to neurodegenerative disorder than, for example, a CC subject.

Preferably, the method is conveniently used to screen for a subject at risk to neurodegenerative disorder correlated with the polymorphism of the G allele at marker rs8004988 in intron 2 of the *ACT* gene.

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As a result of their studies, the inventors identified a number of new polymorphisms in the *ACT* gene which are associated with neurodegenerative disorders. These include:-

- (a) at nucleotide position -12799 of the *ACT* gene (G/T) (referred to herein as ACT 1); (b) at nucleotide position -596 of the *ACT* gene (G/A) (referred to herein as ACT 2); (c) at nucleotide position -501 of the *ACT* gene (A/G) (referred to herein as ACT 3); (d) at nucleotide position 10167 of the *ACT* gene (G/T) (referred to herein as ACT 11); (e) at nucleotide position 11998 of the *ACT* gene (A/C) (referred to herein as ACT 12); and (f) at nucleotide position 12013 of the *ACT* gene (C/T) (referred to herein as ACT 13).

- In addition, there are a number of known polymorphisms in the *ACT* gene which are also associated with neurodegenerative disorders. These include:-
- (a) at nucleotide position -51 of the *ACT* gene (G/T) (referred to herein as ACT 4); (b) at nucleotide position 2076 of the *ACT* gene (A/G) (referred to herein as ACT 5); (c) at nucleotide position 2354 of the *ACT* gene (A/G) (referred to herein as ACT 6); (d) at nucleotide position 6980 of the *ACT* gene (C/T) (referred to herein as ACT 8); (e) at nucleotide position 10074 of the *ACT* gene (A/G) (referred to herein as ACT 9); and (f) at nucleotide position 10120 of the *ACT* gene (G/A) (referred to herein as ACT 10).

Figure 4 shows Global *P*-values for case-control association with various combinations of each of these SNPs in total (solid line), LOAD (dashed line) and EOAD (dotted line) datasets. The inventors believe that a synergy effect occurs between the preferred polymorphism of the G allele at marker rs8004988 in intron 2 of the *ACT* gene, and any one or more of a combination of other genetic polymorphisms found or known to exist in the *ACT* gene, which are associated with neurodegenerative disease, and which are listed in Figure 6.

Hence, preferably, the method according to the invention comprises detecting for the presence of a second genetic polymorphism, wherein the second polymorphism is independently selected from a group of genetic polymorphisms consisting of :-

(i) at nucleotide position -12799 of the ACT gene (G/T) (referred to herein as ACT 1); (ii) at nucleotide position -596 of the ACT gene (G/A) (referred to herein as ACT 2); (iii) at nucleotide position -501 of the ACT gene (A/G) (referred to herein as ACT 3); (iv) at nucleotide position -51 of the ACT gene (G/T) (referred to herein as ACT 4); (v) at nucleotide position 2076 of the ACT gene (A/G) (referred to herein as ACT 5); (vi) at nucleotide position 2354 of the ACT gene (A/G) (referred to herein as ACT 6); (vii) at nucleotide position 6980 of the ACT gene (C/T) (referred to herein as ACT 8); (viii) at nucleotide position 10074 of the ACT gene (A/G) (referred to herein as ACT 9); (ix) at nucleotide position 10120 of the ACT gene (G/A) (referred to herein as ACT 10); (x) at nucleotide position 10167 of the ACT gene (G/T) (referred to herein as ACT 11); (xi) at nucleotide position 11998 of the ACT gene (A/C) (referred to herein as ACT 12); and (xii) at nucleotide position 12013 of the ACT gene (C/T) (referred to herein as ACT 13).

It will be appreciated that a combination of detectable polymorphisms (SNPs), rather than an individual SNP, has much more utility in diagnosing susceptibility to neurodegenerative disorder. In many previous studies, the potential association between *ACT* SNPs and AD has centred on single SNP analyses. However, the inventors suggest that by looking at single SNPs, additive and/or opposing associations with other SNPs are being ignored, and explain some of the conflicting data reported in the past. The present study however, which is based on 1,087 samples, represents the most comprehensive haplotype analysis of *ACT* in AD to date. This approach has 80% power to detect a genotype relative risk of 1.5 to 2.1 associated with alleles or haplotypes with a frequency $\geq 5\%$ at a level of statistical significance of ≤ 0.01 . Hence, when the various SNPs are considered collectively in the present invention, they demonstrate synergy, and therefore provide more accurate diagnosis.

Hence, in a preferred embodiment, any two of said polymorphic regions correlate with susceptibility to a neurodegenerative disorder. Preferably, the second polymorphism is in linkage disequilibrium with the first polymorphism in intron 2 of the ACT gene.

The term "linkage disequilibrium" refers to the co-inheritance of two or more alleles at frequencies greater than would be expected from the separate frequencies of occurrence of each allele in a given control population. The expected frequency of occurrence of two alleles that are inherited independently is the frequency of the first allele in that population multiplied by the frequency of the second allele in that population. Alleles that co-occur at expected frequencies are said to be in "linkage equilibrium". The cause of linkage disequilibrium is often unclear. It can be due to selection for certain allele combinations or to recent admixture of genetically heterogeneous populations. In addition, in the case of markers that are very tightly linked to a disease gene, such as *ACT* and neurodegenerative disorder, an association of an allele (or group of linked alleles) with the disease gene is expected if the disease mutation occurred in the recent past, so that sufficient time has not elapsed for equilibrium to be achieved through recombination events in the specific chromosomal region.

A preferred second polymorphism, which is detected using the method of the first aspect, in addition to the polymorphism in intron 2 of the *ACT* gene comprises either a guanine (G) allele or an adenine (A) allele at nucleotide position 2076 of the ACT gene (referred to as ACT 5). The inventors have found that a subject having the G allele (mutant) in ACT 5 shows a statistically significant propensity to show a decline in cognitive function. The adenine allele is therefore the wild-type allele.

Hence, preferably, the second polymorphism which is detected comprises the G allele at nucleotide position 2076 of the ACT gene (ACT 5).

Accordingly, a combination of the ACT 5 G allele and the ACT 7 G allele therefore provides an accurate assessment of a subject's pre-disposition to neurodegenerative disorder. Accordingly, it is most preferred that the method of the invention comprises detecting for both the G allele of the ACT 7 polymorphism
5 and the G allele of the ACT 5 polymorphism.

It should be appreciated that when referring to polymorphisms that comprise more than one allele, a first allelic pattern is in linkage disequilibrium with a second allelic pattern if at least one of the alleles that comprise the first allelic pattern are
10 in linkage disequilibrium with at least one of the alleles of the second allelic pattern. Such an example of linkage disequilibrium would be that which occurs between the alleles at the ACT 5 and ACT 7 sites.

Preferably, and advantageously, diagnosis of the neurodegenerative disorder may
15 be carried out by detection of the genetic polymorphism in intron 2 of the *ACT* gene with any combination of polymorphisms (i) to (xii) referred to above. By way of example only, the method may therefore comprise detecting for the genetic polymorphism in intron 2 of the *ACT* gene; and the genetic polymorphism in ACT 1, and/or ACT 2, and/or ACT 3, and/or ACT 5, and/or ACT 6, and/or ACT 8, and/or
20 ACT 9, and/or ACT 10, and/or ACT 11, and/or ACT 12, and/or ACT 13. Preferably, diagnosis of the susceptibility to neurodegenerative disorder may be carried out by detecting which alleles of the various polymorphic regions are present.

Advantageously, screening for the presence of at least two alleles of the polymorphic regions in the ACT gene allows for the identification of individuals
25 likely to have a genetic susceptibility to neurodegenerative disorder.

Preferably, in carrying out the method of the invention, an individual's ACT gene genotype is determined by analysis of a polymorphic region of the ACT gene, rather than by analysis of the entire gene sequence. Preferably, in an embodiment
30 of the present invention, the susceptibility to neurodegenerative disorder is assessed by determining whether an individual is homozygous or heterozygous for either or both of the preferred alleles of the polymorphic regions of the ACT gene, ie. the G allele of the ACT 7 polymorphism and the G allele of the ACT 5

polymorphism.

According to the invention, an individual who carries either or both of the G allele of the ACT 7 polymorphism and the G allele of the ACT 5 polymorphism is
5 classified as being at a higher risk for susceptibility to neurodegenerative disorder when compared against an individual who has the Guanine allele of the ACT 7 polymorphism and the Adenine allele of the ACT 5 polymorphism or the C allele of ACT 7 and the G allele of ACT 5. Hence, subjects being homozygous for the G allele in both SNPs will be most pre-disposed to neurodegenerative disorder.

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Preferably, the method of the invention comprises detecting at least one polymorphic region in the ACT gene in a sample taken from a test subject. Preferably, the method according to the first aspect is carried out *in vitro* on a bodily sample from the test subject.

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Preferably, the sample which is tested according to the method comprises a biological sample which, preferably, comprises nucleic acid. Preferably, the nucleic acid encodes at least the ACT gene and, more preferably, at least intron 2 of the ACT gene and, most preferably, the nucleotide 6738 of intron 2 of the ACT gene
20 (ACT 7). Additionally, the sample may also encode at least exon 2 of the ACT gene and, preferably, nucleotide 2076 of exon 2 of the ACT gene (ACT 5). Preferably, the sample comprises genomic DNA and, more preferably, comprises at least nucleotide number 6738 of intron 2, and at least nucleotide number 2076 of exon 2 of the ACT gene.

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Techniques for determining the presence of particular alleles in the sample are known to the skilled technician and include, but are not limited to, nucleic acid techniques based on size or sequence, such as restriction fragment length polymorphism (RFLP), nucleic acid sequencing, or nucleic acid hybridization. The
30 nucleic acid tested may be RNA or DNA. These techniques may also comprise the step of amplifying the nucleic acid before analysis. Amplification techniques are known to those of skill in the art and include, but are not limited to, cloning, polymerase chain reaction (PCR), polymerase chain reaction of specific alleles

(PASA), polymerase chain ligation, nested polymerase chain reaction, and the like. Hence, the methods according to the invention may comprise a step of amplifying nucleic acid in the sample, which nucleic acid harbours the polymorphism to be detected.

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Hence, preferably, said detecting step comprises amplifying at least intron 2 of the ACT gene and, preferably, nucleotide +6738 of the ACT gene, and identifying the allele encoded by said amplified DNA. Existence of the Guanine allele at nucleotide position +6738 is indicative that the subject has or is pre-disposed to neurodegenerative disorder.

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Additionally, said detecting step may comprise amplifying at least exon 2 of the ACT gene and, preferably, nucleotide +2076 of the ACT gene, and identifying the allele encoded by said amplified DNA. Existence of the Guanine allele at nucleotide position +2076 is indicative that the subject has or is pre-disposed to neurodegenerative disorder.

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It will be appreciated that due to the synergistic effect between these two SNPs, that existence of both the Guanine allele at nucleotide position +6738 and also the Guanine allele at nucleotide position +2076 is indicative that the subject has or is pre-disposed to neurodegenerative disorder.

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Preferably, the method comprises use of PCR using suitable primers adapted to amplify at least the genomic site including intron 2 of alpha-1-antichymotrypsin (ACT) gene, and preferably, exon 2 of the ACT gene. Many variations of the basic amplification protocol are well known to the skilled technician. PCR-based detection means may include multiplex amplification of a plurality of markers simultaneously. For example, it is well known to select PCR primers to generate PCR products that do not overlap in size and can be analysed simultaneously. Alternatively, it is possible to amplify different genetic markers with primers that are differentially labelled and thus can each be differentially detected in the same reaction. Of course, hybridization-based detection means allow the differential

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detection of multiple PCR products in a sample. Other techniques are known in the art to allow multiplex analysis of a plurality of markers.

Preferably, said detecting comprises use of at least one oligonucleotide operable to be used for amplification of intron 2 of the ACT gene. Preferably, the amplification step employs at least one primer comprising substantially the nucleotide sequence as set out as SEQ ID No: 59 (5'-ctatgagggactctgggcactt-3'), or SEQ ID No: 60 (5'-cgccctgctcgaccct-3') when detecting for the ACT 7 polymorphism.

Preferably, said detecting comprises use of at least one oligonucleotide operable to be used for amplification exon 2 of the ACT gene. Preferably, the amplification step employs at least one primer comprising substantially the nucleotide sequence as set out as SEQ ID No: 51 (5'-agctttgctttcagagttgagaat-3'), or SEQ ID No: 52 (5'-ctcgtcaagtgggctgtagg-3') when detecting for the ACT 5 polymorphism.

Amplification products may be detected or assayed in a variety of ways, including size analysis, restriction digestion followed by size analysis, detecting specific tagged oligonucleotide primers in the reaction products, allele specific oligonucleotide (ASO) hybridization, allele specific S' exonuclease detection, sequencing, nucleic acid hybridization and the like. Polymorphic variations leading to altered protein sequences or structures may also be detected by analysis of the expressed protein itself.

Said detecting may comprise subjecting the amplified DNA to size analysis, preferably, electrophoresis and, preferably, comparing the results to a positive control and, preferably, a negative control. Said size analysis may be preceded by restriction enzyme digestion. Said detecting may comprise digesting the amplified DNA with a restriction enzyme, preferably, *Stu1* or *Mva1*, and then, preferably, subjecting the resultant digested DNA to electrophoresis and, preferably, comparing the results to a positive and, preferably, a negative control. The enzyme *Stu1* is used to cut the amplification product when detecting for ACT 7 polymorphism, and *Mva1* is used when detecting for the ACT 5 polymorphism.

Preferably, and advantageously, the alleles of a biallelic polymorphism of a single base variation (C/G) at position +6738 may be identified by allele-specific cleavage using a restriction enzyme, preferably, *Stu1*. Preferably, and advantageously, the alleles of a biallelic polymorphism of a single base variation (A/G) at position
5 +2076 may be identified by allele-specific cleavage using a restriction enzyme, preferably, *Mva1*.

After restriction enzyme digestion of the products of the PCR reaction, the DNA may be separated on a gel by electrophoresis. From this gel, the alleles of the
10 polymorphism may be identified. Alternatively, or additionally, the gel may undergo Southern blotting or other hybridization analyses comprising labeling, preferably, radio-labeling a probe. Said detecting may comprise sequencing the DNA encoding the polymorphism to determine the allele or alleles present.

15 Preferably, the detecting comprises a step of probing the product of the amplification step with a first probe which is adapted to bind to one of the alleles of the genetic polymorphism, and preferably, probing with a second probe which is adapted to bind to the other of the alleles of the genetic polymorphism. The probes are preferably nucleic acid sequences designed to bind to one of the alleles. If one
20 of the probes binds to the amplification product, the subject is homozygous for that allele. If however both probes bind to the amplification product, the subject is heterozygous for each allele. Figure 7 illustrates so-called Vic and Fam probes, which are preferred probes used for probing ACT 1, ACT 4, ACT 5, ACT 6 and ACT 7.

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Preferably, the probing step employs at least one probe comprising substantially the nucleotide sequence as set out as SEQ ID No: 61 (5'- cttccgcagcagtg-3'), or SEQ ID No: 62 (5'- ctccccagcagtg-3') when detecting for the ACT 7 polymorphism. SEQ ID No: 62 binds to the mutant G allele and SEQ ID No: 61
30 binds to the wild-type C allele.

Preferably, the probing step employs at least one probe comprising substantially the nucleotide sequence as set out as SEQ ID No: 53 (5'- tacctctcctggctct-3'), or

SEQ ID No: 54 (5'- tggtacctctcctgactc-3') when detecting for the ACT 5 polymorphism. SEQ ID No: 54 binds to the mutant G allele and SEQ ID No: 53 binds to the wild-type C allele.

- 5 The skilled technician will appreciate how to detect binding of the probe to the amplification product in order to determine which allele the subject has. For example, probe binding may be detected by radiolabel or fluorescence.

10 The inventors have realized that their findings may be applied to develop a novel kit for identifying the existence of, or susceptibility to, neurodegenerative disorder in a subject.

15 Hence, according to a second aspect of the invention, there is provided a kit for identifying the existence of, or susceptibility to, neurodegenerative disorder in a subject, the kit comprising means for detecting the presence of a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) in the subject, wherein the polymorphism is associated with neurodegenerative disorder.

20 The kit is preferably used to carry out the method according to the first aspect. Preferably, the kit is used for identifying the existence of, or susceptibility to, Alzheimer's disease, and most preferably early-onset Alzheimer's disease in the subject.

25 Preferably, the kit comprises collecting means for collecting a biological sample from the subject. Preferably, the sample comprises DNA. The sample collecting means may be suitable for isolating a DNA sample from the individual from which DNA may be used for subsequent analysis. The DNA sample may be obtained from a tissue sample, for example, blood, saliva, or urine etc. In a preferred embodiment, the DNA is obtained from blood cells, preferably, obtained from a finger prick of the individual. Preferably, the sample collecting means is operable
30 to isolate blood from the individual. Preferably, the DNA is isolated from dried blood spots. Preferably, the DNA comprises the polymorphism to be detected, which may be amplified, for example, using PCR.

Preferably, the means for determining the presence of the polymorphism comprises analysis of the DNA sample, more preferably, genetic analysis.

Preferably, the kit comprises means for detecting the polymorphism in intron 2 of the ACT gene, and most preferably, the nucleotide +6738 of intron 2 of the ACT gene (ACT 7). Most preferably, the kit comprises means for detecting a Guanine allele in the polymorphism.

Preferably, the kit comprises means to compare the polymorphism to a control sample of known disease severity to determine an individual's susceptibility to neurodegenerative disorder.

Hence, preferably, the kit comprises means for detecting for the presence of a second genetic polymorphism, wherein the second polymorphism is independently selected from a group of genetic polymorphisms consisting of :-

(i) at nucleotide position -12799 of the ACT gene (G/T) (referred to herein as ACT 1); (ii) at nucleotide position -596 of the ACT gene (G/A) (referred to herein as ACT 2); (iii) at nucleotide position -501 of the ACT gene (A/G) (referred to herein as ACT 3); (iv) at nucleotide position -51 of the ACT gene (G/T) (referred to herein as ACT 4); (v) at nucleotide position 2076 of the ACT gene (A/G) (referred to herein as ACT 5); (vi) at nucleotide position 2354 of the ACT gene (A/G) (referred to herein as ACT 6); (vii) at nucleotide position 6980 of the ACT gene (C/T) (referred to herein as ACT 8); (viii) at nucleotide position 10074 of the ACT gene (A/G) (referred to herein as ACT 9); (ix) at nucleotide position 10120 of the ACT gene (G/A) (referred to herein as ACT 10); (x) at nucleotide position 10167 of the ACT gene (G/T) (referred to herein as ACT 11); (xi) at nucleotide position 11998 of the ACT gene (A/C) (referred to herein as ACT 12); and (xii) at nucleotide position 12013 of the ACT gene (C/T) (referred to herein as ACT 13).

Preferably, the kit comprises means for detecting any combination of polymorphisms (i) to (xii). However, a preferred second polymorphism is at nucleotide position 2076 of the ACT gene (A/G) (referred to herein as ACT 5). Therefore, preferably, the kit comprises means for detecting a polymorphism in

exon 2 of the ACT gene and, preferably, nucleotide 2076 of exon 2 of the *ACT* gene (ACT 5). Most preferably, the kit comprises means for detecting a Guanine allele in this polymorphism.

- 5 According to the invention, an individual who carries at least the G allele in the ACT 7 polymorphism, and the G allele in the ACT 5 polymorphism is classified as being at a higher risk for neurodegenerative disorder when compared against an individual who has either the G allele for the ACT 7 polymorphism and the Adenine allele for the ACT 5 polymorphism or the C allele for ACT 7 and the A allele for
10 ACT 5.

Advantageously, the kit according to the invention requires a low concentration of blood. However, other means for collecting DNA and determining polymorphism patterns which are known in the art may be used.

15

Preferably, the kit comprises DNA sampling reagents and, preferably, PCR amplification reagents. Preferably, the PCR amplification reagents comprise Taq Polymerase, and preferably, Ampli-Taq Gold.

- 20 Preferably, said kit comprises at least one oligonucleotide comprising a sequence operable to be used for amplification of intron 2 of the ACT gene. Preferably, the kit comprises at least one primer comprising substantially the nucleotide sequence as set out as SEQ ID No: 59 (5'-ctatgagggactctgggcactt-3'), or SEQ ID No: 60 (5'-cgccctgctcgaccct-3') when detecting for the ACT 7 polymorphism.

25

Preferably, the kit comprises at least one oligonucleotide operable to be used for amplification exon 2 of the ACT gene. Preferably, the kit comprises at least one primer comprising substantially the nucleotide sequence as set out as SEQ ID No: 51 (5'-agctttgcttttcagagttgagaat-3'), or SEQ ID No: 52 (5'-ctcgtcaagtgggctgttagg-3')
30 when detecting for the ACT 5 polymorphism.

Oligonucleotide primers that target the specific polymorphism DNA region within the ACT gene may be prepared so that, in the PCR reaction, amplification of the

target sequences may be achieved. Said primers may comprise a detectable label.

Preferably, the kit comprises a control sample, which comprises one or more alleles corresponding to the genetic polymorphism in intron 2 of alpha-1-

5 antichymotrypsin (*ACT*), wherein existence of the mutant allele in the test subject suggests that the patient is suffering from, or is susceptible to neurodegenerative disorder. Preferably, the control sample comprises one or more alleles corresponding to the *ACT* 7 polymorphism. For example, the kit may comprise a control sample having either the C or G allele for the *ACT* 7 polymorphism.

10 Preferably, the kit comprises a further control sample corresponding to the second genetic polymorphism, for example, one or more alleles corresponding to the *ACT* 5 polymorphism, ie the A or G allele.

The amplified DNA sequences from the target DNA may be analysed using
15 restriction enzyme digestion to determine the polymorphism pattern present in the amplified sequences and thereby provide a genetic polymorphism profile of the individual. The restriction enzyme digestion may comprise using the restriction enzyme *Stu1* to digest the amplified DNA sequences when genotyping the individual for the *ACT* 7 polymorphism, and enzyme *Mva1* to digest the amplified
20 DNA sequences when genotyping the individual for the *ACT* 5 polymorphism.

Preferably, said detecting comprises subjecting the amplified DNA to size analysis, preferably, electrophoresis and, preferably, comparing the results to a positive control and, preferably, a negative control.

25

Preferably, the kit comprises means for probing the product of the amplification step to determine the genotype of the subject under test. The kit preferably comprises a first probe which is adapted to bind to one of the alleles of the genetic polymorphism, and preferably, a second probe which is adapted to bind to the
30 other of the alleles of the genetic polymorphism. The probes are preferably nucleic acid sequences designed to bind to one of the alleles. Figure 7 illustrates so-called Vic and Fam probes, which are preferred probes used for probing *ACT* 1, *ACT* 4, *ACT* 5, *ACT* 6 and *ACT* 7.

A preferred first probe may comprise substantially the nucleotide sequence as set out as SEQ ID No: 61 (5'- cttccgcagcagtg-3'), or SEQ ID No: 62 (5'- ctccccagcagtg-3') when detecting for the ACT 7 polymorphism. A preferred
5 second probe may comprise substantially the nucleotide sequence as set out as SEQ ID No: 53 (5'- tacctctcctggctct-3'), or SEQ ID No: 54 (5'- tggtacctctcctgactc-3') when detecting for the ACT 5 polymorphism.

The inventor believes that the isolation of the novel SNP ACT 7 will also have
10 significant opportunities in the medical and therapeutic fields.

Hence, according to a third aspect, there is provided a method of treating a subject suffering from, or susceptible to, neurodegenerative disorder, the method comprising:-

- 15 (i) detecting the presence of a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) gene, wherein the polymorphism is associated with neurodegenerative disorder; and
(ii) administering to the subject a therapeutic agent that prevents, reduces or delays progression of neurodegenerative disorder.

20

The kit according to the second aspect is useful for providing a prognosis of the subject's condition, such that the clinician can carry out the treatment method according to the third aspect. The kit may also be used to monitor the efficacy of a putative treatment for neurodegenerative disorder. The methods and the kit
25 according to the invention are therefore very useful for guiding a neurodegenerative disorder treatment regime for the clinician, and to monitor the efficacy of such a treatment regime.

A suitable treatment to prevent, reduce or delay progression of renal failure may
30 be hormone replacement therapy or gene therapy. The use of this therapy can thus be commenced in individuals likely to show a predisposition to the neurodegenerative disorder upon detection of the presence of a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) gene.

The skilled technician will appreciate the various types of therapeutic agent that may be administered to the subject in the method of the third aspect for preventing, reducing or delaying progression of neurodegenerative disorder. For example, suitable agents may include an anticholinesterase inhibitor. For example, the agent may be Physostigmine (Synapton (R), Forest Laboratories; New York, NY), Tacrine (Cognex (R); Parke-Davis; Morris Plains, NJ), Donepezil (Aricept(R), Pfizer/Eisai; New York, NY/Tokyo, Japan), Metrifonate (Bayer; Leverkusen, Germany), or Rivastigmine (Exelon(R); Novartis; Basel, Switzerland).

Preferably, the method comprises detecting the G allele of the polymorphism (ACT 7).

The method may comprise detecting additional polymorphisms as well as the genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (ACT) gene. Hence, preferably, the method of the third aspect comprises detecting for the presence of a second genetic polymorphism, wherein the second polymorphism is independently selected from a group of polymorphisms consisting of :-

(i) at nucleotide position -12799 of the ACT gene (G/T) (referred to herein as ACT 1); (ii) at nucleotide position -596 of the ACT gene (G/A) (referred to herein as ACT 2); (iii) at nucleotide position -501 of the ACT gene (A/G) (referred to herein as ACT 3); (iv) at nucleotide position -51 of the ACT gene (G/T) (referred to herein as ACT 4); (v) at nucleotide position 2076 of the ACT gene (A/G) (referred to herein as ACT 5); (vi) at nucleotide position 2354 of the ACT gene (A/G) (referred to herein as ACT 6); (vii) at nucleotide position 6980 of the ACT gene (C/T) (referred to herein as ACT 8); (viii) at nucleotide position 10074 of the ACT gene (A/G) (referred to herein as ACT 9); (ix) at nucleotide position 10120 of the ACT gene (G/A) (referred to herein as ACT 10); (x) at nucleotide position 10167 of the ACT gene (G/T) (referred to herein as ACT 11); (xi) at nucleotide position 11998 of the ACT gene (A/C) (referred to herein as ACT 12); and (xii) at nucleotide position 12013 of the ACT gene (C/T) (referred to herein as ACT 13).

Preferably, the method comprises detecting any combination of polymorphisms (i) to (xii). A preferred second polymorphism is at nucleotide position 2076 of the ACT gene (A/G) (referred to herein as ACT 5). Preferably, the method comprises detecting a polymorphism in exon 2 of the ACT gene and, preferably, nucleotide
5 2076 of exon 2 of the ACT gene (ACT 5). Most preferably, the method comprises detecting a Guanine allele in the ACT 5 polymorphism.

The inventors have realized that the kit of the second aspect may be used to monitor the progression of neurodegenerative disorder, and thereby determine the
10 efficacy of the treatment regime that is being used with the method of the third aspect.

Hence, according to a fourth aspect, there is provided a method of screening for progression of neurodegenerative disorder in a subject, the method comprising
15 detecting the presence or absence of a Guanine allele of a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (ACT) gene in the subject, wherein presence of the Guanine allele of the polymorphism in the ACT gene is indicative of progression of neurodegenerative disorder.

20 Preferably, the method comprises a step of isolating genomic DNA from the patient prior to detecting for the allele.

Preferably, the method comprises detecting for the G allele of the G allele in position +6738 of polymorphism. Preferably, the method comprises detecting for a
25 G allele of a genetic polymorphism in position +2076 of exon 2 of alpha-1-antichymotrypsin (ACT) gene in the subject, wherein presence of the G allele is indicative of progression of neurodegenerative disorder.

The inventors also believe that the identification of the new SNP, ACT 7, may be
30 used to facilitate identification of still further SNPs associated with susceptibility to neurodegenerative disorders.

According to a fifth aspect of the present invention, there is provided a method of

identifying an allele associated with susceptibility to neurodegenerative disorder, said method comprising identifying an allele which is in linkage disequilibrium with a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) gene, wherein the polymorphism is associated with neurodegenerative disorder.

5

Further alleles of newly discovered SNPs using the method of the fourth aspect may be used to provide a more accurate assessment of the disease condition.

10

The inventors also envisage that the nucleic acids encoding the polymorphisms disclosed herein, and peptides encoded thereby, may have direct medical and therapeutic applications.

15

Hence, according to sixth aspect of the present invention, there is provided use of nucleic acid comprising a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) gene, or a peptide encoded thereby, for the preparation of a medicament for the treatment of neurodegenerative disorder.

20

Preferably, the treatment may comprise retarding or preventing the disease, preferably Alzheimer's.

Preferably, the nucleic acid comprises intron 2, and most preferably, nucleotide number +6738. Most preferably, the nucleic acid comprises a Guanine allele at position +6738.

25

Figure 6 sets out the nucleotide sequences, which flank position +6738.

Preferably, the nucleic acid comprises a nucleotide sequence substantially as set out in SEQ ID No: 29 (5'-tctgggcacttccactgctg-3') and/or SEQ ID No: 30 (5'-ggaagcagggctcgagcaggg-3').

30

It will be appreciated that the intron 2 polymorphism is in non-coding DNA and so does not encode a peptide. However, other known and newly discovered polymorphisms in the *ACT* gene are located in coding DNA, and so their peptides may be used in the manufacture of a medicament.

Hence, the use according to the sixth aspect may comprise use of at least a second nucleic acid comprising a second genetic polymorphism, or a peptide encoded thereby, wherein the second polymorphism is independently selected

5 from a group of polymorphisms consisting of :-

(i) at nucleotide position -12799 of the ACT gene (G/T) (referred to herein as ACT 1); (ii) at nucleotide position -596 of the ACT gene (G/A) (referred to herein as ACT 2); (iii) at nucleotide position -501 of the ACT gene (A/G) (referred to herein as ACT 3); (iv) at nucleotide position -51 of the ACT gene (G/T) (referred to herein as ACT 4); (v) at nucleotide position 2076 of the ACT gene (A/G) (referred to
10 herein as ACT 5); (vi) at nucleotide position 2354 of the ACT gene (A/G) (referred to herein as ACT 6); (vii) at nucleotide position 6980 of the ACT gene (C/T) (referred to herein as ACT 8); (viii) at nucleotide position 10074 of the ACT gene (A/G) (referred to herein as ACT 9); (ix) at nucleotide position 10120 of the ACT
15 gene (G/A) (referred to herein as ACT 10); (x) at nucleotide position 10167 of the ACT gene (G/T) (referred to herein as ACT 11); (xi) at nucleotide position 11998 of the ACT gene (A/C) (referred to herein as ACT 12); and (xii) at nucleotide position 12013 of the ACT gene (C/T) (referred to herein as ACT 13).

20 As shown in Figure 6, a preferred second nucleic acid comprises a nucleotide sequence independently selected from a group of nucleotides consisting of: SEQ ID No: 17; SEQ ID No: 18; SEQ ID No: 19; SEQ ID No: 20; SEQ ID No: 21; SEQ ID No: 22; SEQ ID No: 23; SEQ ID No: 24; SEQ ID No: 25; SEQ ID No: 26; SEQ ID No: 27; SEQ ID No: 28; SEQ ID No: 29; SEQ ID No: 30; SEQ ID No: 31; SEQ
25 ID No: 32; SEQ ID No: 33; SEQ ID No: 34; SEQ ID No: 35; SEQ ID No: 36; SEQ ID No: 37; SEQ ID No: 38; SEQ ID No: 39; SEQ ID No: 40; SEQ ID No: 41; and SEQ ID No: 42.

The nucleic acid and/or encoded peptide may have utility in drug research
30 purposes for retarding or preventing neurodegenerative disorder. Preferably, said drug research purposes comprises the generation of a molecular model of said nucleic acid or said peptide. Preferably, the nucleic acid is isolated and, preferably, further comprises functional and/or structural variants thereof. Preferably, the

peptide is isolated and, preferably, comprises functional and/or structural variants thereof.

Preferably, and advantageously, the aspects of the present invention allow for the
5 identification of an individual's genetic polymorphism pattern associated with neurodegenerative disorder, and preferably, Alzheimer's disease. Advantageously, the identification of those at risk allows preventative measures to be initiated prior to development of Alzheimer's.

10 In summary, in the present invention a specific combination of two SNPs in the *SERPINA3* (ie *ACT*) gene are shown to be diagnostic, with a high degree of confidence, for Alzheimer's disease. A combination of SNPs rather than an individual SNP has much more utility in diagnosing susceptibility to Alzheimer's Disease. The use of the SNPs in a diagnostic test could identify individual humans
15 with a high probability of developing early-onset Alzheimer's Disease.

All of the features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except
20 combinations where at least some of such features and/or steps are mutually exclusive.

For a better understanding of the invention, and to show how embodiments of the same may be carried into effect, reference will now be made, by way of example, to the accompanying diagrammatic drawings, in which:-

25 Figure 1 shows a flow-chart of the patient samples used for each phase of this study;

Figure 2 shows a schematic representation of the *ACT* gene showing (A) location of exons and (B) locations of SNPs identified during this study. The previously identified functional promoter SNP is indicated by a star (*), and the signal

sequence polymorphism by a hash (#). The scale bar represents the number of base pairs from the start of chromosome 14;

Figure 3 shows an alternative schematic representation of the *ACT* gene showing (A) location of exons and (B) locations of SNPs identified during this study. Coding exons are represented by shaded regions. SNPs underlined were previously unreported. The previously identified functional promoter SNP is indicated by a star (*), and the single sequences polymorphisms by a hash (#).

Figure 4 shows Global *P*-values for case-control association with all SNP combinations in the total (solid line), LOAD (dashed line) and EOAD (dotted line) datasets. Y axis is shown on a log scale, intercept crosses y-axis at $P=0.05$;

Figure 5 shows a non-linear model of cognitive decline for 129 AD patients. A-E : Interactive effects of individual *ACT* genotype on cognitive decline; F : Effect of age on cognitive decline (old refers to patients > 73.2 years and young to those ≤ 73.2 years);

Figure 6 is a table showing the location of SNPs identified in the *ACT* gene. Numbering is from the only known transcription start site (TSS). Contig number is relative to contig sequence AL049839. ^aSNP excluded from further analysis due to unsuccessful Taqman assay; and

Figure 7 is a table showing primer and probe sequences used in the Taqman genotyping assays. Polymorphic bases are underlined.

Examples

Materials and Methods

Patient Samples

Details of patient samples used in this study are shown in Figure 1. Informed consent was obtained for all samples, which was granted approval by the local

Ethics Committee. For the case-control haplotype association study, the 1,087 samples were obtained from 4 UK centres; University of Nottingham Brain Bank, Oxford Project To Investigate Memory and Ageing (OPTIMA), University of Manchester Neurosciences Centre, and University of Birmingham Institute of Biomedical Research (no controls from this centre). The term "haplotype" refers to a set of alleles that are inherited together as a group (they are in linkage). As used herein, haplotype is defined to include those haplotypes that occur at statistically significant levels ($P_{\text{corr}} < 0.05$). As used herein, the phrase "ACT haplotype" refers to a haplotype at the ACT locus.

As there was no evidence for population stratification of this gene in a large ($N=2000$) study comprising seven centres within Western Europe including England and Scotland (Chappell *et al.*, 2006, *Hum Mutat.* 27, 103-9), or in this study, the samples were pooled. Samples were histopathologically confirmed as definite disease (AD) ($N=420$) or control ($N=419$) using CERAD criteria (Mirra *et al.*, 1993, *Arch Pathol Lab Med.* 117, 132-44). Probable Alzheimer's disease (AD) patients ($N=248$, Manchester and Oxford) were also included since there was 100% concordance between patients diagnosed with probable AD and confirmation of the disease post-mortem ($N=34$) (Nagy *et al.*, 1998, *Dement Geriatr Cogn Disord.* 9, 219-26). All patients with evidence of an autosomal dominant AD trait, or where a first degree relative had been diagnosed with familial AD, were excluded.

In order to determine any effect of age-at-onset, the AD samples from Manchester, Birmingham and Oxford (centres where age-at-onset was known), were subsequently divided into LOAD and EOAD. There was no evidence for an effect of age on ACT SNP allele frequency in the control group and the frequencies in young and old controls were not different, thus all controls were used as a comparator for both LOAD and EOAD. The cognitive decline study comprised 129 AD patients and 156 controls from Oxford for which cognitive scores were available. The pathology association study comprised 123 AD patients from Manchester, for which levels of A β 40, A β 42 and total A β as well as tau and

microglial cell load and degree of astrocytic activity within frontal cortex were measured at death.

Phase 1 - SNP Identification

Forty four control samples were used to identify the SNPs within the *ACT* gene by sequencing known regulatory regions (promoter (Morgan *et al.*, 2001, *Human Genetics*. 109, 303-310) and -13kb enhancer (Kordula *et al.*, 1998, *J Biol Chem*. 273, 4112-8)), all coding regions (exons 1-5) and 1kb of 5' and 3' flanking sequence. This approach has 99% power for the detection of polymorphisms present at a frequency of $\geq 5\%$. Polymorphic sites were identified by multiple sequence alignment using ClustalW software (<http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>) and confirmed by repeat PCR and sequencing from the same or opposite strand. Polymorphisms with a minor allele frequency (MAF) of $\geq 5\%$ were then genotyped in a further 225 control samples to obtain a more accurate estimate of allelic frequency.

PCR and automated sequencing

Genomic DNA was extracted from whole blood or brain tissue using the QIAamp DNA blood mini kit (Qiagen, Crawley, West Sussex, UK). The amplification and sequencing protocols for all *ACT* regions are as follows:-

SNP Identification and determination of frequency

Amplification of the majority of regions was performed in a reaction mix containing 100ng DNA template; 1 μ M each primer; 200 μ M dNTP; 10mM Tris-HCl, pH8.8; 50mM KCl; 1.5mM MgCl₂; 0.08% Nonidet P40; 1U Taq polymerase and water to a final volume of 30 μ l. Exceptions to this were the promoter region and 3' flanking region. The promoter was amplified in a reaction mix containing 100ng DNA template; 1 μ M each primer, 200 μ M dNTP; 10mM Tris-HCl, pH8.8; 20mM (NH₄)₂SO₄; 0.01% Tween 20; 3mM MgCl₂; 1U Taq polymerase and water to a final volume of 30 μ l. The *ACT* 3' flanking region was amplified in a reaction mix containing 100ng DNA template; 1 μ M each primer, 200 μ M dNTP; 10mM Tris-HCl, pH8.8; 20mM (NH₄)₂SO₄; 0.01% Tween 20; 1.5mM MgCl₂; 1U Taq polymerase

and water to a final volume of 30 μ l. Primer sequences, product sizes and optimum annealing temperatures for all *ACT* regions are shown in Table 1.

Table 1 - Sequence of primers used to amplify regions of interest in the *ACT* gene. Product sizes and optimum annealing temperatures are also shown

Region	Forward primer	Reverse primer	Product (bp)	Annealing Temp (°C)
5' enhancer	gaacagggatgattgagaagc (SEQ ID No.1)	atcatccccagggtccagaaga (SEQ ID No.2)	530	60
Promoter	acctgtgctccccgaaagct (SEQ ID No.3)	tcttggaacacagattcctcc (SEQ ID No.4)	1025	66
Exon 1	ttagctggggtcttctctgg (SEQ ID No.5)	taccctcacactccacaca (SEQ ID No.6)	497	66
Exon 2	aggctagcaagaggcagcag (SEQ ID No.7)	cacctcttctgaacccaag (SEQ ID No.8)	799	65
Exon 3	ccctcacccccaataacttt (SEQ ID No.9)	cctggacattggtgagacct (SEQ ID No.10)	499	60
Exon 4	agggtggaggcaggtaggtta (SEQ ID No.11)	tttcccccatggatagtaa (SEQ ID No.12)	500	60
Exon 5	gccagcactaggtgctcaat (SEQ ID No.13)	gggcgcagagtctgatagtc (SEQ ID No.14)	598	62
3' flanking region	cttcagtctggagggtcctg (SEQ ID No.15)	cccattctgaagcattgtcct (SEQ ID No.16)	1149	66

The optimum annealing temperature for each primer pair was determined using the thermal gradient facility on an MJ Research thermal cycler, with temperatures ranging from 50°C to 65°C. Reaction conditions were as follows: 94°C 30s optimised annealing temperature 60s, 72°C 60s for 35 cycles. This was followed by a final extension of 10 minutes at 72°C and quenching at 10°C.

Automated Sequencing

Prior to sequencing, PCR products were incubated in a 5 μ l reaction containing 5U exonuclease I and 1U shrimp alkaline phosphatase (Amersham Life Sciences, Freiburg, Germany) at 37°C for 15 minutes. The enzymes were then inactivated by heating at 80°C for 15 minutes. Cycle sequencing was performed in a reaction mix

containing 4µl Big Dye version 3 (Applied Biosystems, Warrington, Cheshire, UK), approximately 200ng enzyme-treated PCR product, 5pmol sequencing primer and water to a final volume of 10µl. Both sense and antisense amplimers were used to generate sequence in both orientations. The sequencing program consisted of 25 cycles of the following: 96°C 30s, 50°C 15s, 60°C 4 minutes. Excess dye terminators were removed from the sequencing products using gel filtration plates (ABgene, Epsom, Surrey, UK). The samples were then dried at 90°C prior to loading onto an ABI 3100 genetic analyser (Applied Biosystems).

10 Phase 2 - Case-control haplotype association study

Genotyping of Polymorphisms

All 1,087 samples were genotyped at five sites using fluorescently labelled TaqMan probes (Vic or Fam) by Geneservice (Cambridge, UK). Details of the genotyping are as follows.

15

The location of the thirteen SNPs identified in the ACT gene are shown in the Table in Figure 6.

20

The primer and probe sequences used in the Taqman genotyping assays as shown in the Table in Figure 7. The polymorphic bases are underlined.

25

For the case-control study, 1,087 samples were genotyped at five sites using the following TaqMan protocol; 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Primer and probe sequences are shown in Table 2 below.

30

Table 2 - P-values for single SNP Pearson correlation and global P-values from haplotype score tests for all SNP combinations and six pathological features in AD patients (N=123). P-values for haplotype score tests are corrected for confounding variables.

SNPs	Total A-beta	A-beta 40	A-beta 42	Tau	Microglia	Astrocytosis
1	0.26	0.21	0.49	0.33	0.44	0.03
4	0.44	0.45	0.51	0.65	0.21	0.42
5	0.85	0.32	0.57	0.97	0.09	0.77
6	0.35	0.35	0.51	0.40	0.29	0.007
7	0.10	0.29	0.14	0.54	0.86	0.004
1, 4	0.07	0.60	0.11	0.66	0.28	0.15
1, 5	0.34	0.61	0.36	0.40	0.21	0.20
1, 6	0.16	0.43	0.27	0.58	0.80	0.07
1, 7	0.07	0.13	0.24	0.96	0.51	0.02
4, 5	0.14	0.84	0.14	0.93	0.46	0.04
4, 6	0.08	0.51	0.08	0.97	0.29	0.08
4, 7	0.07	0.08	0.11	0.91	0.55	0.07
5, 6	0.32	0.72	0.25	0.44	0.27	0.09
5, 7	0.53	0.10	0.63	0.89	0.37	0.009
6, 7	0.15	0.15	0.32	0.97	0.40	0.03
5, 6, 7	0.51	0.16	0.74	0.72	0.47	0.02
4, 6, 7	0.22	0.04	0.21	1.00	0.36	0.12
4, 5, 7	0.31	0.10	0.37	0.83	0.65	0.01
4, 5, 6	0.18	0.69	0.24	0.86	0.62	0.02
1, 6, 7	0.28	0.26	0.49	0.78	0.61	0.07
1, 5, 7	0.57	0.13	0.71	0.94	0.41	0.01
1, 5, 6	0.40	0.69	0.42	0.32	0.36	0.12
1, 4, 7	0.08	0.09	0.16	0.96	0.46	0.07
1, 4, 6	0.16	0.68	0.16	0.80	0.42	0.21
1, 4, 5	0.31	0.79	0.37	0.98	0.51	0.03
4, 5, 6, 7	0.37	0.13	0.51	0.94	0.65	0.02
1, 5, 6, 7	0.50	0.19	0.73	0.65	0.59	0.03
1, 4, 6, 7	0.21	0.07	0.22	1.00	0.39	0.13
1, 4, 5, 7	0.38	0.12	0.48	0.95	0.55	0.02
1, 4, 5, 6	0.29	0.81	0.37	0.80	0.68	0.04
1, 4, 5, 6, 7	0.37	0.18	0.50	0.96	0.65	0.03

Fifteen percent of the samples assayed were of known genotype, determined by sequencing, which were unknown to Geneservice, but known at source and 10% were genotyped in duplicate as a quality assurance measure. The data were only accepted when there was 100% concordance between duplicate samples.

Haplotype Analyses

The genotyped SNPs were used to estimate the haplotype frequencies in controls and AD patients using the haplo.em function of haplo.stats v1.2.2 (<http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm>). This method

utilises a maximum likelihood analysis approach, which the inventors have previously used to describe the variability of the alpha-1-antitrypsin gene (Chappell *et al.*, 2004, *Hum Mutat.* 24, 535-6). Samples that failed at >50% of the sites were not included in the analyses. The haplo.stats software predicts missing genotypes with a high probability of inferring the correct genotype. In an 'in-house' simulation, with and without this estimation, no significant difference in haplotype frequency was observed, thus validating the approach. For each of the SNPs the failure rates were as follows; ACT 1: 0.7%, ACT 4: 4.1%, ACT 5: 11.5%, ACT 6: 9.8% and ACT 7: 1.7%.

Chi-squared tests of individual SNPs with disease status were performed using Statistical Package for Social Sciences (SPSS) v12.0.1. This was followed by a case-control haplotype association study using haplo.stats for all SNP combinations. The inventors used the haplo.score function of haplo.stats to calculate global and haplotype-specific score statistics (Schaid *et al.*, 2002, *Am J Hum Genet.* 70, 425-34) (and corresponding *P*-values). The global score statistic tests for an overall association of haplotypes (occurring at a frequency of >5%) comprising information from all SNPs in any particular combination with disease status. The haplotype-specific score statistic compares the frequency of each individual haplotype compared to the most frequent haplotype. Rather than correcting for multiple testing, simulated *P*-values were computed for the global and haplotype-specific scores by repeatedly permuting the genotypes (10,000 iterations) among the subjects. The simulated *P*-value is calculated as the number of times the simulated score statistic exceeds the observed, divided by the total number of simulations.

In order to validate the findings, the inventors replicated the haplotype association study using a second software package, UNPHASED, which uses the same approach to generate haplotypes (Dudbridge, 2003, *Genet Epidemiol.* 25, 115-21).

Cognitive decline analysis

Cognitive scores were obtained using the CAMCOG score system (Roth M, 1988). In a previous study using samples from the same OPTIMA collection, the CAMCOG score data could be fitted using a non-linear mixed effects model (Martins *et al.*, 2005, *Neurology*. 65, 1888-93). Consequently, analysis of the CAMCOG data in this study was performed using the same methods. Briefly, the inventors used a three-parameter logistic (S-shaped) function: $\text{CAMCOG} = \text{asymptote} / [1 + \exp([\text{age} - \text{xmid}] / \text{scale})]$. The asymptotic score was set at a CAMCOG score of 95, in common with the previous work. The xmid parameter is the age at which patients reach 50% of the asymptotic score (CAMCOG=47.5) and scale is the time taken to fall from three-fourths to half the asymptotic score. The inventors first modelled the interactive effects of age with *APOE* allelic status (fixed effect) upon the xmid and scale parameters. A random effect was also included for xmid, recognising that different patients will obtain a score of 50% of the asymptotic value at different ages. The inventors then modelled *ACT* single SNP genotypes using a similar model. The model was implemented using the non-linear mixed-effects function 'nlme' of the nlme library v3.1-77 (Pinheiro J, 2000, *Mixed-effects models in s and s-plus*. Edited by Springer).

Pathology association analyses

The percentage area of frontal cortex tissue occupied by amyloid (total A β , A β 40 and A β 42), tau and microglial cells was determined by computer image analysis as described elsewhere (Iwatsubo *et al.*, 1994, *Neuron*. 13, 45-53; Thaker *et al.*, 2003, *Neuropathol Appl Neurobiol*. 29, 35-44; Zhang *et al.*, 2004, *Neuroscience Letters*. 362, 99-102). The degree of astrogliosis within frontal cortex was rated in GFAP immunostained sections on a scale from 0 to 2, where 0 = absent/mild, 1 = moderate, 2 = severe astrogliosis. Of the quantitative variables, A β 40, tau and microglia did not follow a gaussian distribution (assumed by haplo.score and Pearson correlations). This was adjusted for by computing the square root of these values and the transformed variables were used in all subsequent analyses. Pearson correlations were used to test association between individual SNPs and each pathological feature using SPSS. Quantitative trait analysis within the

haplo.score function of haplo stats was used to determine any association between these pathological features and all SNP combinations. Confounding variables were age-at-death (calculated as age-at-onset + duration of illness), duration of illness, *APOE* ϵ 4 status and gender and the score statistics were
5 adjusted using the x.ma function of haplo.score. For haplotypes showing a significant association with a particular trait, the haplo.glm function of haplo stats was used to perform regression in a general linear model. This provides *P*-values for all haplotype frequencies compared to the baseline haplotype. The effects of
10 haplotypes can be modelled as additive (homozygotes for a particular haplotype have a larger effect than heterozygotes), dominant (heterozygotes and homozygotes have equivalent effects), or recessive (homozygotes have an alternative effect on the trait). All *P*-values were adjusted for all confounding variables.

15 Results

Phase 1 - Identification of SNPs

In addition to the *ACT* signal sequence polymorphism (*ACT* 5), a total of twelve other SNPs (*ACT* 1-4 and 6-13) were identified, as illustrated in Figures 2 and 3. The adjacent sequences for these SNPs are available in the supplementary
20 information. Eight of these polymorphisms had been described previously (*ACT* 2 and 7 (HapMap, 2005), 4 (Morgan *et al.*, 2001, *Human Genetics*. 109, 303-310), 5 and 6 (Poller *et al.*, 1993, *Genomics*. 17, 740-3), 8, 9 and 10 (Wang *et al.*, 2002, *Human Genetics*. 110, 356-65)) and five were novel (*ACT* 1,3, 11-13). Of these 13 SNPs, seven had a Minor allele frequency (MAF) of >5% during the mapping of 88
25 alleles.

Referring to Figure 7, TaqMan assays were designed for six SNPs (*ACT* 1, 4, 5, 6, 7 and 8); one site (*ACT* 2) was excluded because a successful TaqMan assay could not be designed. Genotyping was performed in a total of 225 individuals,
30 and this information was used to re-estimate the MAFs. The MAF of *ACT* 8 decreased from 6% to 4% resulting in its exclusion. The remaining 5 SNPs, of which *ACT* 4 and *ACT* 5 were in 88% linkage disequilibrium (LD), were used for

haplotype analyses in the case-control study of 1,087 samples. All polymorphisms were in Hardy-Weinberg equilibrium in controls, LOAD and EOAD patients.

2. Case-control haplotype association analyses

- 5 Using these five SNPs, five common haplotypes were identified, which accounted for over 93% *ACT* variation in controls as shown in Table 3.

Table 3 - Frequencies (%) of the five common haplotypes of the *ACT* gene in 419 controls from Nottingham, Oxford and Manchester

10

Haplotype					Nottingham Controls	Oxford Controls	Manchester Controls	<i>P</i> -value	Global <i>P</i> -value
<i>ACT</i> 1	<i>ACT</i> 4	<i>ACT</i> 5	<i>ACT</i> 6	<i>ACT</i> 7					
G	G	G	A	C	23 (10.3%)	57 (13.8%)	23 (11.4%)	0.43	0.69
G	G	G	A	G	43 (19.2%)	102 (24.7%)	53 (26.4%)	0.24	
G	T	A	A	C	95 (42.4%)	158 (38.2%)	79 (39.5%)	0.50	
G	T	A	A	G	19 (8.5%)	34 (8.2%)	13 (6.7%)	0.56	
T	G	G	G	G	24 (10.7%)	35 (8.4%)	21 (10.5%)	0.54	
All other haplotypes					20 (8.9%)	28 (6.7%)	11 (5.5%)	0.66	
Total chromosomes					224	414	200		

A chi-squared test of the distribution of these haplotypes between the three control sample collections found no significant difference in their frequencies ($P=0.69$);

- 15 hence they were pooled. Table 4 shows the *P*-values for the chi-squared tests of each individual SNP versus disease status.

Table 4 - Single SNP analyses, frequency of minor allele (less frequent allele in controls), performed for AD ($N=668$), LOAD ($N=251$) and EOAD ($N=234$) vs. all controls ($N=419$).

20

SNP	Allele	Controls	AD	<i>P</i> -value	LOAD	<i>P</i> -value	EOAD	<i>P</i> -value
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ACT 1	T	84 (10.0%)	141 (10.7%)	0.70	53 (10.6%)	0.80	52 (11.3%)	0.56
ACT 4	G	398 (49.1%)	624 (49.0%)	0.98	221 (46.2%)	0.34	228 (52.5%)	0.28
ACT 5	G	381 (49.0%)	570 (49.7%)	0.78	221 (48.3%)	0.85	171 (53.1%)	0.24
ACT 6	G	88 (11.0%)	124 (10.7%)	0.90	50 (10.3%)	0.77	43 (12.4%)	0.54
ACT 7	G	362 (43.7%)	518 (39.5%)	0.06	189 (38.9%)	0.10	184 (39.7%)	0.17

The lowest P -value for a single SNP association was for ACT 7 ($P = 0.06$ in total dataset, $P = 0.10$ in LOAD and $P = 0.17$ in EOAD). The global P -values obtained for each multiple SNP combination are shown in Figure 4. The lowest global P -value was obtained with ACT 1 and ACT 7 in the total (global $P=0.08$, global simulated $P=0.06$ (computed over 10,000 iterations), maximum score statistic=0.06) and LOAD datasets (global $P= 0.07$, global simulated $P=0.06$, maximum score statistic=0.13). In EOAD, the lowest global P -value was for ACT 4, 5 and 7 (global $P=0.05$, global simulated $P=0.05$, maximum score statistic $P= 0.02$). These results were consistent with those obtained using the COCAPHASE function of UNPHASED (data not shown).

Effects of ACT genotypes and APOE allelic status on cognitive decline in AD

The effects of APOE allelic status on cognitive decline have been previously described using a larger dataset ($N=218$) of the OPTIMA collection (Martins *et al.*, 2005). The inventors performed the same analysis on 129 OPTIMA patients and found the same trends ($\epsilon 4$ allele predicted earlier cognitive decline and $\epsilon 2$ predicted slower decline), although the findings were not significant in this smaller subset (data not shown). The inventors then investigated the individual ACT genotypes using a similar non-linear model (Figure 5 a-e). The only SNP to show an effect of genotype on cognitive decline was for ACT 7. In comparison with the CC homozygotes, the curve for GG homozygotes showed a significant shift to the left, thus predicting earlier cognitive decline ($P=0.04$), whereas the curve for the GC heterozygotes showed no significant shift ($P=0.69$; Figure 5e). Both the GC (scale=1.65 years) and CC (scale=1.4 years) genotypes predicted more rapid decline than the GG genotype (scale=2.03 years) as evidenced by the steeper slopes ($P=0.009$ and $P<0.0001$ respectively). The average predicted cognitive

decline (xmid parameter) was calculated as 11.6 points per year for GG homozygotes compared with 16.8 points per year for CC homozygotes. The inventors next modelled the effects of age on cognitive decline (Figure 5f). The 920 measurements in their dataset covering patients at different ages were divided into two subsets about the mean age at testing (73.2 years) and the cognitive scores for the two subsets were fit to the model separately. There was no significant difference in rate of cognitive decline between the young (age at testing ≤ 73.2 years, 443 measurements, scale=1.65 years) and old (age at testing > 73.2 years, 477 measurements, scale=1.54 years) individuals. Therefore, the difference in rate of cognitive decline for *ACT 7* genotypes cannot be accounted for by the effect of age.

Analysis of ACT haplotypes with AD pathology

The *P*-values for correlation between individual SNPs and the six pathological parameters are shown in Table 5A.

Table 5A - *P*-values for single SNP Pearson correlations and pathological features in AD patients (*N*=123).

SNP	Total A-beta	A-beta 40	A-beta 42	Tau	Microglia	Astrocytosis
1	0.26	0.21	0.49	0.33	0.44	0.03
4	0.44	0.45	0.51	0.65	0.21	0.42
5	0.85	0.32	0.57	0.97	0.09	0.77
6	0.35	0.35	0.51	0.40	0.29	0.007
7	0.10	0.29	0.14	0.54	0.86	0.004

ACT 6 and *ACT 7* were both correlated with astrocytosis ($P=0.007$ and $P=0.004$ respectively). Haplotype score tests were performed for all SNP combinations and each pathological feature. Global *P*-values for all 2-SNP combinations are shown in Table 5B.

Table 5B Global *P*-values from haplotype score tests for all 2-SNP combinations versus pathological features in AD patients (*N*=123).

SNPs	Total A-beta	A-beta 40	A-beta 42	Tau	Microglia	Astrocytosis
1, 4	0.07	0.60	0.11	0.66	0.28	0.06
1, 5	0.34	0.61	0.36	0.40	0.21	0.23
1, 6	0.16	0.43	0.27	0.58	0.80	0.04
1, 7	0.07	0.13	0.24	0.96	0.51	0.007
4, 5	0.14	0.84	0.14	0.93	0.46	0.40
4, 6	0.08	0.51	0.08	0.97	0.29	0.03
4, 7	0.07	0.08	0.10	0.92	0.56	0.03
5, 6	0.32	0.72	0.25	0.44	0.27	0.04
5, 7	0.53	0.10	0.63	0.89	0.37	0.003
6, 7	0.15	0.15	0.32	0.97	0.40	0.004

Although no SNP combination showed association with A β (total, A β 40 or A β 42), tau load, or proportion of microglial cells a strong association was observed between the SNP combination *ACT* 5 & 7 and astrocytosis (global $P=0.003$, global simulated $P=0.003$, maximum score statistic=0.002). No associations were observed between any 3-, 4- or 5-SNP combination and any pathological feature (data available in supplementary information). Table 5C shows the haplotype frequencies for the *ACT* 5 & 7 combination in individuals grouped by astrocytosis severity.

Table 5C - Haplotype frequencies of the *ACT* 5 and 7 combination associated with the degree of astrocytosis. (P -values in B and C are corrected for confounding variables)

Haplotype		Haplotype Score	Haplotype frequency (%)			Haplotype-specific P -value
<i>ACT</i> 5	<i>ACT</i> 7		Absent/Mild astrocytosis	Moderate astrocytosis	Severe astrocytosis	
G	G	-2.2	36.3	30.5	17.1	0.03
A	G	-1.1	8.4	4.0	6.5	0.28
A	C	0.2	45.6	52.9	44.9	0.87
G	C	3.4	9.7	12.6	31.5	0.0006
Number of patients			38	29	36	

The haplotype showing the strongest association was *ACT* 5 G/ *ACT* 7 C (global $P=0.0006$ and global simulated $P=0.0004$) and was more frequent in patients with severe astrocytosis (31.5%) compared with moderate (12.6%) and absent/mild (9.7%) astrocytosis. This 2-SNP combination was fitted to a dominant effect general linear model with astrocytosis. The P -values for the frequency of all haplotypes compared to *ACT* 5 A/ *ACT* 7 C in the two groups of astrocytosis severity were as follows; *ACT* 5 A/ *ACT* 7 G ($P=0.68$), *ACT* 5 G/ *ACT* 7 G ($P=0.42$) and *ACT* 5 G/ *ACT* 7 C ($P=0.003$). When fitted as an additive model the association was not as strong (*ACT* 5 G/ *ACT* 7 C $P=0.01$). There was no evidence for an *APOE* $\epsilon 4$ effect on the degree of astrocytosis (OR for possession of at least one $\epsilon 4$ allele = 1.17 (95% CI .8-1.8 $P=0.48$) or age-at-death and degree of astrocytosis.

Analysis of this 2-SNP haplotype and the quantitative pathological traits (all except astrocytosis) was replicated using the QTPHASE function of UNPHASED (data not shown). As with haplo.score, no association was seen with this haplotype and any pathological feature. Since UNPHASED cannot perform analysis on ordinal traits such as degree of astrocytosis we were unable to accurately replicate this analysis using UNPHASED.

Discussion

These surprising findings implicate an intronic SNP of the *ACT* gene (rs8004988, *ACT* 7) as a modifier of Alzheimer's disease (AD) clinical and pathological indices. Analysis of cognitive decline in AD patients revealed that possession of one or two copies of the *ACT* 7 C allele predicted a faster rate of cognitive decline but at a later age than GG homozygotes. Although the inventors do not wish to be bound by any hypothesis, they believe that, since cognitive decline is directly correlated with neuronal death, the association of the *ACT* 7 C allele with a later age of onset for cognitive decline possibly indicates a potential protective role of this SNP, albeit that the rate of eventual decline is more rapid. Hence, the mutant *ACT* 7 G allele is suggestive of pre-disposition to AD.

In terms of AD pathology, possession of the *ACT* 7 C allele was associated with more severe astrocytosis at death in a different subset of our AD patients.

Astrocytosis can be used as a direct measure of the brain's response to A β deposition and neuronal death. It is interesting to note that the degree of

5 astrocytosis did not correlate with age-at-death or duration of illness and therefore did not reflect the severity of the disease in this study. Furthermore, the mean age-at-death of AD patients in our astrocytosis study was 72.7 (+/-10.1 years) and according to the inventor's non-linear model, patients of this age possessing at least one copy of the C allele will still be in the early stages of cognitive decline.

10 Although the inventors do not wish to be bound by any hypothesis, they believe that the higher degree of astrocytosis reflects a protective mechanism in some patients. Accordingly, individuals lacking the C allele, ie homozygotes for the G allele will show increased pre-disposition to AD.

15 Surprisingly, the association with severe astrocytosis was strongest in individuals possessing a G allele at the *ACT* signal sequence polymorphism, *ACT* 5 (rs4934), and the C allele at *ACT* 7. Pertinently, a potential function of this 2-SNP haplotype can be inferred, since the G-allele at *ACT* 5 correlates with an increased secretion of mature glycosylated ACT (Nilsson *et al.*, 2001b, *Neurochemistry International*.

20 39, 361-70). Hence, the inventors believe that possession of the *ACT* 5 G/*ACT* 7 C haplotype could result in an increased secretion of mature glycosylated ACT. The *ACT* 7 SNP has no known function to-date. Evidence from animal models has shown that elevated ACT in the brain may play a detrimental role in AD pathophysiology. The inventors suggest that this may be why the cognitive decline

25 in patients possessing one or two copies of the *ACT* 7 C allele was more rapid than GG homozygotes, albeit much later; increased astrocyte secretion of ACT in the brain of AD patients may eventually lead to a more aggressive disease. An alternative explanation could be that one or both of these *ACT* SNPs are in LD with disease-associated polymorphisms in other genes nearby, rather than the

30 *ACT* gene, and that these SNPs are simply acting as 'proxies' for this association. These SNPs are acting as markers for the association and the 'genuine' functional SNP is nearby

In many previous studies, the potential association between *ACT* SNPs and AD has centred on single SNP analyses. However, the inventors suggest that by looking at single SNPs, additive and/or opposing associations with other SNPs are being ignored, and may in part explain some of the conflicting data reported in the past. Such additive or opposing actions may be accounted for by haplotype analysis. The present study, based on 1,087 samples, represents the most comprehensive haplotype analysis of *ACT* in AD to date. This approach has 80% power to detect a genotype relative risk of 1.5 to 2.1 associated with alleles or haplotypes with a frequency $\geq 5\%$ at a level of statistical significance of ≤ 0.01 .

Since the inventors did not find any single SNP or haplotype associations with disease in the whole AD dataset, or separately in EOAD and LOAD, it could be concluded that *ACT* gene variation has little or no association with AD. It is also possible that complex genetic diseases, such as AD, are characterised by multiple gene haplotypes each having a subtle, but significant effect, of a magnitude similar to that observed in this study. However, collectively, they may demonstrate synergy.

CLAIMS

1. A method of identifying the existence of, or susceptibility to, neurodegenerative disorder in a subject, the method comprising:-
- 5 (i) obtaining a sample from a subject; and
 (ii) detecting, in the sample, the presence of a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) gene, wherein the polymorphism is associated with neurodegenerative disorder.
- 10 2. A method according to claim 1, wherein the neurodegenerative disorder includes Parkinson's disease; Lewis body dementia; Fronto Temporal degeneration (FTD); Picks disease; or normal aging, which results in gradual decline of cognitive function or performance.
- 15 3. A method according to either claim 1 or claim 2, wherein the neurodegenerative disorder is Alzheimer's disease.
4. A method according to any preceding claim, wherein the neurodegenerative disorder is early-onset Alzheimer's disease.
- 20 5. A method according to any preceding claim, wherein the polymorphism which is detected comprises the G allele at marker rs8004988 in intron 2 of the *ACT* gene.
6. A method according to any preceding claim, wherein the method comprises
- 25 determining whether the subject is homozygous or heterozygous for alleles at the genetic polymorphism of intron 2 of the *ACT* gene, or other regions genetically linked thereto.
7. A method according to any preceding claim, wherein the method comprises
- 30 detecting for the presence of a second genetic polymorphism, wherein the second polymorphism is independently selected from a group of genetic polymorphisms consisting of :-

- (i) at nucleotide position -12799 of the ACT gene (G/T) (referred to herein as ACT 1); (ii) at nucleotide position -596 of the ACT gene (G/A) (referred to herein as ACT 2); (iii) at nucleotide position -501 of the ACT gene (A/G) (referred to herein as ACT 3); (iv) at nucleotide position -51 of the ACT gene (G/T) (referred to herein as ACT 4); (v) at nucleotide position 2076 of the ACT gene (A/G) (referred to herein as ACT 5); (vi) at nucleotide position 2354 of the ACT gene (A/G) (referred to herein as ACT 6); (vii) at nucleotide position 6980 of the ACT gene (C/T) (referred to herein as ACT 8); (viii) at nucleotide position 10074 of the ACT gene (A/G) (referred to herein as ACT 9); (ix) at nucleotide position 10120 of the ACT gene (G/A) (referred to herein as ACT 10); (x) at nucleotide position 10167 of the ACT gene (G/T) (referred to herein as ACT 11); (xi) at nucleotide position 11998 of the ACT gene (A/C) (referred to herein as ACT 12); and (xii) at nucleotide position 12013 of the ACT gene (C/T) (referred to herein as ACT 13).
8. A method according to any preceding claim, wherein the second polymorphism, which is detected comprises the G allele at nucleotide position 2076 of the ACT gene (ACT 5).
9. A method according to any preceding claim, wherein the method is carried out *in vitro* on a bodily sample from the test subject.
10. A method according to claim 9, wherein the sample which is tested comprises nucleic acid.
11. A method according to either claim 9 or claim 10, wherein the sample comprises at least intron 2 of the ACT gene, and ideally nucleotide 6738 of intron 2 of the ACT gene (ACT 7).
12. A method according to any one of claims 9 to 11, wherein the sample comprises at least exon 2 of the ACT gene and, ideally nucleotide 2076 of exon 2 of the ACT gene (ACT 5).

13. A method according to any preceding claim, wherein said detecting step comprises amplifying at least intron 2 of the ACT gene and, ideally nucleotide +6738 of the ACT gene, and identifying the allele encoded by said amplified DNA.

5 14. A method according to any preceding claim, wherein said detecting step comprises amplifying at least exon 2 of the ACT gene and, ideally nucleotide +2076 of the ACT gene, and identifying the allele encoded by said amplified DNA.

10 15. A method according to any preceding claim, wherein said detecting comprises use of at least one oligonucleotide operable to be used for amplification of intron 2 of the ACT gene.

16. A method according to claim 15, wherein the amplification step employs at least one primer comprising substantially the nucleotide sequence as set out as
15 SEQ ID No: 59 (5'-ctatgagggactctgggcactt-3'), or SEQ ID No: 60 (5'-cgccctgctcgaccct-3') when detecting for the ACT 7 polymorphism.

17. A method according to any preceding claim, wherein said detecting comprises use of at least one oligonucleotide operable to be used for amplification of exon 2 of
20 the ACT gene.

18. A method according to claim 17, wherein the amplification step employs at least one primer comprising substantially the nucleotide sequence as set out as
SEQ ID No: 51 (5'-agctttgctttcagagttgagaat-3'), or SEQ ID No: 52 (5'-
25 ctcgtcaagtgaggctgttagg-3') when detecting for the ACT 5 polymorphism.

19. A method according to any one of claims 13 to 18, wherein said detecting comprises subjecting the amplified DNA to size analysis.

30 20. A method according to any one of claims 13 to 19, wherein the detecting comprises a step of probing the product of the amplification step with a first probe which is adapted to bind to one of the alleles of the genetic polymorphism.

21. A method according to either claim 21, wherein the probing step employs at least one probe comprising substantially the nucleotide sequence as set out as SEQ ID No: 61 (5'- cttccgcagcagtg-3'), or SEQ ID No: 62 (5'- ctccccagcagtg-3') when detecting for the ACT 7 polymorphism.

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22. A method according to either claim 20 or claim 21, wherein the method comprises probing the product of the amplification step with a second probe which is adapted to bind to the other of the alleles of that genetic polymorphism.

10 23. A method according to any one of claims 13 to 22, wherein the detecting comprises a step of probing the product of the amplification step with a first probe which is adapted to bind to one of the alleles of the second genetic polymorphism.

15 24. A method according to either claim 23, wherein the probing step employs at least one probe comprising substantially the nucleotide sequence as set out as SEQ ID No: 53 (5'- tacctctcctggctct-3'), or SEQ ID No: 54 (5'- tggctacctctcctgactc-3') when detecting for the ACT 5 polymorphism.

20 25. A kit for identifying the existence of, or susceptibility to, neurodegenerative disorder in a subject, the kit comprising means for detecting the presence of a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) in the subject, wherein the polymorphism is associated with neurodegenerative disorder.

25 26. A kit according to claim 25, the kit adapted to carry out the method according to any one of claims 1 to 24.

27. A kit according to either claim 25 or 26, wherein the kit comprises means for detecting the polymorphism in intron 2 of the *ACT* gene, and ideally, the nucleotide +6738 of intron 2 of the *ACT* gene (*ACT* 7).

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28. A kit according to any one of claims 25 to 27, wherein the kit comprises means for detecting for the presence of a second genetic polymorphism, wherein the

second polymorphism is independently selected from a group of genetic polymorphisms consisting of :-

- (i) at nucleotide position -12799 of the ACT gene (G/T) (referred to herein as ACT 1); (ii) at nucleotide position -596 of the ACT gene (G/A) (referred to herein as ACT 2); (iii) at nucleotide position -501 of the ACT gene (A/G) (referred to herein as ACT 3); (iv) at nucleotide position -51 of the ACT gene (G/T) (referred to herein as ACT 4); (v) at nucleotide position 2076 of the ACT gene (A/G) (referred to herein as ACT 5); (vi) at nucleotide position 2354 of the ACT gene (A/G) (referred to herein as ACT 6); (vii) at nucleotide position 6980 of the ACT gene (C/T) (referred to herein as ACT 8); (viii) at nucleotide position 10074 of the ACT gene (A/G) (referred to herein as ACT 9); (ix) at nucleotide position 10120 of the ACT gene (G/A) (referred to herein as ACT 10); (x) at nucleotide position 10167 of the ACT gene (G/T) (referred to herein as ACT 11); (xi) at nucleotide position 11998 of the ACT gene (A/C) (referred to herein as ACT 12); and (xii) at nucleotide position 12013 of the ACT gene (C/T) (referred to herein as ACT 13).

29. A kit according to any one of claims 25 to 28, wherein the kit comprises means for detecting a second polymorphism at nucleotide position 2076 of the ACT gene (A/G) (referred to herein as ACT 5).

30. A kit according to any one of claims 25 to 29, wherein said kit comprises at least one oligonucleotide comprising a sequence operable to be used for amplification of intron 2 of the ACT gene.

31. A kit according to any one of claims 25 to 30, wherein the kit comprises at least one primer comprising substantially the nucleotide sequence as set out as SEQ ID No: 59 (5'-ctatgagggactctgggcactt-3'), or SEQ ID No: 60 (5'-cgccctgctcgaccct-3') when detecting for the ACT 7 polymorphism.

32. A kit according to any one of claims 25 to 31, wherein the kit comprises at least one oligonucleotide operable to be used for amplification exon 2 of the ACT gene.

33. A kit according to any one of claims 25 to 32, wherein the kit comprises at least one primer comprising substantially the nucleotide sequence as set out as SEQ ID No: 51 (5'-agctttgcttttcagagttgagaat-3'), or SEQ ID No: 52 (5'-ctcgtcaagtgggctgttagg-3') when detecting for the ACT 5 polymorphism.

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34. A kit according to any one of claims 30 to 33, wherein the kit comprises means for probing the product of the amplification step to determine the genotype of the subject under test.

10 35. A kit according to any one of claims 25 to 28, wherein the kit comprises a first probe which is adapted to bind to one of the alleles of the genetic polymorphism, and a second probe which is adapted to bind to the other of the alleles of the genetic polymorphism.

15 36. A kit according to claim 35, wherein the first probe comprises substantially the nucleotide sequence as set out as SEQ ID No: 61 (5'-cttccgcagcagtg-3'), or SEQ ID No: 62 (5'-ctccccagcagtg-3') when detecting for the ACT 7 polymorphism.

20 37. A kit according to either claim 35 or claim 36, wherein the second probe comprises substantially the nucleotide sequence as set out as SEQ ID No: 53 (5'-tacctctcctggctct-3'), or SEQ ID No: 54 (5'-tgttacctctcctgactc-3') when detecting for the ACT 5 polymorphism.

25 38. A kit according to any one of claims 25 to 37, wherein the kit comprises a control sample, which comprises one or more alleles corresponding to the genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*), wherein existence of the mutant allele in the test subject suggests that the patient is suffering from, or is susceptible to neurodegenerative disorder.

30 39. A method of treating a subject suffering from, or susceptible to, neurodegenerative disorder, the method comprising:-

- (i) detecting the presence of a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) gene, wherein the polymorphism is associated with neurodegenerative disorder; and
- (ii) administering to the subject a therapeutic agent that prevents, reduces or delays progression of neurodegenerative disorder.

40. A method according to claim 39, wherein the therapeutic agent includes an anticholinesterase inhibitor.

41. A method of screening for progression of neurodegenerative disorder in a subject, the method comprising detecting the presence or absence of a Guanine allele of a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) gene in the subject, wherein presence of the Guanine allele of the polymorphism in the *ACT* gene is indicative of progression of neurodegenerative disorder.

42. A method of identifying an allele associated with susceptibility to neurodegenerative disorder, said method comprising identifying an allele which is in linkage disequilibrium with a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) gene, wherein the polymorphism is associated with neurodegenerative disorder.

43. Use of nucleic acid comprising a genetic polymorphism in intron 2 of alpha-1-antichymotrypsin (*ACT*) gene, or a peptide encoded thereby, for the preparation of a medicament for the treatment of neurodegenerative disorder.

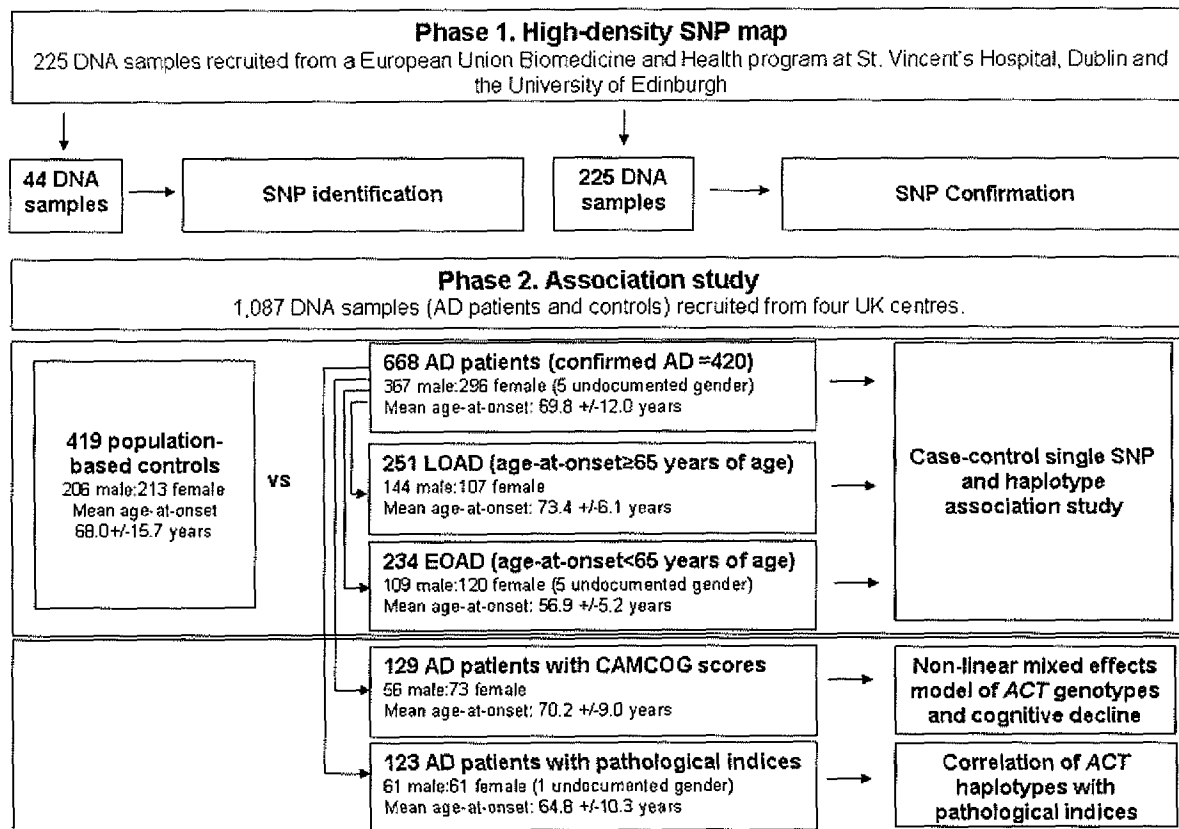
44. Use according to claim 43, wherein the treatment comprises retarding or preventing the disease, such as Alzheimer's disease.

45. Use according to either claim 43 or claim 44, wherein the nucleic acid comprises a nucleotide sequence substantially as set out in SEQ ID No: 29 (5'-tctgggcacttccactgctg-3') and/or SEQ ID No: 30 (5'-ggaagcagggctcgagcaggg-3').

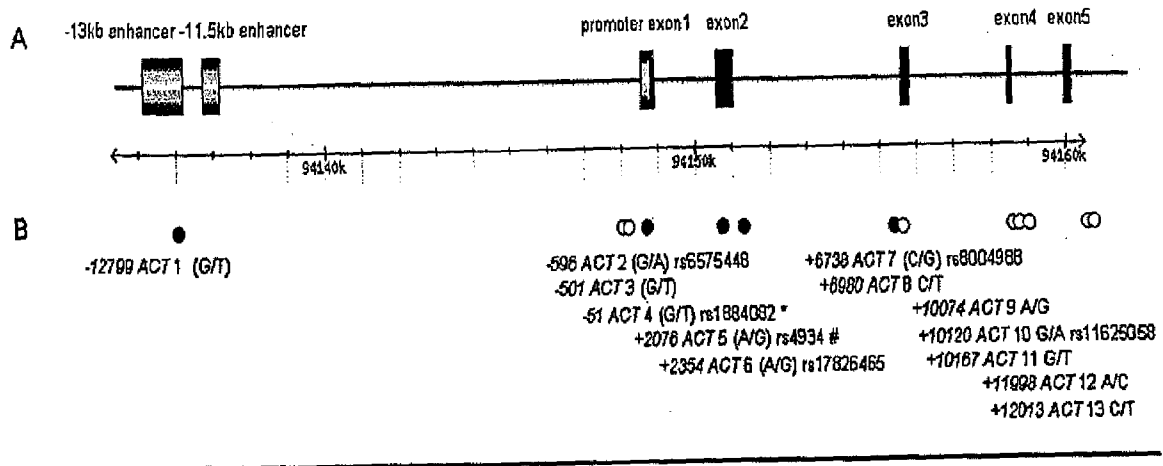
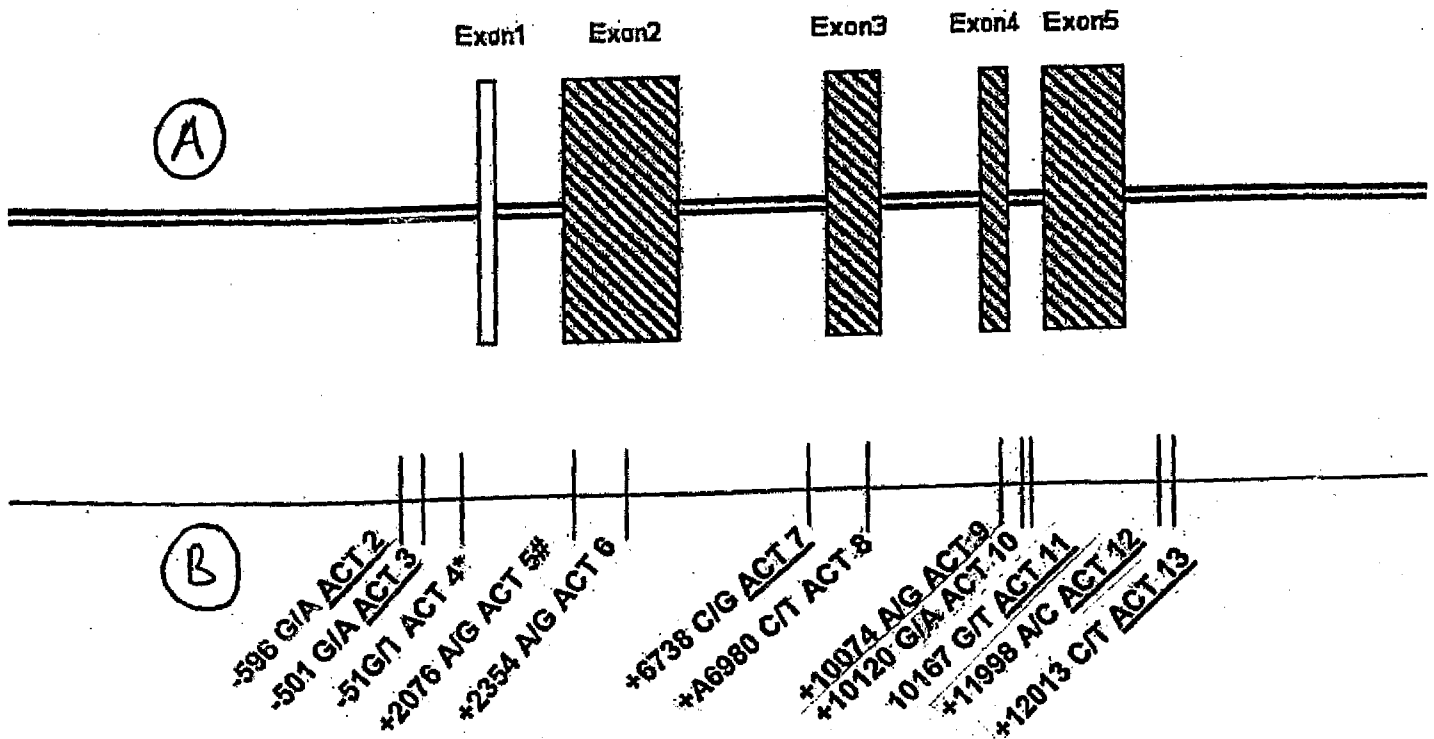
46. Use according to any one of claims 43 to 45, comprising use of at least a second nucleic acid comprising a second genetic polymorphism, or a peptide encoded thereby, wherein the second polymorphism is independently selected from a group of polymorphisms consisting of :-

- 5 (i) at nucleotide position -12799 of the ACT gene (G/T) (referred to herein as ACT 1); (ii) at nucleotide position -596 of the ACT gene (G/A) (referred to herein as ACT 2); (iii) at nucleotide position -501 of the ACT gene (A/G) (referred to herein as ACT 3); (iv) at nucleotide position -51 of the ACT gene (G/T) (referred to herein as ACT 4); (v) at nucleotide position 2076 of the ACT gene (A/G) (referred to
10 herein as ACT 5); (vi) at nucleotide position 2354 of the ACT gene (A/G) (referred to herein as ACT 6); (vii) at nucleotide position 6980 of the ACT gene (C/T) (referred to herein as ACT 8); (viii) at nucleotide position 10074 of the ACT gene (A/G) (referred to herein as ACT 9); (ix) at nucleotide position 10120 of the ACT gene (G/A) (referred to herein as ACT 10); (x) at nucleotide position 10167 of the
15 ACT gene (G/T) (referred to herein as ACT 11); (xi) at nucleotide position 11998 of the ACT gene (A/C) (referred to herein as ACT 12); and (xii) at nucleotide position 12013 of the ACT gene (C/T) (referred to herein as ACT 13).

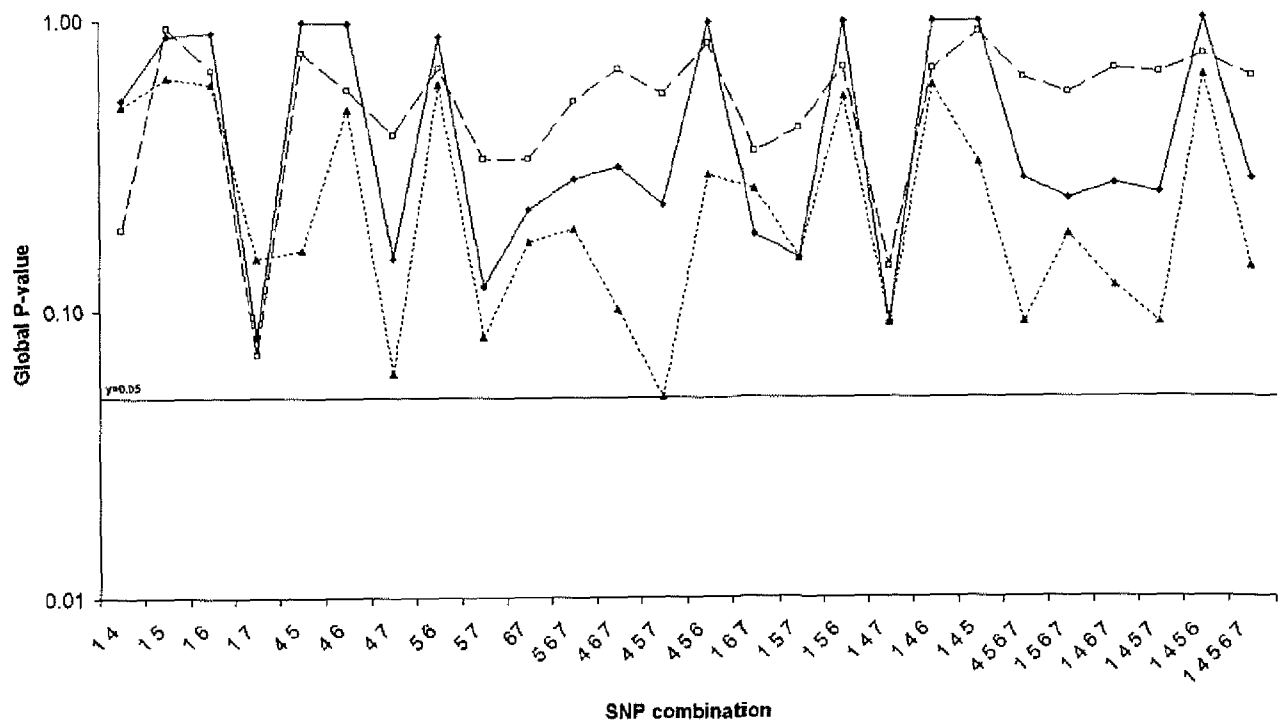
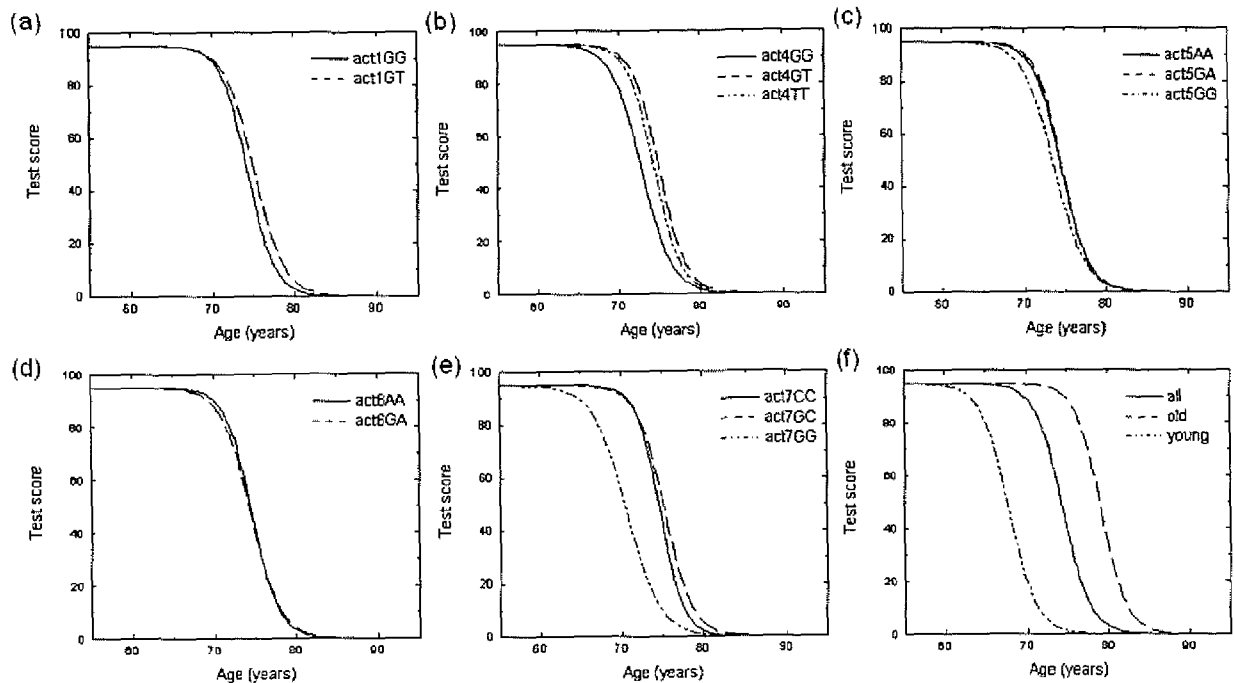
47. Use according to claim 46, wherein the second nucleic acid comprises a
20 nucleotide sequence independently selected from a group of nucleotides consisting of: SEQ ID No: 17; SEQ ID No: 18; SEQ ID No: 19; SEQ ID No: 20; SEQ ID No: 21; SEQ ID No: 22; SEQ ID No: 23; SEQ ID No: 24; SEQ ID No: 25; SEQ ID No: 26; SEQ ID No: 27; SEQ ID No: 28; SEQ ID No: 29; SEQ ID No: 30; SEQ ID No: 31; SEQ ID No: 32; SEQ ID No: 33; SEQ ID No: 34; SEQ ID No: 35;
25 SEQ ID No: 36; SEQ ID No: 37; SEQ ID No: 38; SEQ ID No: 39; SEQ ID No: 40; SEQ ID No: 41; and SEQ ID No: 42.

Figure: 1

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Figure: 2**Figure: 3**

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Figure: 4**Figure 5**

SNP	Region	Position		5' flanking sequence	3' flanking sequence	Minor allele frequency	
		From TSS	Contig			N = 44	N = 225
ACT 1	5' enhancer	-12799 G/T	44695	ctgggcctcctagctgcaga (SEQ ID No.17)	attggtggggtgtgcaga (SEQ ID No.18)	0.14	0.09
ACT 2	Promoter	-596 G/A	56898	gatacagaggtcaggagttc (SEQ ID No.19)	agaccagtcggaccaacaag (SEQ ID No.20)	0.41	---
ACT 3	Promoter	-501 G/A	56993	gccctagtcacagctactc (SEQ ID No.21)	ggaggctgaggcagaaagt (SEQ ID No.22)	0.02	---
ACT 4	Promoter	-51 G/T	57443	tctggcaattcccaagcag (SEQ ID No.23)	gggaggagtctctgcagga (SEQ ID No.24)	0.44	0.49
ACT 5	Exon 2	2076 A/G	59569	agagaatgtaccctctctg (SEQ ID No.25)	ctctggggtcttggcggct (SEQ ID No.26)	0.41	0.46
ACT 6	Exon 2	2354 A/G	59847	accctgacagagattctcaa (SEQ ID No.27)	ggcctcaagtcaacctcac (SEQ ID No.28)	0.14	0.09
ACT 7	Intron 2	6738 C/G	64231	tctgggcacttcacacgctg (SEQ ID No.29)	ggaagcagggtcagagcagg (SEQ ID No.30)	0.49	0.43
ACT 8	Exon 3	6980 C/T	64473	aagtaacacaggcaatgccag (SEQ ID No.31)	gcactctcatctctcctga (SEQ ID No.32)	0.06	0.04
ACT 9	Exon 4	10074 A/G	67567	gctgacctgtcagggaatcac (SEQ ID No.33)	ggggccaggaaaccttagcagt (SEQ ID No.34)	0.01	---
ACT 10	Intron 4	10120 G/A	67613	aggtagtcttagacttgg (SEQ ID No.35)	tcaattcatcttctgtatcc (SEQ ID No.36)	0.03	---
ACT 11	Intron 4	10167 G/T	67660	gaattgtgaaggccagatg (SEQ ID No.37)	actttgggtactcttgaac (SEQ ID No.38)	0.03	---
ACT 12	3' FR	11998 A/C	69491	gagctctagccctgaccac (SEQ ID No.39)	tctggcacagcccc(c/t)ggact (SEQ ID No.40)	0.01	---
ACT 13	3' FR	12013 C/T	69506	accac(a/c)tctggcacagcccc (SEQ ID No.41)	ggactctgtctctgcagc (SEQ ID No.42)	0.02	---

Figure 6

Figure 7

Polymorphism		Forward primer	Reverse Primer	Vic Probe	Fam Probe
Name	Position				
ACT 1	-12799 G/T	agggagggtccggctcta (SEQ ID No.43)	agaccccccttctggacaa (SEQ ID No.44)	ctagctgcagagatt (SEQ ID No.45)	ctagctgcagatatt (SEQ ID No.46)
ACT 4	-51 G/T	cacttggtgtcctggcaatt (SEQ ID No.47)	tgctggagtagtggatttcaiga (SEQ ID No.48)	actctccccctgct (SEQ ID No.49)	tgaactctctccactgc (SEQ ID No.50)
ACT 5	2076 A/G	agctttgcttttcagagttgagaat (SEQ ID No.51)	ctcgcaagtgggcgtgttagg (SEQ ID No.52)	tacctctctctggctct (SEQ ID No.53)	tgttacctctctgactc (SEQ ID No.54)
ACT 6	2354 A/G	gcccataataccaccctgaca (SEQ ID No.55)	tcagaagctctccgtgagggtgaa (SEQ ID No.56)	tcctcaaggcctcaag (SEQ ID No.57)	tcctcaaggcctcaa (SEQ ID No.58)
ACT 7	6738 C/G	ctatgagggaactctgggcactt (SEQ ID No.59)	cgccctgtctgacct (SEQ ID No.60)	cttccgcagcagtg (SEQ ID No.61)	ctccccagcagtg (SEQ ID No.62)