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(54) CIRCULATING SERUM MICRORNA BIOMARKERS AND METHODS FOR DETERMINING PARKINSON'S DISEASE

ZIRKULIERENDE SERUM MIKRORNA BIOMARKER UND VERFAHREN ZUR BESTIMMUNG VON PARKINSON'SCHER KRANKHEIT

MIRNA LIBRES SÉRIQUES BIOMARQUEURS ET PROCÉDÉS DE DÉTERMINATION DE LA MALADIE DE PARKINSON

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Remarks:

The complete document including Reference Table(s) and the Sequence Listing(s) can be downloaded from the EPO website

Description

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0001] The present invention relates to methods for determining Parkinson's disease in human patients.

2. Brief Description of the Background Art

[0002] Parkinson's Disease (PD) is a highly specific degeneration of dopamine-containing cells of the substantia nigra of the midbrain, causing a dopamine deficiency in the striatum. PD currently affects about 10 million people worldwide. Effective management of a patient with PD is possible in the first 5-7 years of treatment, after which time a series of often debilitating complications, together referred to as Late Motor Fluctuations (LMF), occur. It is believed that treatment with levodopa ((-)-L-α-amino-beta-(3,4-dihydroxybenzene) propanoic acid), or L-dopa, the most effective anti-Parkinson drug, may facilitate or even promote the appearance of LMF. Dopamine agonists are employed as a treatment alternative, but they do not offer the same degree of symptomatic relief to patients as L-dopa does.

[0003] Symptomatic therapies improve signs and symptoms without affecting the underlying disease state. Levodopa increases dopamine concentration in the striatum, especially when its peripheral metabolism is inhibited by a peripheral decarboxylase inhibitor (PDI). Levodopa/PDI therapy is widely used for symptomatic therapy for Parkinson's disease, such as combinations with levodopa, with carbidopa ((-)-L-α-hydrazino-α-methyl-beta-(3,4-dihydroxybenzene) propanoic acid monohydrate), levodopa and controlled release carbidopa, levodopa and benserazide, levodopa plus controlled release benserazide (2-Amino-3-hydroxy-propionic acid N'-(2,3,4-trihydroxy-benzyl)-hydrazide).

[0004] Catechol-O-methyltransferase (COMT) inhibitors enhance levodopa treatment as they inhibit levodopa's metabolism, enhancing its bioavailability and thereby making more of the drug available in the synaptic cleft for a longer period of time. Examples of COMT inhibitors include tolcapone (3,4-dihydroxy-4'-methyl-5-nitrobenzophenone) and entacapone ((E)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-N,N-diethyl-2-propenamide).

[0005] Dopamine agonists provide symptomatic benefit by directly stimulating post-synaptic striatal dopamine receptors. Examples include bromocriptine ((5α)-2-Bromo-12'-hydroxy-2'-(1-methylethyl)-5'-(2-methylpropyl)erg- otaman-3',6',18-trione), pergolide (8B-[(Methylthio)methyl]-6-propylergoline), ropinirole (4-[2-(Dipropylamino)ethyl]-1,3-dihydro-2H-indol-2-one), pramipexole ((S)-4,5,6,7-Tetrahydro-N^6-propyl-2,6-benzothiazolediamine), lisuride (N'-[(8α)-9,10-didehydro-6-methylergolin-8-yl]-N,N-diethyl- urea), cabergoline ((8β)-N-[3-(Dimethylamino)propyl]-N-[(ethylamino)carbonyl]-6-(2-propenyl)ergoline-8-carboxamide), apomorphine ((6aR)-5,6,6a,7-Tetrahydro-6-methyl-4 H-dibenzo[de,g]quinoline-10,11-diol), sumanirole (5-(methylamino)-5,6-dihydro-4H-imidazo {4,5,1-ij}quinolin-2(1H)-one), rotigotine ((-)(S)-5,6,7,8-tetrahydro-6-[propyl[2-(2-thienyl)ethyl]amino]-1-naphthol-), talipexole (5,6,7,8-Tetrahydro-6-(2-propenyl)-4H-thiazolo[4,5-d]azepin-2-amine), and dihydroergocriptine (ergotaman-3 ',6', 18-trione,9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl) (5' cc)). Dopamine agonists are effective as monotherapy early in the course of Parkinson's disease and as an adjunct to levodopa in more advanced stages. Unlike levodopa, dopamine agonists directly stimulate post-synaptic dopamine receptors. They do not undergo oxidative metabolism and are not thought to accelerate the disease process.

[0006] Amantidine (1-Aminotricyclo (3,3,1,$1^{3,7}$) decane) is an antiviral agent that was discovered by chance to have anti-Parkinsonian activity. Its mechanism of action in PD has not been established, but is believed to work by increasing dopamine release. Patients who receive amantidine either as monotherapy or in combination with levodopa show improvement in akinesia, rigidity and tremor.

[0007] Other medications used in the treatment of Parkinson's disease include MAO-B inhibitors. Inhibition of L-dopa metabolism through inactivation of the monoamino oxidase type B (MAO-B) is an effective means of enhancing the efficacy of both endogenous residual dopamine and that exogenously derived from its precursor, L-dopa. Selegiline (methyl-(1-methyl-2-phenyl-ethyl)-prop-2-ynyl-amine) is a MAO-B inhibitor. There is evidence that treatment with selegiline may slow down disease progression in PD by blocking formation of free radicals derived from the oxidative metabolism of dopamine. Other examples of MAO B inhibitors include lazabemide (N-(2-Aminoethyl)-5-chloro-2-pyridinecarboxamide), rasagiline (N-propargyl-1-(R)aminoindan and caroxazone (2-oxo-2H-1,3-benzoxazine-3(4H)-acetamide).

[0008] It is imperative to diagnose individuals with PD at an early stage to increase the efficacy of therapeutic agents. However, there are neither any objective tests nor established biomarkers for diagnosing PD. Moreover, the heterogeneity, subtypes and progression of the disease make it difficult to develop specific therapeutic candidates.

[0009] MicroRNAs ("miRNAs) are a class of non-coding RNAs that play key roles in the regulation of gene expression. miRNAs act at the post-transcriptional level and fine-tune the expression of as much as 30% of all mammalian protein-encoding genes. Mature miRNAs are short, single-stranded RNA molecules approximately 22 nucleotides in length.

miRNAs may be encoded by multiple loci, and may be organized in tandemly co-transcribed clusters. miRNA genes are transcribed by RNA polymerase II as large primary transcripts (pri-microRNA) that are processed by a protein complex containing the RNase III enzyme Drosha, DGCR8 and other cofactors, to form an approximately 70 nucleotide precursor microRNA (pre-miRNA). (Cathew RW, Cell, 2009; Kim VN, Nat Rev Mol Cel Biol, 2009; Siomi H, Mol Cel, 2010; Bartel DP, Cell, 2004; Lee Y, Nature 2003; Han J, Genes Dev, 2004.) Pre-miRNA is transported to the cytoplasm by Exportin-5 where it is processed by DICER, a second RNase III enzyme, together with TRBP, PACT and Ago2 in the RNA Induced Silencing Complex resulting in miRNA duplexes (Kim VN, Nat Rev Mol Cel Biol, 2009; Gregory RI, Nature 2004; MAcRae IJ, PNAS, 2008). The guide strands of miRNA duplexes separate and associate with Ago 2 for incorporation into a ribonuclear particle to form the RNA-induced silencing complex RISC that mediates gene silencing. The mechanisms of miRNA range from direct degradation or silencing of mRNA and repression of translation to post-transcriptional upregulations. (MacRae IJ, PNAS, 2008.)

[0010] The presence of miRNAs has been reported in body fluids including blood, cerebrospinal fluid (CSF), plasma, serum and saliva at detectable levels. The tissue-specificity of miRNAs suggests their vital and integral role in various physiological processes. The tissue-enrichment promises a new but less explored role as diagnostic biomarker and potential therapeutic target. Circulating miRNAs are understood to originate from passive leakage from damaged tissue as a result of cell lysis or apoptosis, active transport from cells via microvesicles, such as exosomes, or bound within RISC protein complexes (Etheridge et al, 2011). Exosome and osmotic pump-mediated delivery of small RNA molecules to the brain and CNS, respectively, provides a solution to overcoming the limitations of miRNA-based therapies (Alvarez-Erviti et al., 2011; Koval et al, 2013, Hum. Mol. Gen). miRNA has been demonstrated to be exceptionally stable and thus present as powerful candidates to be potential biomarkers (Chen et al, 2008; Grasso, 2014).

SUMMARY OF THE INVENTION

[0011] It is an object of the present invention to identify miRNAs relevant to patients suffering from Parkinson's disease.

[0012] It is another object of the present invention to provide methods for determining patients suffering from Parkinson's disease.

[0013] These objects and others are achieved by the present invention, which provides miRNA biomarkers that may be used in pairs or in combination to determine patients suffering from Parkinson's disease. The scope of the invention is defined by the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014]

Figure 1 shows the mean fold change of three PARKmiRNAs between PD patients and healthy controls;
Figure 2A is a ROC analysis based on predicted probabilities from the model and compared to true disease status;
Figure 2B is a ROC analysis based on predicted prohibition from the model;
Figure 3 is a ROC analysis based on predicted probabilities from the model and compared to true disease status;
Figure 4 is a ROC analysis based on predicted probabilities from the model and compared to true disease status;
Figure 5 is a ROC analysis based on predicted probabilities from the model and compared to true disease status;
Figure 6(A)-(H) illustrate microRNAs targeting Parkinson's Disease proteins;
Figure 7 is a ROC analysis based on predicted probabilities from the model and compared to true disease status;
Figure 8 is a ROC analysis based on predicted probabilities from the model and compared to true disease status;
Figure 9 is a ROC analysis based on predicted probabilities from the model and compared to true disease status; and
Figure 10 is a ROC analysis based on predicted probabilities from the model and compared to true disease status.

DETAILED DESCRIPTION OF THE INVENTION

METHODS

Serum samples handling and classification

[0015] All patients and controls participated in the Norwegian ParkWest project or the Swedish NYPUM study, which are ongoing prospective population-based longitudinal cohort studies investigating the incidence, neurobiology and prognosis of PD. The Norwegian ParkWest study is a prospective longitudinal multicenter cohort study of patients with incident **Park**inson's disease (PD) from **West**ern and Southern Norway. Between November 1st 2004 and 31 st of August 2006 it was endeavored to recruit all new cases of Parkinson Disease within the study area. Since the start of

the study 212 of 265 (80 %) of these patients and their age-/sex-matched control group have been followed. Further information about this project can be found at http://www.parkvest.no. The NYPUM study began in 2004 and endeavours to identify all new cases with idiopathic parkinsonism within the Umeå catchment area and follow them in their disease progression for at least five years. Further information about this study can be found at http://www.ufbi.umu.se/english/collaborations/current-projects/nypum/.

[0016] All possible efforts were undertaken to establish an unselected and population-representative cohort of patients with PD. Patients were included if they had provided serum at study entry and fulfilled diagnostic criteria for PD of the National Institute of Neurological Disorders and Stroke (http://www.ninds.nih.gov/disorders/parkinsons_disease/parkinsons_disease.ht m) and UK Brain Bank (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/GetPdf.cgi?id=phd000042) at latest follow-up. Patients with secondary parkinsonism at study entry were excluded from this study. Control subjects were recruited from multiple sources, including friends, spouses, and public organizations for elderly and were included in this study if they had provided serum. All patients and controls were Caucasian.

[0017] In this study of possible biomarkers for PD we applied a two-stage procedure. For the first discovery phase serum from 16 patients and 8 controls were selected at random. The remaining 164 patients with PD and 182 controls that were eligible for this study were selected for verification purposes.

[0018] Serum samples were collected at the same day as the clinical examinations and then stored frozen at -70 degrees Celsius until transported to the facilities in New York on dry ice.

Example 1: Analyses of differentially expressed human miRNA by qPCR

RNA Isolation from serum samples and QC

[0019] After thawing on ice, twenty-four (eight control, sixteen PD samples) serum samples were spun down for 5 mins at 3000xg to remove debris. The supernatant was used to perform small RNA isolation using miRCURY RNA Isolation Kit - Biofluids (Exiqon, MA). Before RNA Isolation, the lysis buffer was spiked with 0.267fmol/ul of spike-in control cel-miR-39-3p (Qiagen, CA). The remaining part of the RNA isolation was performed following manufacturer's protocol and the isolated RNA was quantified on a Nanodrop 2000 (Thermo Scientific, MA). The RNA was used for running Affymetrix v4 microRNA microarray chips and for subsequent cDNA synthesis and qPCR. RNA from 434 serum samples (22 control and 42 PD from NYPUM study in addition to 190 control and 180 PD from ParkWest project) was isolated as described above, they were not quantified by Nanodrop, but the qPCR data resulting from these samples were normalized by a reference small RNA scaRNA17.

miRNA microarray and data analysis

[0020] The isolated RNA from twenty-four patient serum samples were quantified and subjected to Affymetrix GeneChip® miRNA 4.0 Array by the Yale Center for Genome Analysis (http://medicine.yale.edu/keck/ycga/index.aspx). The normalized .CEL files obtained from Affymetrix Expression Console software were imported into Partek Genomics Suite version 6.6 Copyright © 2012 (Partek, MO) for analysis. The 'microRNA Expression Workflow' was employed to detect differentially expressed miRNAs employing ANOVA resulting in lists of miRNAs significantly ($p<0.05$) expressed between control versus PD cohorts. The miRNAs detected were used for further qPCR verification.

Quantitative Polymerase Chain Reaction

[0021] cDNA for miRNA specific qPCR was synthesized using qScript™ microRNA cDNA Synthesis kit (Quanta Biosciences, MD) following manufacturer's protocol and subsequent qPCRs were performed using miRNA specific forward primers (Table#) and PerfeCTa®Universal PCR primer (Quanta Biosciences, MD). scaRNA17 and U6 were used reference small RNAs for normalizing qPCR Cq values whereas cel-miR-39-3p was used as spike-in control. PerfeCTa® SYBR® GREEN SuperMix for IQ™ (Quanta Biosciences, MD) was used for all qPCRs in a MyiQ™ Single color Real-Time PCR Detection System (Bio-Rad, CA). Standard curve for cel-miR-39-3p was analyzed in MS Excel with R^2 = 0.97882 and PCR efficiency 92.96%. No Template Control (NTC) was implied wherever needed.

Data analysis based on PD model

[0022] The discriminative ability of miRNAs with regard to PD diagnosis was assessed from ROC analysis using IBM SPSS Statistics, version 21; for combinations of miRNAs the test variable was the predicted probability from logistic regression with PD diagnosis (yes/no) as outcome. To minimize the influence of outlying values on the fit, logistic regression was performed with log transformed miRNA values.

[0023] Differentially expressed human miRNAs in Parkinson's disease patients' serum samples from The Norwegian

ParkWest study were determined employing miRNA microarray. Provided below are the miRNAs with >1.2 fold differential expression.

85 differentially expressed human pre- and mature miRNAs with >1.2 fold change

[0024] hsa-miR-548ac, hsa-miR-335-5p, hsa-miR-548x-3p, hsa-miR-520g, hsa-miR-520h, hsa-miR-548ae, hsa-miR-3910-1, hsa-miR-4708-3p, hsa-miR-16-2-3p, hsa-miR-603, hsa-miR-3613-3p, hsa-miR-4797-5p, hsa-miR-548aj-3p, hsa-miR-450b-5p, hsa-miR-548ap-3p, hsa-miR-1184, hsa-miR-2277-5p, hsa-miR-1323, hsa-miR-548aa, hsa-miR-548t-3p, hsa-miR-221-5p, hsa-miR-190a-3p, hsa-miR-6873-5p, hsa-miR-155-3p, hsa-miR-510-5p, hsa-miR-4313, hsa-miR-3616, hsa-miR-8075, hsa-miR-4306, hsa-miR-6776, hsa-miR-6075, hsa-miR-8052, hsa-miR-532, hsa-miR-4791, hsa-miR-320b-1, hsa-miR-548y, hsa-miR-7973, hsa-miR-3136-5p, hsa-miR-606, hsa-miR-500a-3p, hsa-miR-4788, hsa-miR-4769-3p, hsa-miR-299-5p, hsa-miR-4431, hsa-miR-6749-5p, hsa-miR-138-2-3p, hsa-miR-1289-2, hsa-miR-548au, hsa-miR-6850, hsa-miR-561, hsa-miR-34b-5p, hsa-miR-3934-5p, hsa-miR-6739-5p, hsa-miR-4325, hsa-miR-4672, hsa-miR-215-5p, hsa-miR-4685-5p, hsa-miR-3160-1, hsa-miR-3160-2, hsa-miR-6793-5p, hsa-miR-8089, hsa-miR-6081, hsa-miR-892b, hsa-miR-936, hsa-miR-548ag, hsa-miR-345, hsa-miR-548k, hsa-miR-3188, hsa-miR-181b-5p, hsa-let-7e, hsa-miR-4487, hsa-miR-509-3p, hsa-miR-3689a-3p, hsa-miR-4771, hsa-miR-520a-5p, hsa-miR-3150b, hsa-miR-6782-5p, hsa-miR-937-5p, hsa-miR-455-3p, hsa-miR-6865-3p, hsa-miR-4749-5p, hsa-miR-378b, hsa-miR-7706, hsa-miR-4445 and hsa-miR-2355-5p.

57 differentially expressed mature miRNAs with >1.2 fold change

[0025] hsa-miR-548ac, hsa-miR-335-5p, hsa-miR-548x-3p, hsa-miR-548ae, hsa-miR-4708-3p, hsa-miR-16-2-3p, hsa-miR-603, hsa-miR-3613-3p, hsa-miR-4797-5p, hsa-miR-548aj-3p, hsa-miR-450b-5p, hsa-miR-548ap-3p, hsa-miR-1184, hsa-miR-2277-5p, hsa-miR-1323, hsa-miR-548aa, hsa-miR-548t-3p, hsa-miR-221-5p, hsa-miR-190a-3p, hsa-miR-6873-5p, hsa-miR-155-3p, hsa-miR-510-5p, hsa-miR-4313, hsa-miR-4306, hsa-miR-8052, hsa-miR-4791, hsa-miR-7973, hsa-miR-3136-5p, hsa-miR-606, hsa-miR-500a-3p, hsa-miR-4769-3p, hsa-miR-299-5p, hsa-miR-6749-5p, hsa-miR-138-2-3p, hsa-miR-34b-5p, hsa-miR-3934-5p, hsa-miR-6739-5p, hsa-miR-4325, hsa-miR-215-5p, hsa-miR-4685-5p, hsa-miR-6793-5p, hsa-miR-936, hsa-miR-548ag, hsa-miR-548k, hsa-miR-181b-5p, hsa-let-7e, hsa-miR-509-3p, hsa-miR-3689a-3p, hsa-miR-4771, hsa-miR-520a-5p, hsa-miR-6782-5p, hsa-miR-937-5p, hsa-miR-455-3p, hsa-miR-6865-3p, hsa-miR-4749-5p, hsa-miR-378b and hsa-miR-2355-5p.

28 differentially expressed premature miRNAs with >1.2 fold change

[0026] hsa-miR-520g, hsa-miR-520h, hsa-miR-3910-1, hsa-miR-3616, hsa-miR-8075, hsa-miR-6776, hsa-miR-6075, hsa-miR-532, hsa-miR-320b-1, hsa-miR-548y, hsa-miR-4788, hsa-miR-4431, hsa-miR-1289-2, hsa-miR-548au, hsa-miR-6850, hsa-miR-561, hsa-miR-4672, hsa-miR-3160-1, hsa-miR-3160-2, hsa-miR-8089, hsa-miR-6081, hsa-miR-892b, hsa-miR-345, hsa-miR-3188, hsa-miR-4487, hsa-miR-3150b, hsa-miR-7706 and hsa-miR-4445.

[0027] These differentially expressed miRNA sequences are illustrated below in Table 1, along with the reference/house-keeping small RNAs cel-miR-39-3p, U6 and ScaRNA17 used as controls. Cel-miR-39-3p is a spike-in control that demonstrates the stability of the RNA samples. U6 and ScaRNA17 are used as internal controls to normalize the readings of the rest of the miRNAs or candidate miRNAs.

Table 1

microRNA/small RNA name	microRNA Sequence
cel-miR-39-3p	UCACCGGGUGUAAAUCAGCUUG (SEQ ID NO:1)
hsa-let-7e	UGAGGUAGGAGGUUGUAUAGUU (SEQ ID NO:2)
hsa-miR-1184	CCUGCAGCGACUUGAUGGCUUCC (SEQ ID NO:3)
hsa-miR-1289-2	CCACGGUCCUAGUUAAAAAGGCACAUUCCUAGACCCUGCCUC AGAACUACUGAACAGAGUCACUGGGUGUGGAGUCCAGGAAUC UGCAUUUUUACCCCUAUCGCCCCCGCC (SEQ ID NO:4)
hsa-miR-1323	UCAAAACUGAGGGGCAUUUUCU (SEQ ID NO:5)
hsa-miR-138-2-3p	GCUAUUUCACGACACCAGGGUU (SEQ ID NO:6)
hsa-miR-155-3p	CUCCUACAUAUUAGCAUUAACA (SEQ ID NO:7)
hsa-miR-16-2-3p	CCAAUAUUACUGUGCUGCUUUA (SEQ ID NO:8)
hsa-miR-181b-5p	AACAUUCAUUGCUGUCGGUGGGU (SEQ ID NO:9)

(continued)

microRNA/small RNA name	microRNA Sequence
hsa-miR-190a-3p	CUAUAUAUCAAACAUAUUCCU (SEQ ID NO:10)
hsa-miR-215-5p	AUGACCUAUGAAUUGACAGAC (SEQ ID NO:11)
hsa-miR-221-5p	ACCUGGCAUACAAUGUAGAUUU (SEQ ID NO:12)
hsa-miR-2277-5p	AGCGCGGGCUGAGCGCUGCCAGUC (SEQ ID NO:13)
hsa-miR-2355-5p	AUCCCCAGAUACAAUGGACAA (SEQ ID NO:14)
hsa-miR-299-5p	UGGUUUACCGUCCCACAUACAU (SEQ ID NO:15)
hsa-miR-3136-5p	CUGACUGAAUAGGUAGGGUCAUU (SEQ ID NO:16)
hsa-miR-3150b	GAGGGAAAGCAGGCCAACCUCGAGGAUCUCCCCAGCCUUGGC GUUCAGGUGCUGAGGAGAUCGUCGAGGUUGGCCUGCUUCCCC UC (SEQ ID NO:17)
hsa-miR-3160-1	GGACCUGCCCUGGGCUUUCUAGUCUCAGCUCUCCUCCAGCUC AGCUGGUCAGGAGAGCUGAGACUAGAAAGCCCAGGGCAGGUU C (SEQ ID NO:18)
hsa-miR-3160-2	ACCUGCCCUGGGCUUUCUAGUCUCAGCUCUCCUGACCAGCUG AGCUGGAGGAGAGCUGAGACUAGAAAGCCCAGGGCAGGU (SEQ ID NO:19)
hsa-miR-3188	GGCGCCUCCUGCUCUGCUGUGCCGCCAGGGCCUCCCCUAGCGC GCCUUCUGGAGAGGCUUUGUGCGGAUACGGGGCUGGAGGCCU (SEQ ID NO:20)
hsa-miR-320b-1	AAUUAAUCCCUCUCUUUCUAGUUCUUCCUAGAGUGAGGAAAA GCUGGGUUGAGAGGGCAAACAAAUUAACUAAUUAAUU (SEQ ID NO:21)
hsa-miR-3 3 5-5p hsa-miR-3 45	UCAAGAGCAAUAACGAAAAAUGU (SEQ ID NO:22)
	ACCCAAACCCUAGGUCUGCUGACUCCUAGUCCAGGGCUCGUG AUGGCUGGUGGGCCCUGAACGAGGGGUCUGGAGGCCUGGGUU UGAAUAUCGACAGC (SEQ ID NO:23)
hsa-miR-34b-5p	UAGGCAGUGUCAUUAGCUGAUUG (SEQ ID NO:24)
hsa-miR-3613-3p	ACAAAAAAAAAAGCCCAACCCUUC (SEQ ID NO:25)
hsa-miR-3616	UGUCACUCCGCCAGCAUCAUGAAGUGCACUCAUGAUAUGUUU GCCCCAUCAGCGUGUCACGAGGGCAUUUCAUGAUGCAGGCGG GGUUGGCA (SEQ ID NO:26)
hsa-miR-3689a-3p	CUGGGAGGUGUGAUAUCGUGGU (SEQ ID NO:27)
hsa-miR-378b	ACUGGACUUGGAGGCAGAA (SEQ ID NO:28)
hsa-miR-3 910-1	CUUUUGCUGUCAGUUUUUCUGUUGCUUGUCUUGGUUUUAUGC CUUUUAUAUCAAGGCACAUAAAAGGCAUAAAACCAAGACAAG CAACAAAAAAAGGAUUGAUCACAGAAG (SEQ ID NO:29)
hsa-miR-3934-5p	UCAGGUGUGGAAACUGAGGCAG (SEQ ID NO:30)
hsa-miR-4306	UGGAGAGAAAGGCAGUA (SEQ ID NO:31)
hsa-miR-4313	AGCCCCCUGGCCCCAAACCC (SEQ ID NO:32)

(continued)

microRNA/small RNA name	microRNA Sequence
hsa-miR-4325	UUGCACUUGUCUCAGUGA (SEQ ID NO:33)
hsa-miR-4431	UGGUUUGCGACUCUGAAAACUAGAAGGUUUAUGACUGGGCA UUUCUCACCCAAUGCCCAAUAUUGAACUUUCUAGUUGUCAGA GUCAUUAACCC (SEQ ID NO:34)
hsa-miR-4445	UUCCUGCAGAUUGUUUCUUUUGCCGUGCAAGUUUAAGUUUUU GCACGGCAAAAGAAACAAUCCAGAGGGU (SEQ ID NO:35)
hsa-miR-4487	ACUGUCCUUCAGCCAGAGCUGGCUGAAGGGCAGAAGGGAACU GUCCUUCAGCCAGAGCUGGCUGAAGGGCAGA (SEQ ID NO:36)
hsa-miR-450b-5p	UUUUGCAAUAUGUUCCUGAAUA (SEQ ID NO:37)
hsa-miR-455-3p	GCAGUCCAUGGGCAUAUACAC (SEQ ID NO:38)
hsa-miR-4672	GGCUGCUUCUCGCCUCUGUCCAGCUGUGUGGCCUUGGACAAG CCUCUUGGUUACACAGCUGGACAGAGGCACGAAACAGCC (SEQ ID NO:39)
hsa-miR-4685-5p	CCCAGGGCUUGGAGUGGGGCAAGGUU (SEQ ID NO:40)
hsa-miR-4708-3p	AGCAAGGCGGCAUCUCUCUGAU (SEQ ID NO:41)
hsa-miR-4749-5p	UGCGGGGACAGGCCAGGGCAUC (SEQ ID NO:42)
hsa-miR-4769-3p	UCUGCCAUCCUCCCUCCCCUAC (SEQ ID NO:43)
hsa-miR-4771	AGCAGACUUGACCUACAAUUA (SEQ ID NO:44)
hsa-miR-4788	AAUGAAGGAUUACGGACCAGCUAAGGGAGGCAUUAGGAUCCU UAUUCUUGCCUCCCUUAGUUGGUCCCUAAUCCUUCGUU (SEQ ID NO:45)
hsa-miR-4791	UGGAUAUGAUGACUGAAA (SEQ ID NO:46)
hsa-miR-4797-5p	GACAGAGUGCCACUUACUGAA (SEQ ID NO:47)
hsa-miR-500a-3p	AUGCACCUGGGCAAGGAUUCUG (SEQ ID NO:48)
hsa-miR-509-3p	UGAUUGGUACGUCUGUGGGUAG (SEQ ID NO:49)
hsa-miR-510-5p	UACUCAGGAGAGUGGCAAUCAC (SEQ ID NO:50)
hsa-miR-520a-5p	CUCCAGAGGGAAGUACUUUCU (SEQ ID NO:51)
hsa-miR-520g	UCCCAUGCUGUGACCCUCUAGAGGAAGCACUUUCUGUUUGUU GUCUGAGAAAAAACAAAGUGCUUCCCUUUAGAGUGUUACCGU UUGGGA (SEQ ID NO:52)
hsa-miR-520h	UCCCAUGCUGUGACCCUCUAGAGGAAGCACUUUCUGUUUGUU GUCUGAGAAAAAACAAAGUGCUUCCCUUUAGAGUUACUGUUU GGGA (SEQ ID NO:53)
hsa-miR-5 3 2	CGACUUGCUUUCUCUCCUCCAUGCCUUGAGUGUAGGACCGUU GGCAUCUUAAUUACCCUCCCACACCCAAGGCUUGCAGAAGAG CGAGCCU (SEQ ID NO:54)
hsa-miR-548aa	AAAAACCACAAUUACUUUUGCACCA (SEQ ID NO:55)
hsa-miR-548ac	CAAAAACCGGCAAUUACUUUUG (SEQ ID NO:56)
hsa-miR-548ae	CAAAAACUGCAAUUACUUUCA (SEQ ID NO:57)
hsa-miR-548ag	AAAGGUAAUUGUGGUUUCUGC (SEQ ID NO:58)
hsa-miR-548aj-3p	UAAAAACUGCAAUUACUUUUA (SEQ ID NO:59)

(continued)

microRNA/small RNA name	microRNA Sequence
hsa-miR-548ap-3p	AAAAACCACAAUUACUUUU (SEQ ID NO:60)
hsa-miR-548au	AAAAGUAAUUGCGGUUUUUGCUAUUGGUUUUAAUGGCAGUU ACUUUUGCACCAG (SEQ ID NO:61)
hsa-miR-548k	AAAAGUACUUGCGGAUUUUGCU (SEQ ID NO:62)
hsa-miR-548t-3p	AAAAACCACAAUUACUUUUGCACCA (SEQ ID NO:63)
hsa-miR-548x-3p	UAAAAACUGCAAUUACUUUC (SEQ ID NO:64)
hsa-miR-548y	GCCUAAACUAUUAGGUUGGUGCAAAAGUAAUCACUGUUUUU GCCAUUACUCUCAGUGGCAAAAACCGUGAUUACUUUUGCACC AACCUAGUAACACCUUCACUGUGGGGG (SEQ ID NO:65)
hsa-miR-561	CUUCAUCCACCAGUCCUCCAGGAACAUCAAGGAUCUUAAACU UUGCCAGAGCUACAAAGGCAAAGUUUAAGAUCCUUGAAGUUC CUGGGGGAACCAU (SEQ ID NO:66)
hsa-miR-603	CACACACUGCAAUUACUUUUGC (SEQ ID NO:67)
hsa-miR-606	AAACUACUGAAAAUCAAAGAU (SEQ ID NO:68)
hsa-miR-6075	GACACCACAUGCUCCUCCAGGCCUGCCUGCCCUCCAGGUCAU GUUCCAGUGUCCCACAGAUGCAGCACCACGGCCCAGGCGGCA UUGGUGUCACC (SEQ ID NO:69)
hsa-miR-6081	CCACCACGGUGCUGGCACCAGGGCCUCUGCCCCGUAGGACAC CGAGGCUUAUGAAUAGGAGCAGUGCCGGCCAAGGCGCCGGCA CCAUCUUGGUGAU (SEQ ID NO:70)
hsa-miR-6739-5p	UGGGAAAGAGAAAGAACAAGUA (SEQ ID NO:71)
hsa-miR-6749-5p	UCGGGCCUGGGGUUGGGGGAGC (SEQ ID NO:72)
hsa-miR-6776	CGGGCUCUGGGUGCAGUGGGGGUUCCCACGCCGCGGCAACCA CCACUGUCUCUCCCCAG (SEQ ID NO:73)
hsa-miR-6782-5p	UAGGGGUGGGGGAAUUCAGGGGUGU (SEQ ID NO:74)
hsa-miR-6793-5p	UCCCCAACCCCUGCCCGCAG (SEQ ID NO:75)
hsa-miR-6850	GUGCGGAACGCUGGCCGGGGCGGGAGGGGAAGGGACGCCCGG CCGGAACGCCGCACUCACG (SEQ ID NO:76)
hsa-miR-6865-3p	ACACCCUCUUUCCCUACCGCC (SEQ ID NO:77)
hsa-miR-6873-5p	CAGAGGGAAUACAGAGGGCAAU (SEQ ID NO:'; 8)
hsa-miR-7706	UGGAGCUGUGUGCAGGGCCAGCGCGGAGCCCGAGCAGCCGCG GUGAAGCGCCUGUGCUCUGCCGAGA (SEQ ID NO:79)
hsa-miR-7973	UGUGACCCUAGAAUAAUUAC (SEQ ID NO:80)
hsa-miR-8052	CGGGACUGUAGAGGGCAUGAGC (SEQ ID NO:81)
hsa-miR-8075	CCUUGCUGAUGGCAGAUGUCGGAUCUGCCUCGCUUAUACGUG CCCUUGCUGAUGGCAGAUGUCGGGUCUGCCUCGCUUAU (SEQ ID NO:82)
hsa-miR-8089	AAGGAGCACUCACUCCAAUUUCCCUGGACUGGGGGCAGGCUG CCACCUCCUGGGGACAGGGGAUUGGGGCAGGAUGUUCCAG (SEQ ID NO:83)

(continued)

microRNA/small RNA name	microRNA Sequence
hsa-miR-892b	UGCAAUGCCCUACUCAGAAAGGUGCCAUUUAUGUAGAUUUUA UGUCACUGGCUCCUUUCUGGGUAGAGCAAGGCUCA (SEQ ID NO:84)
hsa-miR-936	ACAGUAGAGGGAGGAAUCGCAG (SEQ ID NO:85)
hsa-miR-937-5p	GUGAGUCAGGGUGGGGCUGG (SEQ ID NO:86)
scaRNA17	AGAGGCUUGGGCCGCCGAGCUGGACCCGGACCGGUUUUGGGU ACUGUACUGGGGGCAGGGCAGAGAGGG (SEQ ID NO:87)
U6	GUGCUCGCUUCGGCAGCACAUAUACUAAAAUUGGAACGAUAC AGAGAAGAUUAGCAUGGCCCCUGCGCAAGGAUGACACGCAAA UUCGUGAAGCGUUCCAUAUUUU (SEQ ID NO:88)

Example 2: Verification of human mature miRNAs by qPCR in sample cohort of 16 patients and 8 controls

[0028] The mean fold change for hsa-miR-335-5p, hsa-miR-3613-3p and hsa-miR-6865-3p PARKmiRs between PD patients and healthy controls are shown below in Table 2 and illustrated in Figure 1.

Table 2

PARKmiR	Fold change	Significance
hsa-miR-335-5p	1.64	0.02
hsa-miR-3613-3p	2.16	0.004
hsa-miR-6865-3p	1.65	0.03

Example 3: Analyses of hsa-miR-335-5p and hsa-miR-6865-3p in a cohort of 346 individuals (182 control and 164 PD serum samples) from

Norwegian ParkWest study

[0029] The qPCR technique of Example 2 was used to identify potential diagnostic biomarkers. It was determined that combinations of hsa-miR-335-5p and hsa-miR-6865-3p show high predictability for PD diagnosis. The results of the model with hsa-miR-335-5p and hsa-miR-6865-3p, Outcome = PD (YES/NO), n = 164 cases + 182 controls are shown below in Table 3.

Table 3: Statistical analysis of individual and combination of PARKmiRs from 164 PD patients and 182 controls

miRNA(s)	Patients (n=164) median (IQR)	Controls (n=182) median (IQR)	p^1	AUC (95% CI)	p^2
335	1.4 (0.5 to 2.7)	0.12 (0.06 to 0.22)	< 0.001	0.90 (0.87 to 0.93)	< 0.001
6865	2.7 (1.1 to 6.9)	1.0 (0.8 to 1.5)	< 0.001	0.74 (0.69 to 0.80)	< 0.001
3613	0.41 (0.19 to 0.92)	0.21 (0.09 to 0.49)	< 0.001	0.65 (0.59 to 0.71)	< 0.001
335/6865				0.90 (0.87 to 0.93)	< 0.001
335/3613				0.90 (0.87 to 0.94)	< 0.001

[0030] ROC analysis based on predicted probabilities compared to true disease status is depicted in Figure 2a, and show strong discriminating ability. The area under the curve of Figure 2a is provided in Table 3 above.

Example 4: Analyses of hsa-miR-335-5p and hsa-miR-3613-3p in a cohort of 346 individuals (182 control and 164 PD serum samples)

[0031] Following the protocol of Example 3 it was determined that combinations of hsa-miR-335-5p and hsa-miR-3613-3p also show high predictability for PD diagnosis. The results of the model with hsa-miR-335-5p and hsa-miR-3613-3p, Outcome = PD (YES/NO), n = 164 cases + 182 controls are shown above in Table 3.

[0032] ROC analysis based on predicted probabilities from the model showing strong discriminating ability are depicted in Figure 2b. The area under the curve of Figure 2b is provided in Table 3 above.

[0033] From the foregoing Examples 1-4 it is evidenced that any combination of two or more microRNAs from the list of 85 identified miRNAs can be used to diagnose the occurrence of PD in patients.

Example 5: hsa-miR-335-5p

[0034] Table 3 above illustrates that hsa-miR-335-5p shows high predictability for PD diagnosis for Outcome = PD (YES/NO), n = 164 cases + 182 controls.

[0035] ROC analysis based on probabilities from the model and compared to true disease status showing strong discriminating ability is shown in Figure 3. The area under the curve of Figure 3 is provided in Table 3 above.

Example 6: has-miR-3613-3p

[0036] hsa-miR-3613-3p also shows high predictability for PD diagnosis as illustrated in Table 3 above. ROC analysis based on probabilities from the model and compared to true disease status showing strong discriminating ability is shown in Figure 4. The area under the curve of Figure 4 is provided in Table 3 above.

Example 7: has-miR-6865-3p

[0037] Similarly, hsa-miR-6865-3p also shows high predictability for PD diagnosis as shown in Table 3 above. ROC analysis based on probabilities from the model and compared to true disease status showing strong discriminating ability is shown in Figure 5. The area under the curve of Figure 5 is provided above in Table 3.

[0038] From the foregoing Examples 5-7, it is evidenced that hsa-miR-335-5p, hsa-miR-3613-3p and hsa-miR-6865-3p may be used individually for accurate diagnosis of PD.

Example 8: Analyses of hsa-miR-335-5p and hsa-miR-6865-3p in a cohort of 64 individuals (22 control and 42 PD serum samples) from

Swedish NYPUM study

[0039] The qPCR technique of Example 2 was used to validate the diagnostic biomarkers of Example 2. It was determined that combinations of hsa-miR-335-5p and hsa-miR-6865-3p show high predictability for PD diagnosis. The results of the model with hsa-miR-335-5p and hsa-miR-6865-3p, Outcome = PD (YES/NO), n = 42 cases + 22 controls are shown below in Table 4.

Table 4: Statistical analysis of individual and combinations of PARKmiRs from 42 PD patients and 22 controls from the NYPUM study.

miRNA(s)	Patients (n=42) median (IQR)	Controls (n=22) median (IQR)	p^1	AUC (95% CI)	p^2
335	1.3 (0.79 to 2.2)	1.1 (0.71 to 1.4)	0.125	0.62(0.48to 0.75)	0.127
3613	2.1 (1.2 to 3.3)	1.2 (1.0 to 1.6)	0.012	0.74(0.62to 0.86)	0.002
6865	1.5 (1.2 to 2.2)	1.2 (1.0 to 1.4)	0.002	0.69(0.56to 0.82)	0.012

(continued)

miRNA(s)	Patients (n=42) median (IQR)	Controls (n=22) median (IQR)	p^1	AUC (95% CI)	p^2
335/3613	n/a	n/a	n/a	0.75(0.63to 0.87)	0.001
335/6865	n/a	n/a	n/a	0.71(0.59to 0.84)	0.006
3613/6865	n/a	n/a	n/a	0.75(0.63to 0.87)	0.001
335/3613/6865	n/a	n/a	n/a	0.76(0.64to 0.87)	0.001

[0040] ROC analysis based on predicted probabilities compared to true disease status is depicted in Figure 7, and show strong discriminating ability. The area under the curve of Figure 7 is provided in Table 4 above.

Example 9: Analyses of hsa-miR-335-5p and hsa-miR-3613-3p in a cohort of 64 individuals (22 control and 42 PD serum samples)

[0041] Following the protocol of Example 3 it was determined that combinations of hsa-miR-335-5p and hsa-miR-3613-3p also show high predictability for PD diagnosis. The results of the model with hsa-miR-335-5p and hsa-miR-3613-3p, Outcome = PD (YES/NO), n = 42 cases + 22 controls are shown above in Table 4.

[0042] ROC analysis based on predicted probabilities from the model showing strong discriminating ability are depicted in Figure 8. The area under the curve of Figure 8 is provided in Table 4 above.

Example 10: Analyses of hsa-miR-3613-3p and hsa-miR-6865-5p in a cohort of 64 individuals (22 control and 42 PD serum samples)

[0043] Following the protocol of Example 3 it was determined that combinations of hsa-miR-3613-3p and hsa-miR-6865-5p also show high predictability for PD diagnosis. The results of the model with hsa-miR-3613-3p and hsa-miR-6865-5p, Outcome = PD (YES/NO), n = 42 cases + 22 controls are shown above in Table 4.

[0044] ROC analysis based on predicted probabilities from the model showing strong discriminating ability are depicted in Figure 9. The area under the curve of Figure 9 is provided in Table 4 above.

Example 11: Analyses of hsa-miR-335-5p, hsa-miR-3613-3p and hsa-miR-6865-5p in a cohort of 64 individuals (22 control and 42 PD serum samples)

[0045] Following the protocol of Example 3 it was determined that combinations of hsa-miR- hsa-miR-335-5p, hsa-miR-3613-3p and hsa-miR-6865-5p also show high predictability for PD diagnosis. The results of the model with hsa-miR-335-5p, hsa-miR-3613-3p and hsa-miR-6865-5p, Outcome = PD (YES/NO), n = 42 cases + 22 controls are shown above in Table 4.

[0046] ROC analysis based on predicted probabilities from the model showing strong discriminating ability are depicted in Figure 10. The area under the curve of Figure 10 is provided in Table 4 above.

[0047] From the foregoing Example 10 it is evidenced that any combination of three or more microRNAs from the list of 85 identified miRNAs can be used to diagnose the occurrence of PD in patients.

Example 12

[0048] Analysis of hsa-miR-335-5p, hsa-miR-3613-3p and hsa-miR-6865-3p targets using multiple bioinformatics tools show that among others, LRRK2 and Parkin are predicted targets of hsa-miR-335-5p, and SNCA is a predicted target of hsa-miR-3613-3p. The regulation of LRRK2 expression in SHSY-5Y cells as a result of modulation in hsa-miR-335-5p levels was confirmed by western blot analysis. hsa-miR-335-5p was overexpressed (Figure 6A) and inhibited (Figure 6A) using mimic and antagomir of hsa-miR-335-5p transfected into neuroblastoma cells. The cells were lysed after 48 hours post-transfection and used for western blot analysis. hsa-miR-335-5p mimic showed downregulation of LRRK2 and hsa-miR-335-5p antagomir showed upregulation of LRRK2 (Figure 6B, C). The hsa-miR-3613-3p regulated SNCA expression in SH-SY5Y cells in moderation. A similar experimental approach like hsa-miR-335-5p was adopted for hsa-

miR-3613-3p (Figure 6D) and the results showed moderate SNCA upregulation with hsa-miR-3613-3p mimic and a moderate SNCA downregulation with hsa-miR-3613-3p antagomir at protein level (Figure 6E, F) and transcript level (Figure 6G).

[0049] The target discovery using LC-MS was performed to find novel targets for hsa-miR-335-5p, hsa-miR-3613-3p and hsa-miR-6865-3p.

a. The proteins with differential expression pattern as a result of hsa-miR-335-5p modulation include acadsb, slc4a7, lnp/kiaa1715, supt5h, sdhd. Wdr1, cmpk1, slc25a1, hmgcs1, twf2, ppp1r18, exoc8, tm9sf4, kif16b, dnajc2, sel1l, hectd1, gmppb.

b. The proteins with differential expression pattern as a result of hsa-miR-3613-3p modulation include wdr1, gmppb, hmbs, eml4, hebp1, apmap/c20orf3, sord, pcyt2, stat3, top2a, skiv2l2, cdc20, myo1e, ttll 12, atad2, carm1, arfgap1, ppp4r1, nde1/ndel1.

c. The proteins with differential expression pattern as a result of hsa-miR-6865-3p modulation include wdr1, ppp1r18, ppp4r1, ube2h, ube3c, stx16, ube4h, gtf2f1, map1b, ube2a, dusp3, arhgap1, nsun2, acox1, fkbp10, fam107b, pofut1, tomm22, hspb8, sbds.

Example 13

[0050] Measurement of levels of a combination of two or more miRNAs in serum from patients can assist in distinctly differentiating between a potential PD patient and a healthy individual. A serum sample is obtained from blood withdrawn from patients suspected of PD. The serum is used for total microRNA isolation and enrichment. This RNA would then be tested using qPCR to measure the levels of any two or more of the 85 miRNAs mentioned in Example 1, or any one of three miRNAs mentioned in Examples 5-7. Detectable levels of any two or more of the 85 miRNAs or any one of the three miRNAs confirms the patient has PD. If desired, other sample fluids may be utilized, including plasma, venous or arterial blood, or CSF samples withdrawn by lumbar puncture. Such plasma, blood or CSF samples are processed as above. It will be understood that measurement of more than two miRNAs in combination or a set of combinations used in a test matrix may desirably increase the accuracy of PD diagnosis.

Example 14

[0051] Since a combination of miRNA can be used for diagnosis it may be advisable to test all the candidates to eliminate any cohort-based variation. It is understood that any detectable amounts of relevant miRNA will indicate PD pathology. However, those of ordinary skill in the art recognize it may be clinically helpful to use values of 164 v 182 samples to set an artificial threshold for diagnosis. Differential miRNA levels can be used to develop diagnostic biomarker kits that can be used by clinicians in diagnosis as well as in clinical trials. In this study the presence and quantification of miRNA from serum was determined by qRT-PCR which amplifies and quantifies the RNA is question. Other suitable techniques known to those of ordinary skill herein may be alternatively utilized, including use of labeled antisense sequences and labeled antibodies. Suitable antibodies are preferentially selective, referring to a binding reaction between two molecules that is typically more than 10 to 100 times background molecular associations under measurement conditions. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular miRNA sequence, thereby identifying its presence. Specific binding to an antibody under such conditions requires an antibody that is selected for its specificity for a particular miRNA. For example, antibodies raised against a particular miRNA can be selected by subtracting out antibodies that cross-react with other molecules. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular miRNA including solid-phase ELISA immunoassays (see, e.g., Harlow & Lane, Antibodies, A Laboratory Manual (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Methods for determining whether two molecules specifically interact are disclosed therein, and methods of determining binding affinity and specificity are well known in the art (see, for example, Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor Laboratory Press, 1988); Friefelder, "Physical Biochemistry: Applications to biochemistry and molecular biology" (W.H. Freeman and Co. 1976)). The term "antibody" as used herein encompasses naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof, (e.g., Fab', F(ab')2, Fab, Fv and rIgG). *See also,* Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, IL). *See also,* e.g., Kuby, J., Immunology, 3rd Ed., W.H. Freeman & Co., New York (1998). Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as described by Huse et al., Science, Vol. 246 (1989) 1275-81. These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today, Vol. 14 (1993) 243-46; Ward et al., Nature, Vol.

341 (1989) 544-46; Harlow and Lane, supra, 1988; Hilyard et al., Protein Engineering: A practical approach (IRL Press 1992); Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995). Methods for producing both monoclonal and polyclonal antibodies from identified RNA sequences are well known in the art.

Example 15

[0052] Many neurodegenerative diseases are closely related to each other when it comes to symptoms as well as pathological markers. The circulating diagnostic markers for one neurodegenerative disease can be useful for diagnosis of other disease. A method to diagnose other neurodegenerative diseases like Dementia with Lewy body (DLB), Amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Multiple system atrophy (MSA), CorticoBasal Degeneration (CBD), Progressive Supranuclear Palsy (PSP) can also be developed using similar miRNA measurements of candidates mentioned above. Disease specific kits can be developed similar to one mentioned in [0037] with various combinations of miRNAs listed in [0019].

Example 16

[0053] The miRNAs detected in one or more combinations can regulate several proteins in the cells. Novel protein targets for PD can be discovered using these microRNAs and their combinations. The involvement of these proteins in PD etiology can be further established to target them for therapy.

Example 17

[0054] We have experimentally confirmed the predicted regulation of LRRK2 by hsa-miR-335-5p and SNCA by hsa-miR-3613-3p in dopaminergic neuronal cell lines. Therapeutic intervention to regulate the novel targets mentioned can be achieved by RNA interference technologies.

Example 18

[0055] Small nucleic acid molecules derived from miRNAs mentioned in [0019] will be designed to therapeutically intervene by specifically targeting genes in PD brains to achieve complete or partial remedy. The effects shown in [0040] will be achieved for precise targeting in brain cells.

Claims

1. A method for determining Parkinson's disease in a human patient, comprising the steps of:
   determining in a sample from said human patient a differential level of:

   (a) at least three miRNAs selected from the group consisting of SEQ ID NOS: 2-86 within said sample, wherein at least two miRNAs are selected from the group consisting of SEQ ID NOS: 22, 25 and 77; or
   (b) at least two miRNAs selected from the group consisting of SEQ ID NOS: 22, 25 and 77 within said sample.

2. The method of claim 1, wherein said sample is serum, plasma or whole blood.

3. The method according to claim 1 or claim 2, wherein the differential level of said miRNAs is determined using qRT-PCR.

4. The method according to any one of claims 1 to 3, wherein the differential level of said miRNAs is determined using labeled antisense nucleotide sequences.

5. The method according to any one of claims 1 to 4, wherein the differential level of said miRNAs is determined using labeled antibodies.

6. The method according to claim 5, wherein the labeled antibodies are monoclonal.

7. The method according to any one of claims 1 to 6, wherein said at least two miRNAs comprise SEQ ID NOS: 22 and 25.

8. The method according to any one of claims 1 to 7, wherein said at least two miRNAs comprise SEQ ID NOS: 22 and 77.

9. The method according to any one of claims 1 to 8, wherein said at least two miRNAs comprise SEQ ID NOS: 25 and 77.

10. The method according to any one of claims 1 to 9, comprising determining the differential level of each of SEQ ID NOS: 22, 25 and 77 within said sample.

11. The method according to any one of claims 1 to 10, wherein the differential level of said miRNAs is determined using microarray profiling.

12. The method according to any one of claims 1 to 11, wherein the differential level of said miRNAs is determined using high throughput NGS sequencing.

Patentansprüche

1. Verfahren zum Bestimmen von Parkinsonkrankheit bei einem menschlichen Patienten, umfassend die Schritte: Bestimmen, in einer Probe von dem menschlichen Patienten, eines differentiellen Spiegels von:

   (a) mindestens drei miRNAs, ausgewählt aus der Gruppe, bestehend aus SEQ ID NOs: 2-86, innerhalb der Probe, wobei mindestens zwei miRNAs ausgewählt sind aus der Gruppe, bestehend aus SEQ ID NOs: 22, 25 und 77; oder
   (b) mindestens zwei miRNAs, ausgewählt aus der Gruppe, bestehend aus SEQ ID NOs: 22, 25 und 77, innerhalb der Probe.

2. Verfahren nach Anspruch 1, wobei die Probe Serum, Plasma oder Vollblut ist.

3. Verfahren nach Anspruch 1 oder Anspruch 2, wobei der differentielle Spiegel der miRNAs unter Verwendung von qRT-PCR bestimmt wird.

4. Verfahren nach einem der Ansprüche 1 bis 3, wobei der differentielle Spiegel der miRNAs unter Verwendung markierter Antisense-Nukleotidsequenzen bestimmt wird.

5. Verfahren nach einem der Ansprüche 1 bis 4, wobei der differentielle Spiegel der miRNAs unter Verwendung markierter Antikörper bestimmt wird.

6. Verfahren nach Anspruch 5, wobei die markierten Antikörper monoklonal sind.

7. Verfahren nach einem der Ansprüche 1 bis 6, wobei die mindestens zwei miRNAs SEQ ID NOs: 22 und 25 umfassen.

8. Verfahren nach einem der Ansprüche 1 bis 7, wobei die mindestens zwei miRNAs SEQ ID NOs: 22 und 77 umfassen.

9. Verfahren nach einem der Ansprüche 1 bis 8, wobei die mindestens zwei miRNAs SEQ ID NOs: 25 und 77 umfassen.

10. Verfahren nach einem der Ansprüche 1 bis 9, umfassend Bestimmen der differentiellen Spiegel von jeder von SEQ ID NOs: 22, 25 und 77 innerhalb der Probe.

11. Verfahren nach einem der Ansprüche 1 bis 10, wobei der differentielle Spiegel der miRNAs unter Verwendung von Mikroarray-Profilierung bestimmt wird.

12. Verfahren nach einem der Ansprüche 1 bis 11, wobei der differentielle Spiegel der miRNAs unter Verwendung von Hochdurchsatz-NGS-Sequenzierung bestimmt wird.

Revendications

1. Procédé de détermination de la maladie de Parkinson chez un patient humain, comprenant les étapes consistant à : déterminer dans un échantillon dudit patient humain un niveau différentiel :

   (a) d'au moins trois miARN sélectionnés dans le groupe consistant en SEQ ID NO : 2-86 dans ledit échantillon,

dans lequel au moins deux miARN sont sélectionnés dans le groupe consistant en SEQ ID NO : 22, 25 et 77 ; ou (b) d'au moins deux miARN sélectionnés dans le groupe consistant en SEQ ID NO : 22, 25 et 77 dans ledit échantillon.

2. Procédé selon la revendication 1, dans lequel ledit échantillon est du sérum, du plasma ou du sang entier.

3. Procédé selon la revendication 1 ou la revendication 2, dans lequel le niveau différentiel desdits miARN est déterminé par qRT-PCR.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel le niveau différentiel desdits miARN est déterminé au moyen de séquences nucléotidiques antisens marquées.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel le niveau différentiel desdits miARN est déterminé au moyen d'anticorps marqués.

6. Procédé selon la revendication 5, dans lequel les anticorps marqués sont monoclonaux.

7. Procédé selon l'une quelconque des revendications 1 à 6, dans lequel lesdits au moins deux miARN comprennent les SEQ ID NO : 22 et 25.

8. Procédé selon l'une quelconque des revendications 1 à 7, dans lequel lesdits au moins deux miARN comprennent les SEQ ID NO : 22 et 77.

9. Procédé selon l'une quelconque des revendications 1 à 8, dans lequel lesdits au moins deux miARN comprennent les SEQ ID NO : 25 et 77.

10. Procédé selon l'une quelconque des revendications 1 à 9, comprenant la détermination du niveau différentiel de chacune des SEQ ID NO : 22, 25 et 77 dans ledit échantillon.

11. Procédé selon l'une quelconque des revendications 1 à 10, dans lequel le niveau différentiel desdits miARN est déterminé par profilage sur microréseau.

12. Procédé selon l'une quelconque des revendications 1 à 11, dans lequel le niveau différentiel desdits miARN est déterminé par séquençage NGS à haut débit.

Figure 1

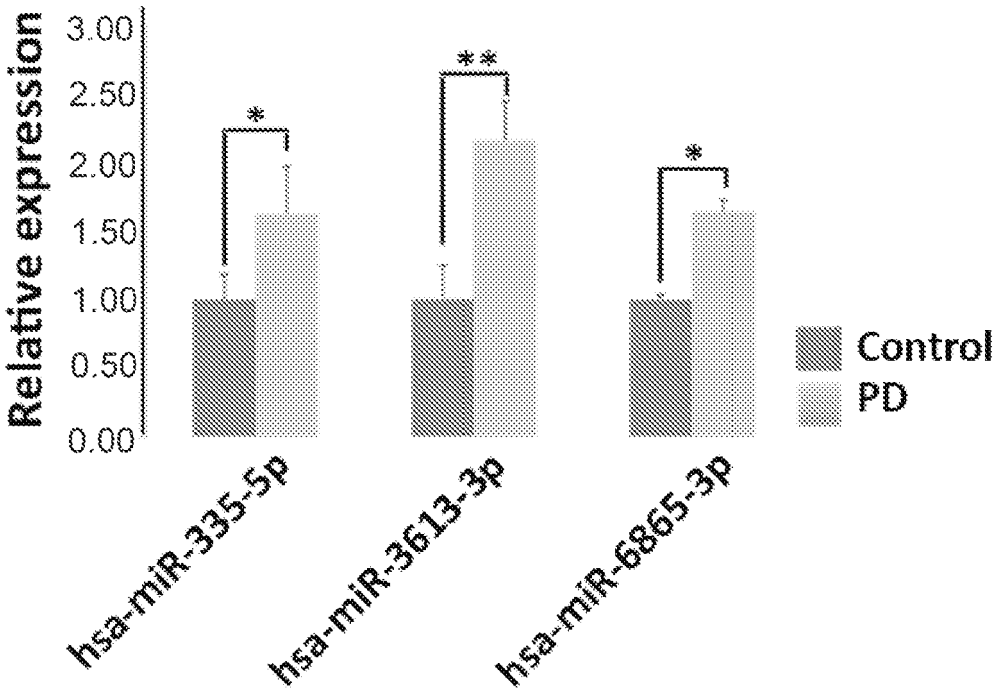

Relative expression
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
**
*
Control
PD
hsa-miR-335-5p
hsa-miR-3613-3p
hsa-miR-6865-3p

Figure 2A

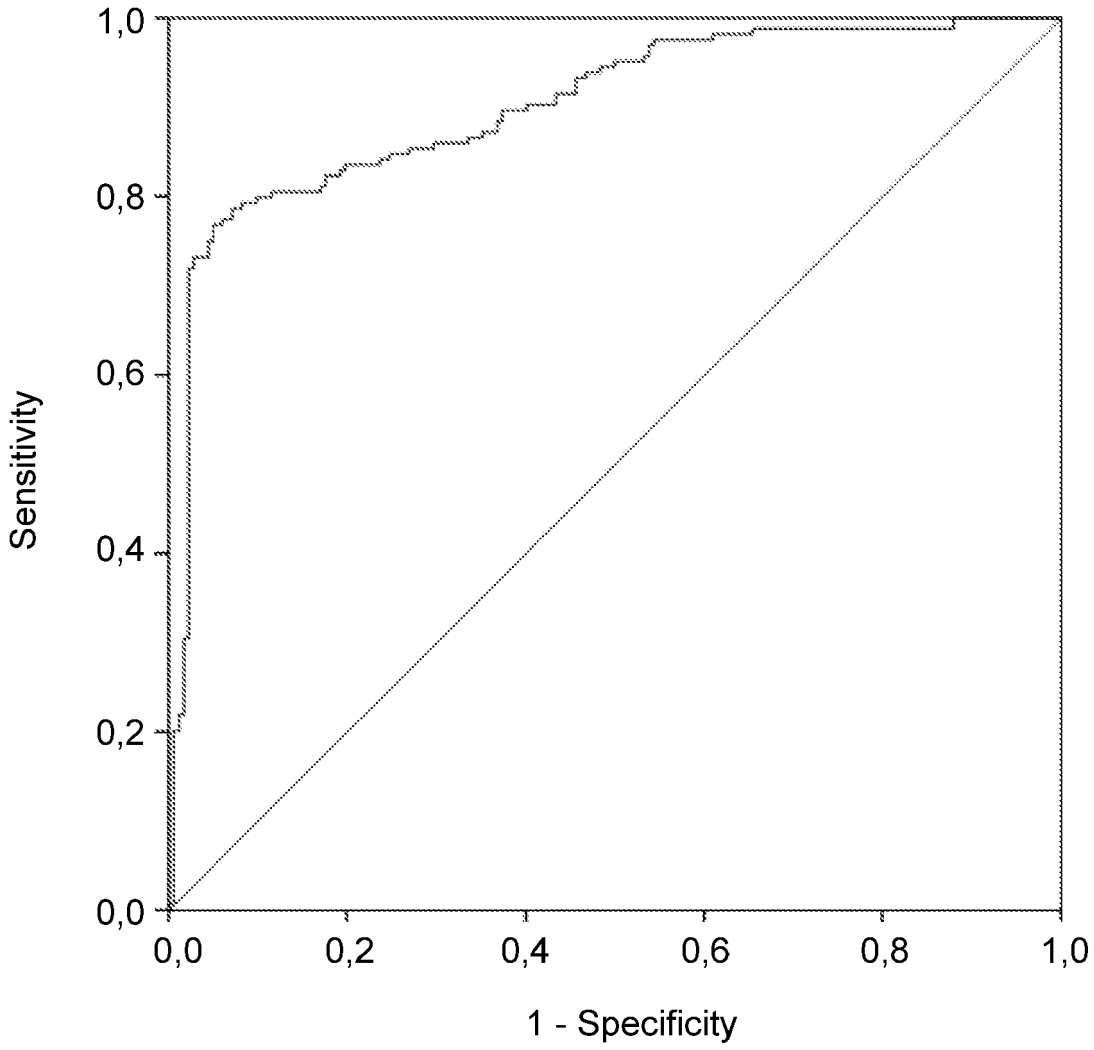


Figure 2B

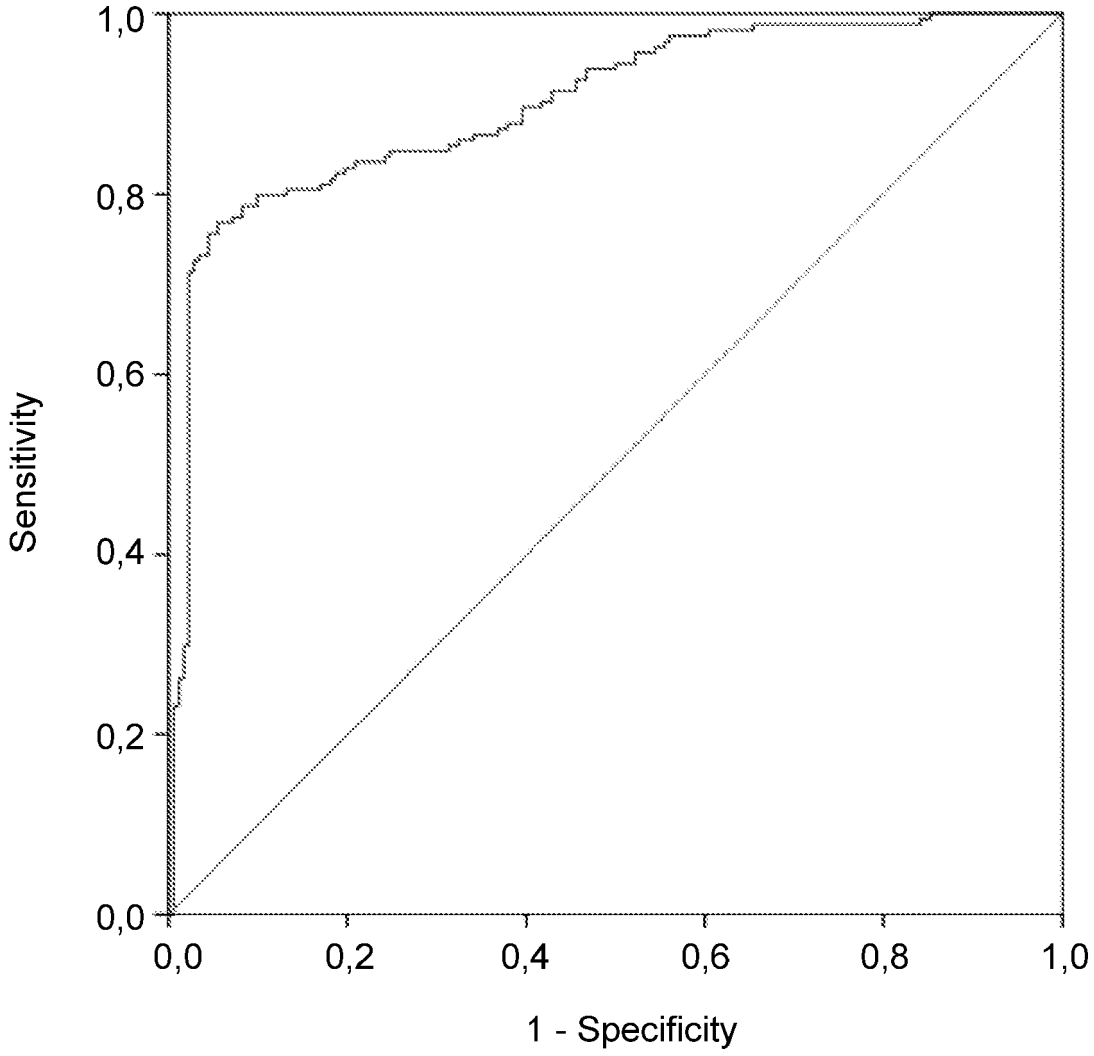


Figure 3

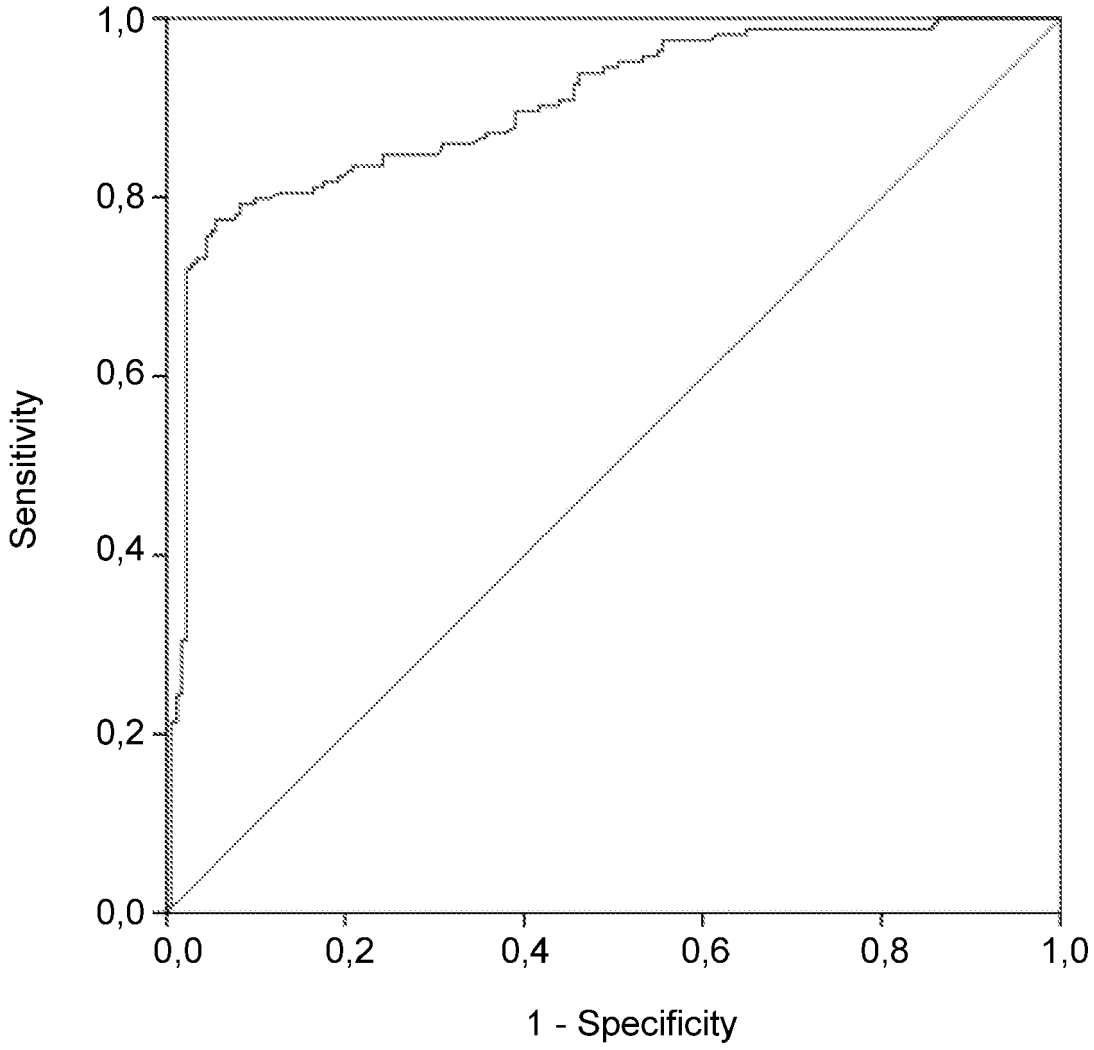


Figure 4

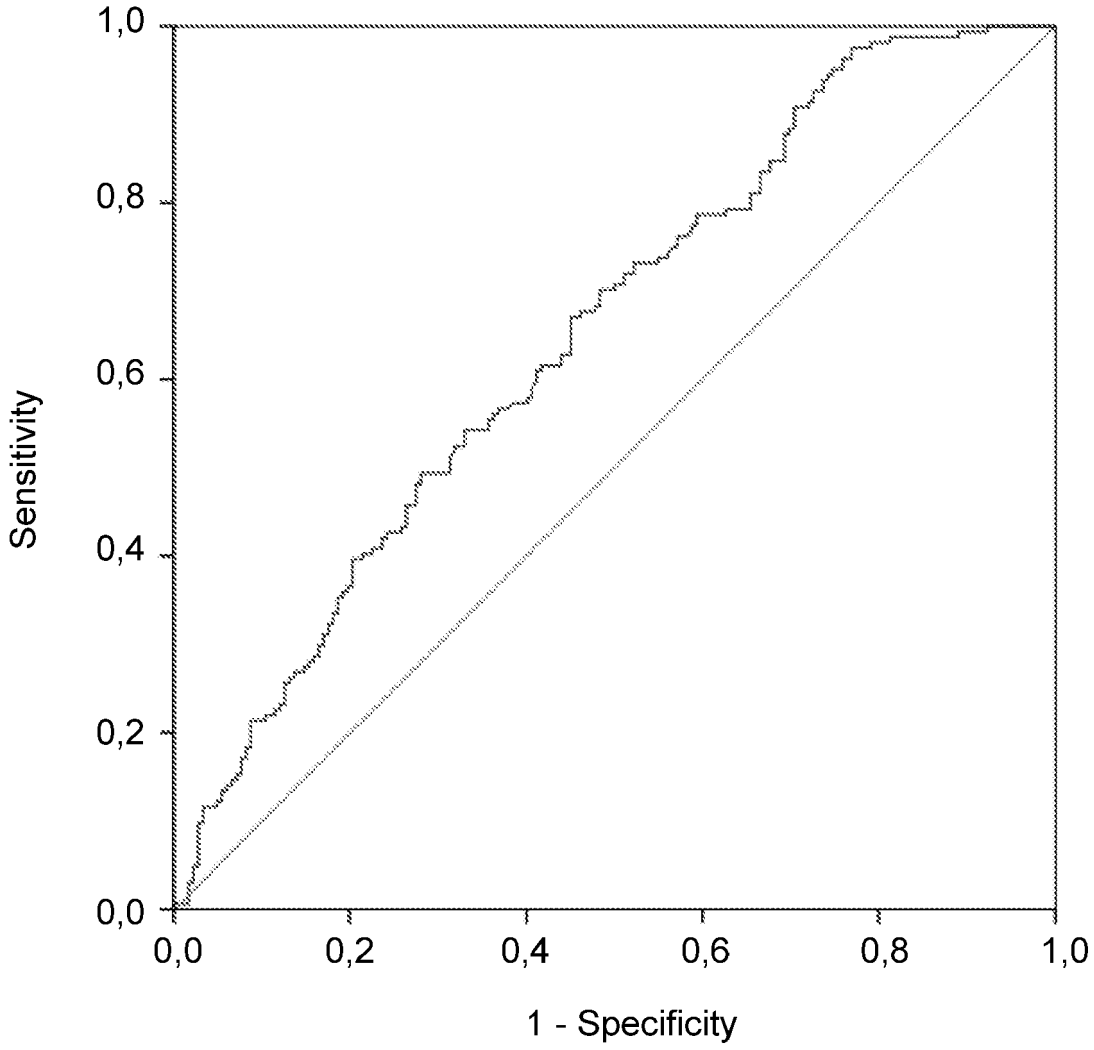


Figure 5

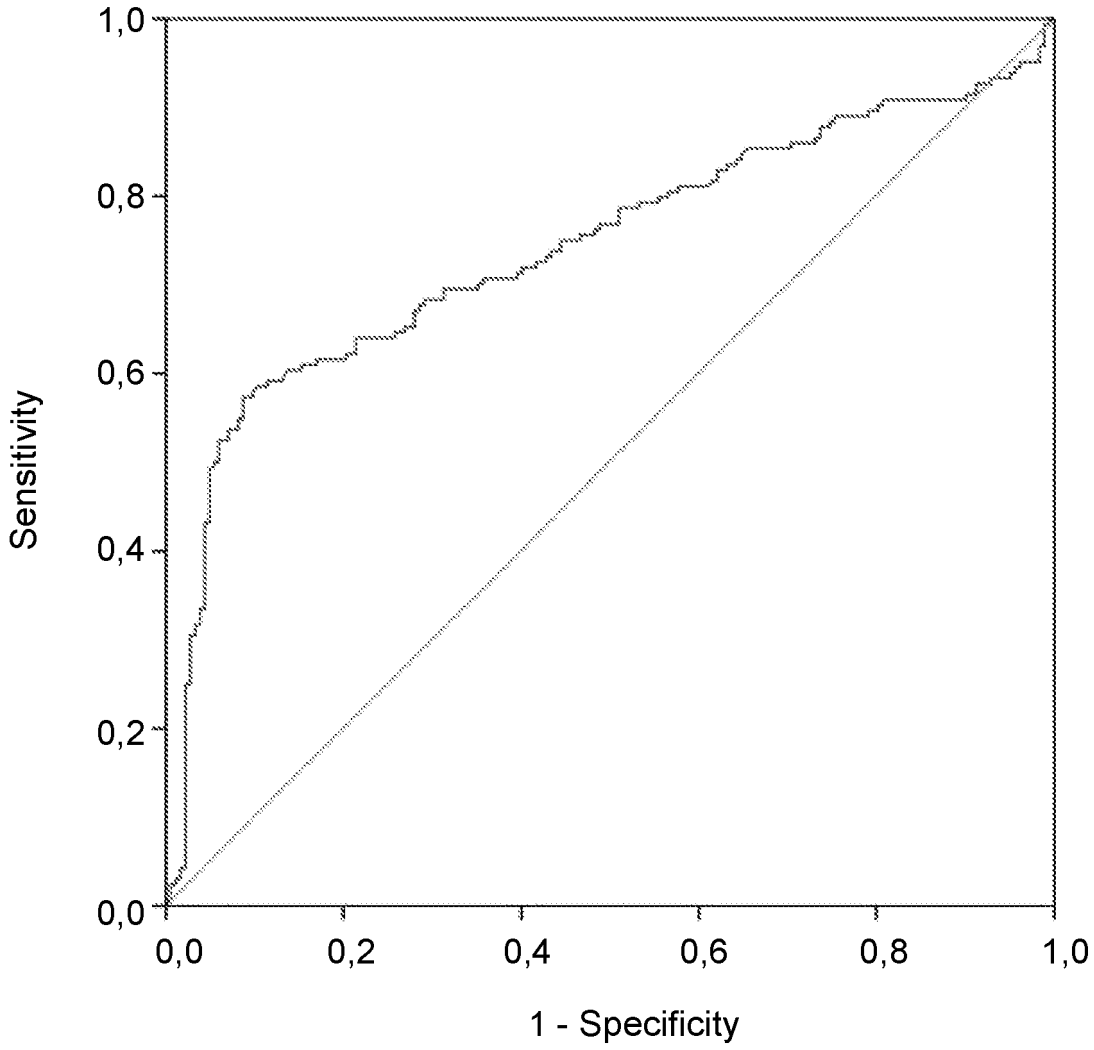


Figure 6A

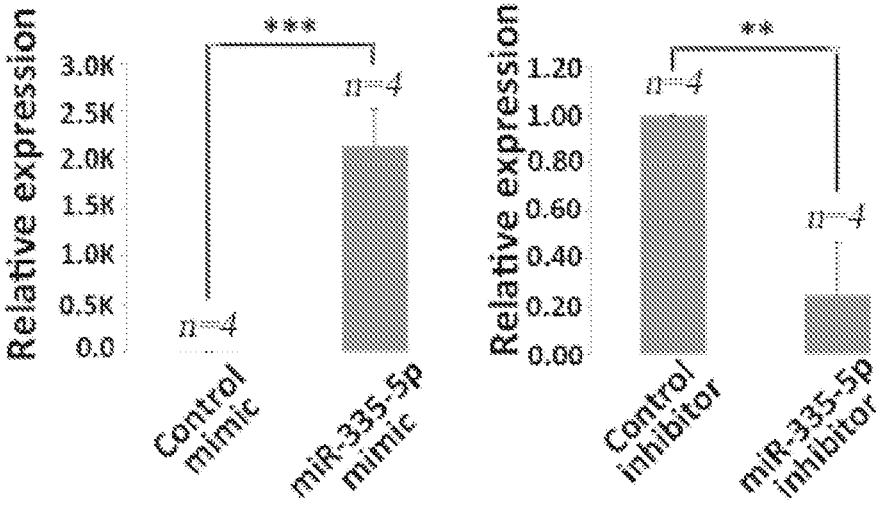

Relative expression
3.0K
2.5K
2.0K
1.5K
1.0K
0.5K
0.0
***
n=4
n=4
Control mimic
miR-335-5p mimic
Relative expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
**
n=4
n=4
Control inhibitor
miR-335-5p inhibitor

Figure 6B

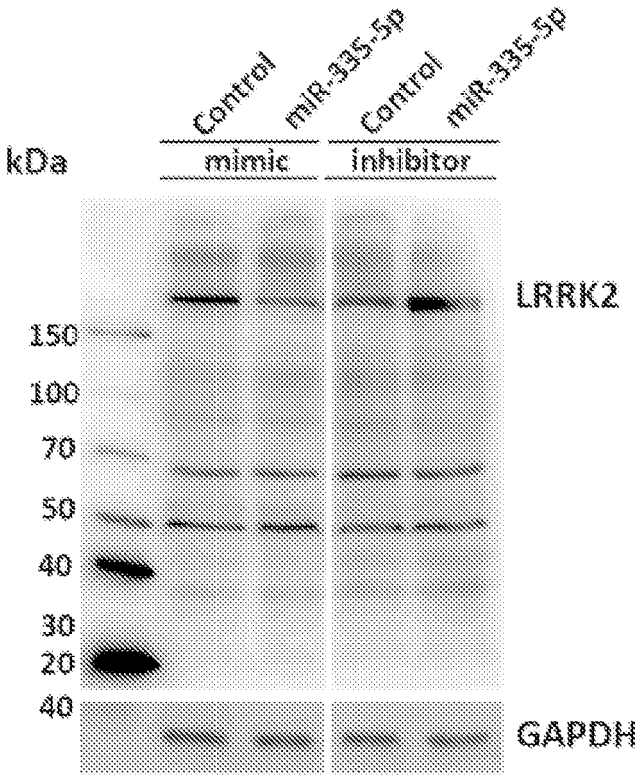

Control
miR-335-5p
Control
miR-335-5p
mimic
inhibitor
kDa
150
100
70
50
40
30
20
40
LRRK2
GAPDH

Figure 6C

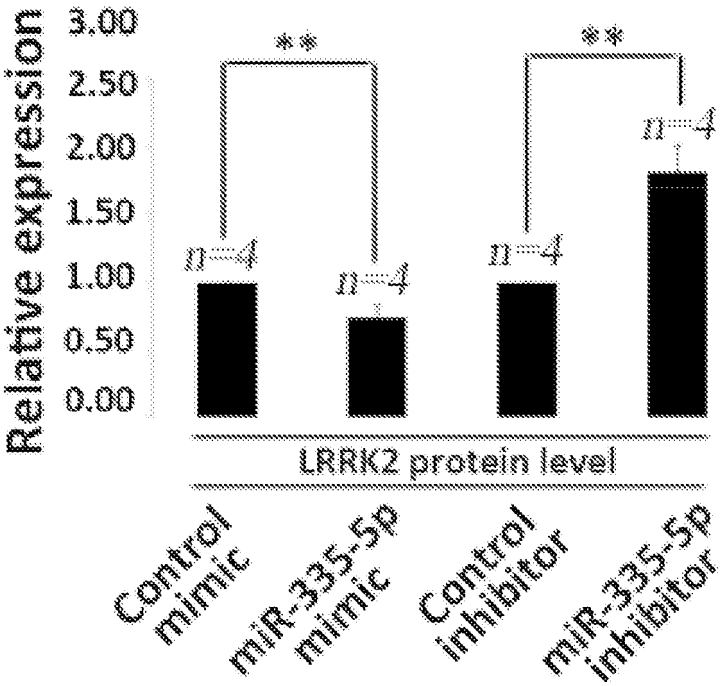

Relative expression
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
**
n=4
n=4
n=4
n=4
LRRK2 protein level
Control mimic
miR-335-5p mimic
Control inhibitor
miR-335-5p inhibitor

Figure 6D

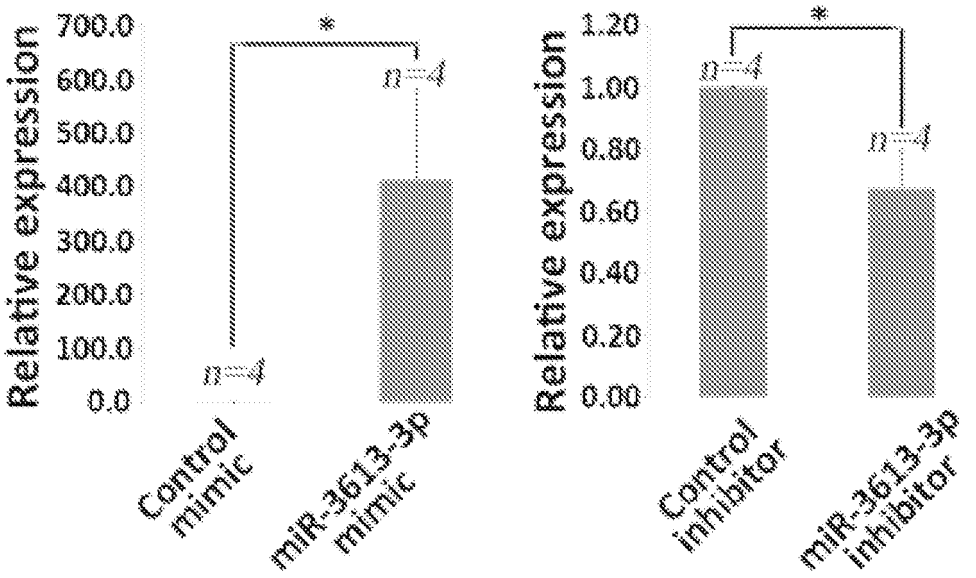

Relative expression
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
*
n=4
n=4
Control mimic
miR-3613-3p mimic
Relative expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
n=4
n=4
Control inhibitor
miR-3613-3p inhibitor

Figure 6E

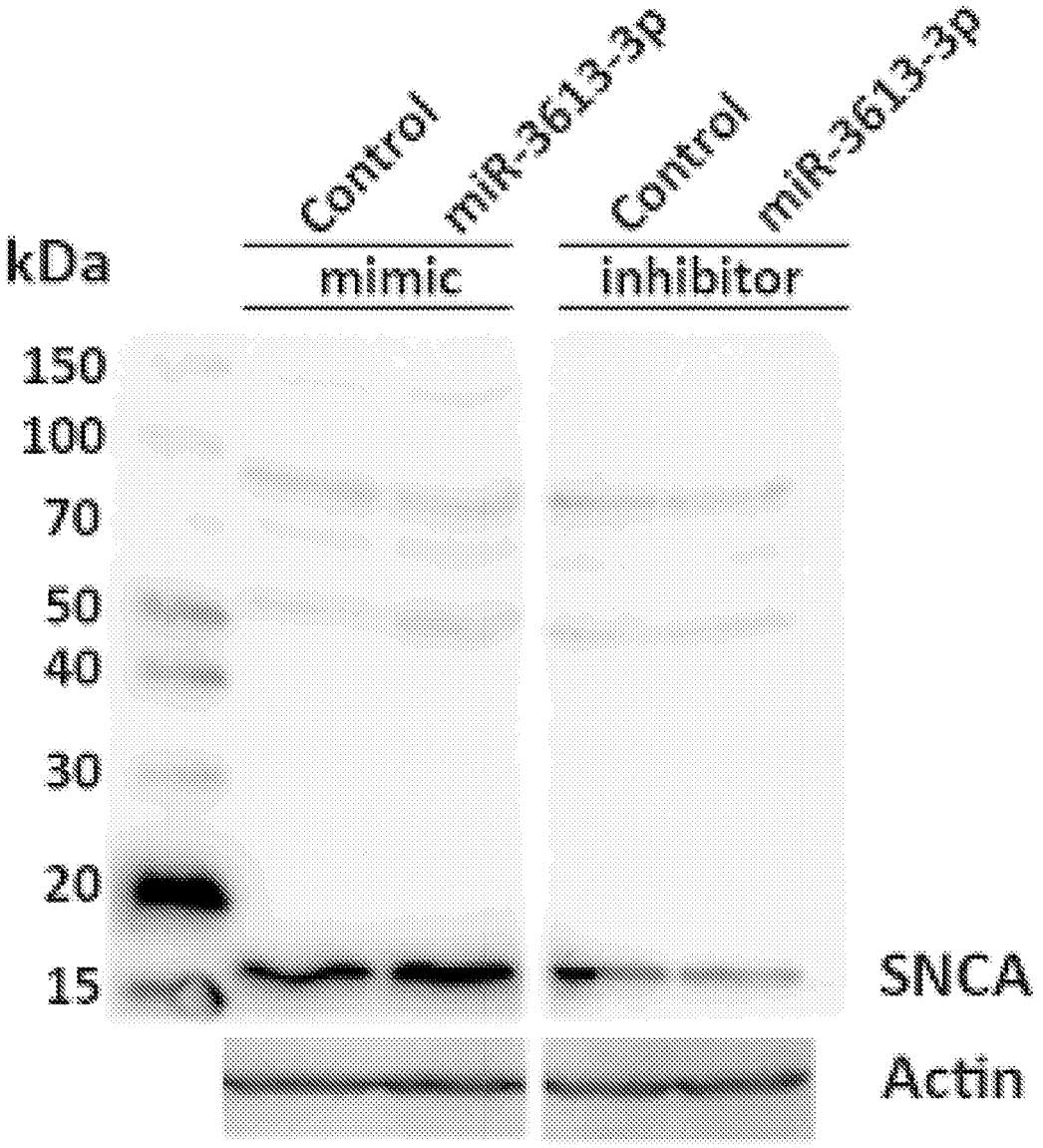

Control
miR-3613-3p
Control
miR-3613-3p
kDa
mimic
inhibitor
150
100
70
50
40
30
20
15
SNCA
Actin

Figure 6F

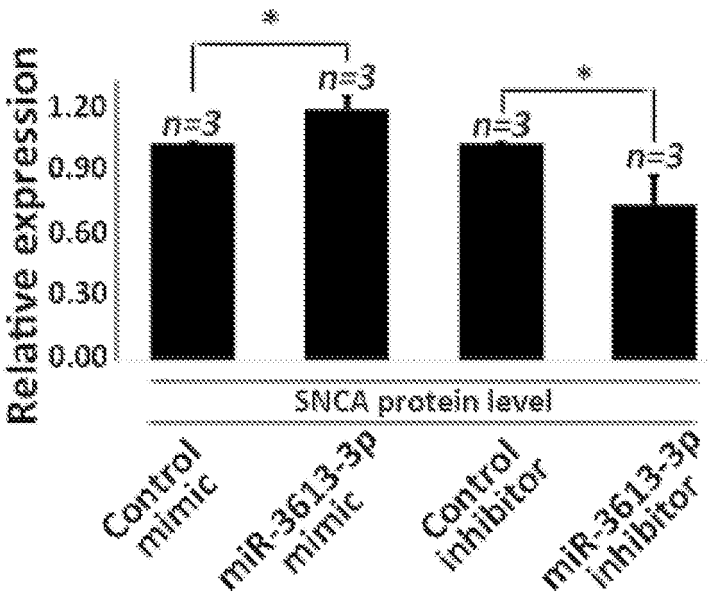

*
*
n=3
n=3
n=3
n=3
Relative expression
1.20
0.90
0.60
0.30
0.00
SNCA protein level
Control mimic
miR-3613-3p mimic
Control inhibitor
miR-3613-3p inhibitor

Figure 6G

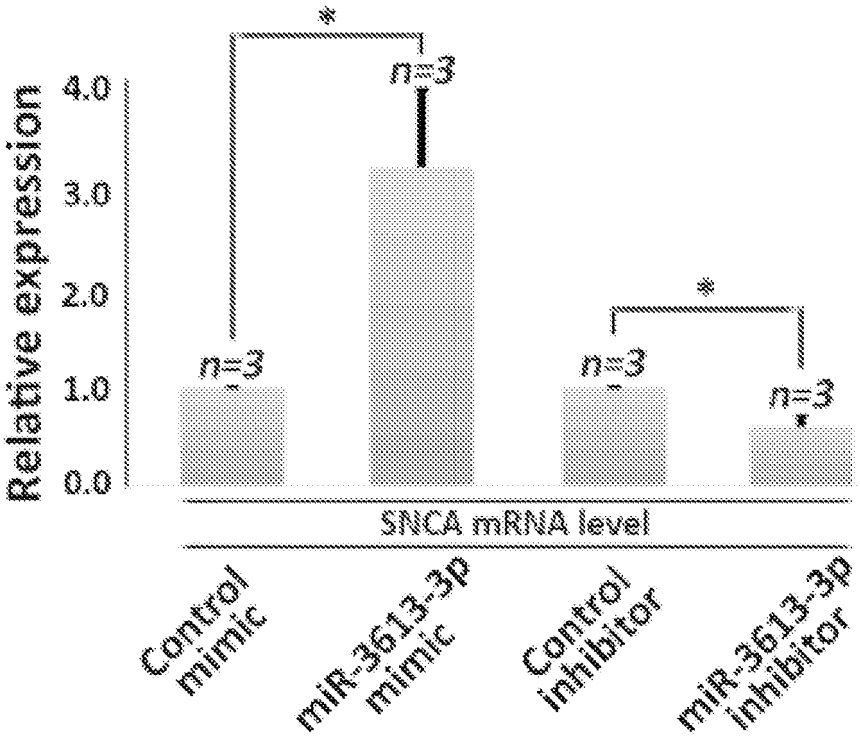

*
n=3
*
n=3
n=3
n=3
Relative expression
4.0
3.0
2.0
1.0
0.0
SNCA mRNA level
Control mimic
miR-3613-3p mimic
Control inhibitor
miR-3613-3p inhibitor

Figure 6H

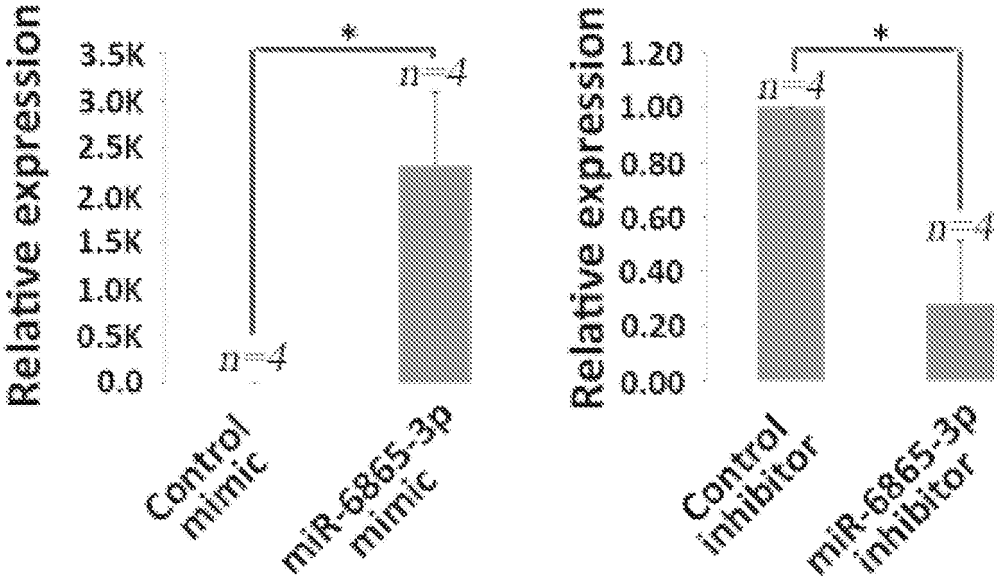

Relative expression
3.5K
3.0K
2.5K
2.0K
1.5K
1.0K
0.5K
0.0
*
n=4
n=4
Control mimic
miR-6865-3p mimic
Relative expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
n=4
n=4
Control inhibitor
miR-6865-3p inhibitor

Figure 7

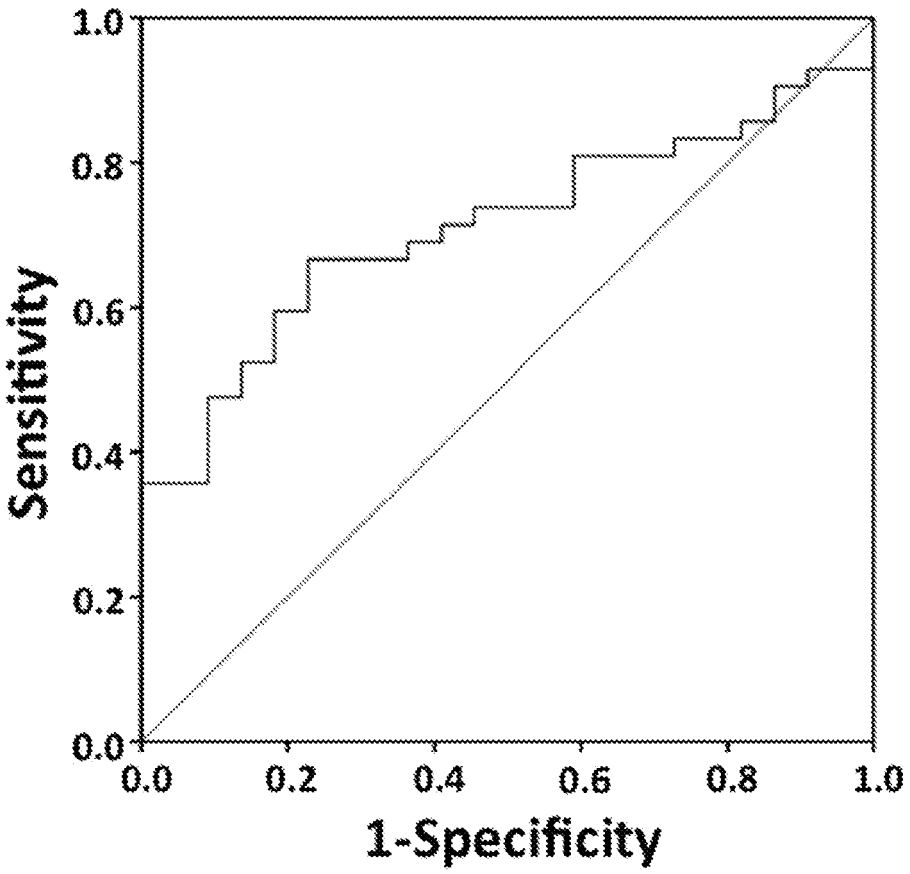


Figure 8

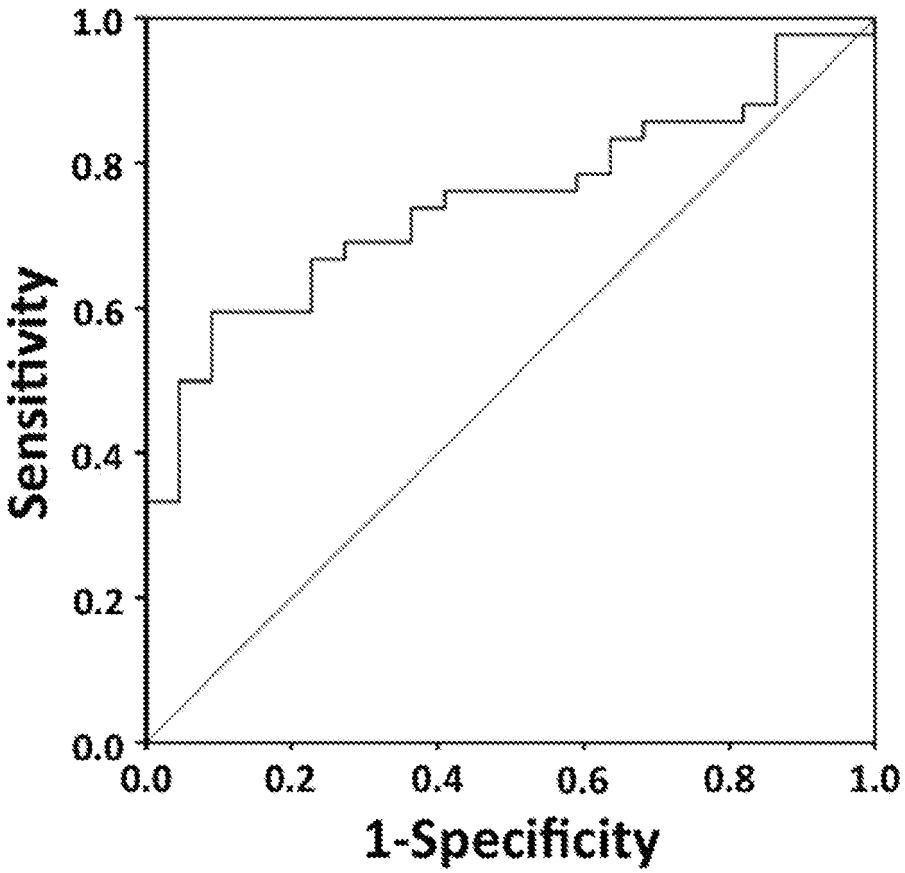


Figure 9

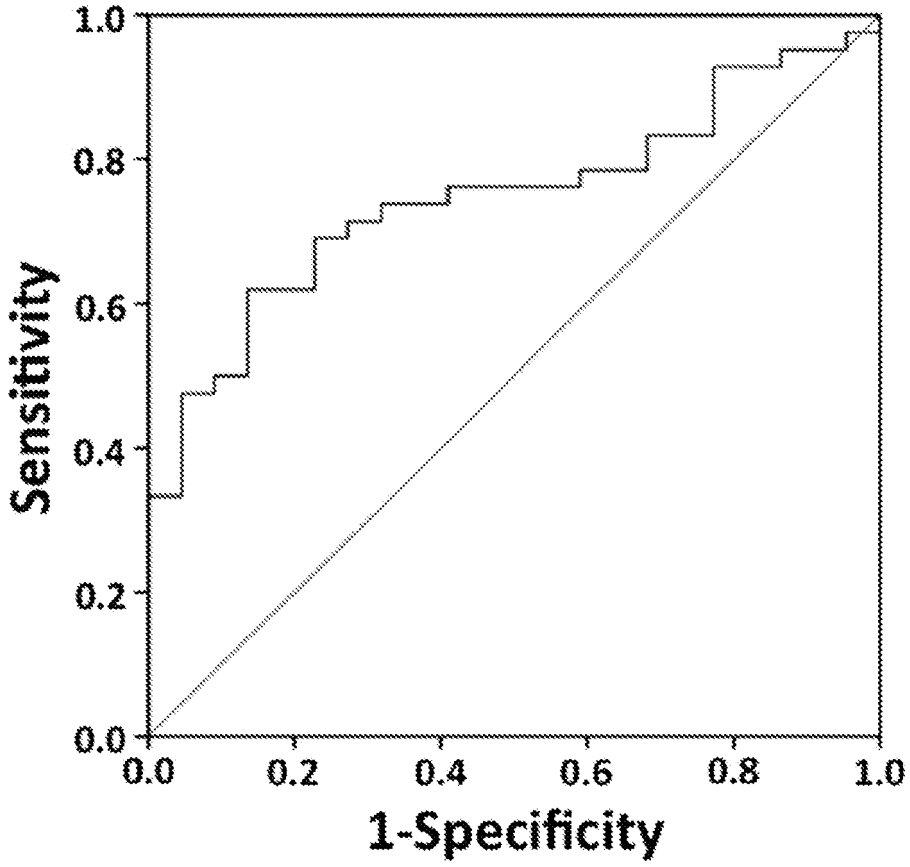
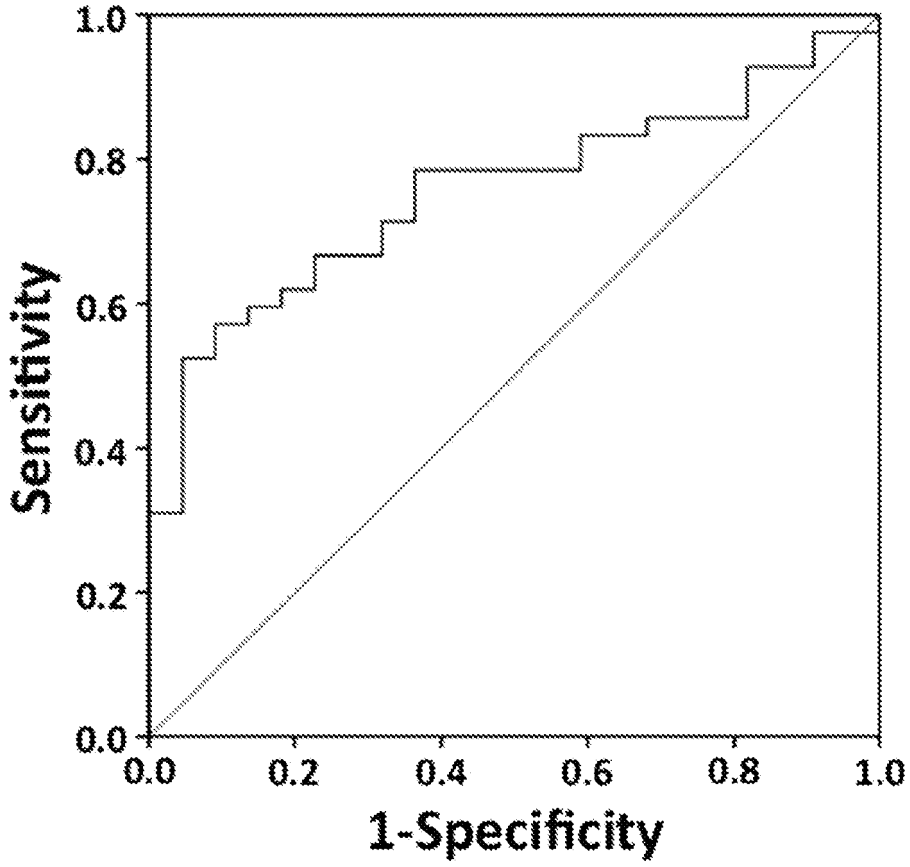


Figure 10



REFERENCES CITED IN THE DESCRIPTION

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