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(54) Benævnelse: **FREMGANGSMÅDER TIL ANVENDELSE AF MIRNA TIL DETEKTION AF IN VIVO CELLEDØD**

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DESCRIPTION

FIELD OF THE INVENTION

[0001] The invention provides non-invasive methods for isolation and detection of cell-free small RNA, in particular microRNA (miRNA) sequences in bodily fluid selected from blood, serum and urine. More specifically, the present invention encompasses methods of detecting *in vivo* cell death by analyzing urine blood or serum for miRNA levels for clinical diagnosis and treatment monitoring.

BACKGROUND OF THE INVENTION

[0002] Cell death is a normal component of development and functioning of multicellular organisms. Being a natural process, cell death is involved in the pathology of numerous diseases caused by internal factors. Cell death also accompanies diseases caused by external physical, chemical, of biological agents.

[0003] There exist two major types of cell death, necrosis and apoptosis, marked by different morphological and molecular characteristics (Kerr et al., Br. J. Cancer. 26, 239-257 (1972); Umansky, Theor. Biol. 97, 591-602 (1982); Umansky et al., Adv Pharmacol. 41, 383-407 (1997); Ameisen, Cell Death Differ. 11, 4-10 (2004); Lockshin et al. Int J Biochem Cell Biol. 36, 2405-19 (2004); G. Kroemer, et al., Cell Death and Differentiation 12,1463-1467 (2005)). Necrosis is considered to be catastrophic metabolic failure resulting directly from severe molecular and/or structural damage and leads to inflammation and secondary damage to surrounding cells. Apoptosis is a much more prevalent biological phenomenon than necrosis and can be induced by specific signals such as hormones, cytokines, by absence of specific signal such as growth or adhesion factors, or by molecular damage that does not cause catastrophic loss of integrity. Apoptosis is a result of an active cellular response involving initiation of an orderly and specific cascade of molecular events. Apoptosis leads to the appearance of distinctive chromatin condensation and margination, nuclear fragmentation, cell shrinkage, membrane blebbing and enzymatic internucleosomal fragmentation of nuclear DNA (Umansky et al., Biochim Biophys Acta. 655, 9-17 (1981); Arends et al., Am J Pathol. 136, 593-608(1990)). Other more rare forms of cell death, characterized by specific morphology, for example, so called autophagic cell death have also been described (Bredesen et al., Stroke. 38(2 Suppl):652-660 (2007).

[0004] Independent of a specific mechanism and type of cell death, methods to detect dying cell types are important for diagnosis of various diseases, critical for disease and treatment monitoring, and helpful for differential diagnosis. Besides, the methods capable of detection of specific cell death *in vivo* are useful for developing drugs aiming at prevention or induction of cell death as well as for analysis of the cytotoxicity of the newly developed drugs.

[0005] There are some clinical tests for diagnosis of disease-related excessive cell death based on detection of tissue specific markers, such as for example antigens, enzymes and other proteins in blood or in other bodily fluids. Measurement of the activity of liver-specific enzymes in blood, for example, is a widely used method for evaluation of hepatocyte death (Amacher, et al., Regul Toxicol Pharmacol. Apr; 27(2):119-130 (1988); Salaspuro, et al., Enzyme.37:87-107 (1987); Herlong, Hosp. Pract. (Off Ed).29(11):32-38 (1994)). Evaluation of the level of cardiomyocyte specific antigens has also been used for diagnosis of the myocardial infarction (Mair et al., Clin Chem Lab Med. 37:1077-1084 (1999); Nunes et al., Rev Port Cardiol. 20:785-788 (2001)). However, the number of such techniques is limited to diseases in which a marker and a method of detection are known in order for the analysis to provide meaningful, tissue-specific results. (Oh S et al., Curr Gastroenterol Rep. 3:12-18 (2001); Rochling et al., Clin Cornerstone. 3(6):1-12 (2001)). Other methods require invasive biopsy of specific tissues suspected of having a diseased condition to get a specimen for analysis. However, biopsy of some organs and tissues, for example brain is highly invasive and often difficult to perform.

[0006] It is well known that apoptosis, or programmed cell death, which is a major form of cell death in the mamalian organism, is accompanied by internucleosomal fragmentation of nuclear DNA. Many laboratories have demonstrated that a portion of this DNA appears in blood (Lo Y.M. Ann N Y Acad Sci. 945: 1-7 (2001); Lichtenstein et al., Ann N Y Acad Sci. 945:239-249 (2001); Taback et al., Curr Opin Mol Ther. 6:273-278 (2004); Bischoff et al., Hum Reprod Update. 8:493-500,(2002)). It has also been shown that this fragmented DNA, called transrenal DNA (Tr-DNA) crosses the kidney barrier and can be detected in the urine. (Botezatu et al., Clin Chem. 46:1078-1084,(2000); Su et al., J Mol Diagn.6: 101-107 (2004); Su et al., Ann N Y Acad Sci. 1022:81-89(2004).

[0007] Su, Y. et al., (2003) Annals of the New York Academy of Sciences vol. 3rd. pp 81-89 describes that a small portion of DNA from apoptotic cells escapes complete degradation, appears in blood as oligonucleosomal-size fragments, is excreted in the

urine, and can be used for diagnostic purposes.

[0008] Swarup V. et al., (2007) FEBS Letters vol. 581, no. 5 pp 795-799, describes how increased levels of plasma circulating (cell-free) nucleic acids have been reported in a number of clinical disorders and suggests that this could provide a non-invasive tool for the early detection of several human diseases.

[0009] Although both cell-free plasma DNA and Tr-DNA may be used as diagnostic tools, they provide a rather limited approach when evaluating tissue specific events, such as cell death. Thus analytical methods that are non-invasive, and provide a broader range of indications of specific pathology, due to their ability to detect levels of dying cells in particular tissues and organs, would be useful for diagnosing and monitoring the state of various diseases or pathological conditions in patients. In addition, tissue specific analytical methods that provide the means for monitoring the response of a patient to a disease therapy would be useful to determine the therapy effectiveness, and in the case of drug treatment, the optimum dosage required for drug administration.

[0010] To address these problems, the instant invention is focused on the use of micro RNA (miRNA) as a diagnostic tool to monitor in *vivo* cell death in bodily fluids, such as for example serum and urine. Unlike cell-free plasma DNA and Tr-DNA, many miRNAs exhibit cell, tissue and organ specific expression profiles (Liang et al, Genomics, 8: 166 (2007); Lukiw et al, Neuroreport. 18:297-300 (2007); Lagos-Ouintana et al, Curr Biol. 12:735-739 (2002); Chen et al, Nat Genet. 38:228-233 (2006); Beuvink et al, J. Nucleic Acids Res. 35:e52 (2007)). Furthermore, correlation of miRNA cell and tissue specific profiles with different pathologies and tumor types have been demonstrated (Visone R., et al Oncogene. 26:7590-7595 (2007); Nelson et al, Neuropathol Exp Neural. 66:461-468 (2007); Negrini et al, J Cell Sci.120:1833-1840 (2007); Chang et al, Annu Rev Genomics Hum Genet. 8:215-239 (2007); Jay et al, Cell Bioi. 26:293-300(2007)).

[0011] Thus, the instant invention provides methods for measuring *in vivo* cell death by detection of tissue-specific miRNAs, characteristic of a specific pathology, in body fluids, selected from blood, serum and urine. The instant methods based on detection of miRNAs in bodily fluids are used for further development of diagnostic or monitoring tests.

SUMMARY OF THE INVENTION

[0012] The instant invention relates to a novel method for detecting and measuring *in vivo* cell-death by analyzing levels of specific miRNA sequences in cell-free nucleic acids obtained from bodily fluids selected from blood, serum and urine, said miRNA originating from cells dying throughout the body, and using the obtained analytical result to determine state of a disease or abnormal medical condition in a patient.

[0013] The methods of the instant invention are based on adsorption of cell-free nucleic acids on and elution from anion-exchangers, which makes it possible to concentrate and isolate nucleic acid fragments larger than 10 nucleotides. Specifically, the instant invention demonstrates: (i) the presence of miRNA in said body fluids; (ii) detection in urine of miRNA that originated from organs located outside of urinary system, which means that they have crossed the kidney barrier, such as for example, transrenal miRNA (Tr-miRNA); iii) detection of miRNA in serum (iv) pathology associated with cell death in a particular cell, tissue and/or organ is accompanied by changes in levels of miRNA specific for the said organ.

[0014] The present invention provides a method of detecting and quantitating cell, tissue and/or organ-specific cell-free miRNAs in said body fluids for evaluation of *in vivo* cell death in various tissue and organs, wherein *in vivo* cell death is associated with a disorder of a particular tissue and/or organ comprising obtaining a body fluid sample from a subject; and analyzing said body fluid sample for one or more specific sequences of miRNA, wherein said analyzing comprises the step of detecting said miRNA with a primer and/or probe that is substantially complementary to a part of said specific miRNA sequences, wherein said disorder is: a pathogen infection, preferably wherein said pathogen is a virus, and more preferably wherein said virus is an Epstein-Barr virus; a brain stroke; Alzheimer's disease; or Parkinson's disease. In some embodiments of the present invention, excessive or in sufficient in *vivo* cell death is associated with a disorder of particular tissue.

[0015] In one embodiment of the present invention, the body fluid is urine. In another embodiment, the present method of analysis of a urine sample includes a technique selected from the group consisting of hybridization, cycling probe reaction, polymerase chain reaction, nested polymerase chain reaction, PCR to analyze single strand conformation polymorphisms and ligase chain reaction. In yet another embodiment, nucleic acid degradation in said urine sample is reduced.

[0016] The method of the present invention includes reducing nucleic acid degradation comprising inhibiting nuclease activity by addition of RNAse inhibitor(s), heat inactivation, or by treating said urine sample with a compound selected from the group

consisting of: guanidine-HCl, guanidine isothiocyanate, N-lauroylsarcosine, and sodium dodecylsulphate. In one embodiment of the present invention, urine sample has been held in the bladder less than 12 hours.

[0017] In one embodiment of the present invention, the body fluid is serum. The method of the present invention includes analysis of a serum sample including a technique selected from the group consisting of hybridization, cycling probe reaction, polymerase chain reaction, nested polymerase chain reaction, PCR to analyze single strand conformation polymorphisms and ligase chain reaction.

[0018] The present invention provides a method of detecting in urine cell-free miRNAs, originating in different organs and tissues, including areas other than urinary system, in a subject as a result of disorder associated with excessive cell death in a tissue or organ, wherein the method is for evaluation of in vivo cell death in various tissues and organs comprising: obtaining a urine sample from a subject; and analyzing said urine sample for one or more specific sequences of miRNA wherein said analyzing comprises the step of detecting said miRNA with a primer and/or probe that is substantially complementary to a part of said specific miRNA sequences, wherein said disorder is: a pathogen infection, preferably wherein said pathogen is a virus and more preferably wherein said virus is an Epstein-Barr virus; a brain stroke; Alzheimer's disease; or Parkinson disease..

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] The foregoing and other objects, features, and advantages of the invention will be apparent from the more particular description of embodiments of the invention, as illustrated in the accompanying drawings. The drawings are not necessary to scale, emphasis instead being placed upon illustrating the principles of the invention.

Figure 1 is a photograph of a polyacrylamide gel electrophoresis of nucleic acids extracted from filtered urine using Q-Sepharose rM.

Figure 2 is a photograph of a polyacrylamide gel analysis of EBV derived BART1 miRNA specific RT-PCR product.

Figures 3A to 3G are dot plot representations of the normalized concentrations of miRNA in urine samples of patients at 12 and 24 hour time points after brain stroke.

Figure 4 is a diagram representing correlation between changes in miRNAs 129 and 219 concentrations and brain stroke outcome. The patients labeled as O and D improved their clinical status a month after stroke, and the clinical status of the patient labeled x has deteriorated a month after a stroke.

Figure 5 is a dot plot representation of the normalized concentrations of miRNA in unfiltered urine samples of patients with Alzheimer's disease and age matched controls.

Figure 6 is a dot plot representation of the normalized concentrations of miRNA in filtered urine samples of patients with Alzheimer's disease and age matched controls.

Figure 7 is a dot plot representation of the normalized concentrations of miRNA in serum samples of patients with Alzheimer's disease and age matched controls.

Figure 8 is a dot plot representation of the normalized concentrations of miRNA in urine samples of patients with Parkinson's disease and age matched controls.

Comparative Figure 9 is a dot plot representation of the normalized concentration ofmiRNA-9 in urine samples of women pregnant with Down syndrome and normal fetuses.

DETAILED DESCRIPTION OF THE INVENTION

[0020] The technology of this invention is based on the discovery that small RNAs, in particular specific micro RNAs (miRNAs), including transrenal miRNA (Tr-miRNA), are presented in bodily fluids and their concentrations reflect cell death associated with organ damage or other pathology. The presence of these nucleic acid sequences at levels lower or higher than that of a control group is therefore an indication that an abnormality or pathological condition is likely present in the patient from whom the sample was obtained.

[0021] The methods of the present invention offer improvements over previous methods of diagnosis, detection and monitoring due to their inherently non-invasive nature.

[0022] To facilitate the understanding of the invention, a number of terms are defined below.

The term "primer" refers to an oligonucleotide which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. An oligonucleotide "primer" can occur naturally, as in a purified restriction digest or be produced synthetically.

[0023] A primer is selected to be "substantially" complementary to a strand of specific sequence of the template. A primer must be sufficiently complementary to hybridize with a template strand for primer elongation to occur. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridize and thereby form a template primer complex for synthesis of the extension product of the primer.

[0024] A "target" nucleic acid is a miRNA sequence to be evaluated by hybridization, amplification or any other means of analyzing a nucleic acid sequence, including a combination of analysis methods.

[0025] "Hybridization" methods involve the annealing of a complementary sequence to the target nucleic acid (the sequence to be analyzed). The ability of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interaction is a well-recognized phenomenon. The initial observations of the "hybridization" process by Marmur and Lane, Proc. Natl. Acad. Sci. USA 46:453 (1960) and Doty et al., Proc. Natl. Acad. Sci. USA 46:461 (1960) have been followed by the refinement of this process into an essential tool of modern biology. Hybridization encompasses, but not be limited to, slot, dot and blot hybridization techniques.

[0026] It is important for some diagnostic applications to determine whether the hybridization represents complete or partial complementarity. For example, where it is desired to detect simply the presence or absence of pathogen miRNA, it is only important that the hybridization method ensures hybridization when the relevant sequence is present; conditions can be selected where both partially complementary probes and completely complementary probes will hybridize. Other diagnostic applications, however, may require that the hybridization method distinguish between partial and complete complementarity. It may be of interest to detect genetic polymorphisms.

[0027] The term "probe" as used herein refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, which forms a duplex structure or other complex with a sequence of another nucleic acid, due to complementarity or other means of reproducible attractive interaction, of at least one sequence in the probe with a sequence in the other nucleic acid. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labeled with any "reporter molecule," so that it is detectable in any detection system, including, but not limited to, enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is further contemplated that the oligonucleotide of interest *(i.e.,* to be detected) will be labeled with a reporter molecule. It is also contemplated that both the probe and oligonucleotide of interest will be labeled. It is not intended that the present invention be limited to any particular detection system or label.

[0028] As used herein, the term "miRNA" is a subclass of small non-coding single stranded RNA, approximately 18-23 nucleotides in length which plays an important role in regulation of metabolic processes, particularly due to their involvement in regulation of stability and translation of mRNA encoding specific proteins. miRNA also participate in other important processes, like heterochromatin formation and genome rearrangement.

[0029] The terms "excessive" and "insufficient" *in vivo* cell death describe the situation when the number of cells dying in a particular organ or tissue is respectively higher or lower than in age and gender matched controls.

[0030] As used herein, the terms "purified", "decontaminated" and "sterilized" refer to the removal of contaminant(s) from a sample.

[0031] As used herein, the terms "substantially purified" and "substantially isolated" refer to nucleic acid sequences that are removed from their natural environment, isolated or separated, and are preferably 60% free, more preferably 75% free, and most preferably 90% free from other components with which they are naturally associated. An "isolated polynucleotide" is therefore a substantially purified polynucleotide. It is contemplated that to practice the methods of the present invention polynucleotides can be, but need not be substantially purified. A variety of methods for the detection of nucleic acid sequences in unpurified form are known in the art.

[0032] As used herein, the terms "PCR product" and "amplification product" referto the resultant mixture of compounds aftertwo or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

[0033] The term "urinary tract" as used herein refers to the organs and ducts which participate in the secretion and elimination of urine from the body.

[0034] "Patient" or "subject" as the terms are used herein, refer to the recipient of the treatment. Mammalian and non-mammalian patients are included. In a specific embodiment, the patient is a mammal, such as a human, canine, murine, feline, bovine, ovine, swine, or caprine. In a particular embodiment, the patient is a human.

[0035] In another embodiment of the present invention, the detected miRNAs originate from and are specifically expressed in a specific cell type, tissue, or organ in the body, wherein alterations in the level of said miRNAs are indicative of chronic pathology of said tissue, such as for example Alzheimer's disease and Parkinson disease

[0036] In yet another embodiment of the present invention, the detected miRNAs originate from and are specifically expressed in a specific cell type, tissue, or organ in the body and can be used for prognosis of disease outcome. Changes in the levels of respective miRNAs, that are indicative of disease progression/regression, success of therapeutic or surgical intervention, are used for disease and treatment monitoring.

[0037] In another embodiment of the invention, the detected miRNAs originate from a pathogen and are used for infection diagnosis and monitoring. In a specific embodiment of the instant invention, the pathogen is a virus, for example Epstein-Barr virus.

[0038] In yet another embodiment of the invention, the detected miRNAs originate from cells of an infected organ and can be used for diagnosis support, evaluation of infected tissue damage, and further disease and treatment monitoring.

[0039] In some embodiments, the levels of cell- and/or tisue-specific miRNAs are normalized using the levels of ubiquitous miRNA in serum, the levels of albumin or creatinine in urine, or the levels of placenta-specific miRNAs for normalization of other tissue-specific fetal miRNAs.

[0040] In one aspect of the invention, the step of analyzing said urine sample to detect specific miRNAs includes a technique selected from the group consisting of hybridization, cycling probe reaction, polymerase chain reaction, nested polymerase chain reaction, PCR to analyze single strand conformation polymorphisms and ligase chain reaction.

[0041] In certain aspects of the invention, the nucleic add degradation in said urine sample is reduced. The method of reducing nucleic acid degradation comprises inhibiting nuclease activity by use of RNAse inhibitors, or by treating said urine sample with a compound selected from the group consisting of: guanidine-HCl, guanidine isothiocyanate, N-lauroylsarcosine, and sodium dodecylsulphate. In another aspect of the invention, said urine sample has been held in the bladder less than 12 hours.

[0042] In one embodiment of the present invention, the miRNA sequences measured are specifically related to tissues in the body, which may be selected from but are not limited to, lung, heart, liver, nervous system, brain, blood, kidney, bone, eye or pancreas.

[0043] The instant methods include isolation of miRNAs from the bodily fluids of the patients wherein a miRNA of interest may be detected in a body fluid selected from blood, plasma, serum, and urine. In one aspect of the instant invention, the miRNA is detected in urine. In another embodiment, the miRNA is detected in serum.

[0044] The instant method of the miRNA isolation of the instant invention can utilize commercially available anion exchange materials. Either strong or weak anion exchangers may be employed. By utilizing selected solutions for adsorption and elution, the

miRNA can be purified, concentrated, and substantially isolated.

[0045] By employing a solution at known ionic strength for the initial binding of the miRNA to the anion exchange column materials, most of the water soluble components including other electronegative molecules such as proteins (weakly-bound contaminants) can be washed through the column. For elution, the required ionic strength is reached by using known concentrations of a salt such as NaCl, which may be mixed with a buffer to control pH, ideally corresponding to the lowest ionic strength at which the nucleic acids will completely elute. Contaminating substances bound to the anion exchange resin with higher stringency than the nucleic acids may thereby be left within the column, *i.e.*, stronger bound contaminants are separated away from the nucleic acids.

[0046] A preferred weak exchanger is one in which primary, secondary, or tertiary amine groups (i.e., protonatable amines) provide exchange sites. The strong base anion exchanger has quaternary ammonium groups (i.e., not protonatable and always positively charged) as exchange sites. Both exchangers are selected in relation to their respective absorption and elution ionic strengths and/or pH for the miRNA being separated. The solution strengths are higher than the binding strengths.

[0047] In one aspect of the invention, a method is provided for isolation tranrenal miRNA from urine, the method comprising providing urine from a subject; optionally separating cells and cell debris from the urine by filtration or centrifugation; adding EDTA and Tris-HCl to the urine, adding silica free anion exchange resin to urine, incubating the mixture, removing the anion exchange medium from the urine, and eluting miRNA from the resin.

[0048] In one embodiment of the method of isolating miRNA from urine, the concentration of EDTA and Tris-HCl after it is added to the urine is in a range of 10-100 mM, and the pH of the solution is between about 8.0 and about 8.5.

[0049] In a further embodiment, the body fluid is optionally pre-filtered through a membrane prior to adsorption onto the anion-exchange medium.

[0050] In a further embodiment, the anion exchange medium is a sepharose-based resin functionalized with cationic quaternary ammonium groups. Examples of sepharose-based resins, functionalized with cationic ammonium groups include Q-Sepharose™ ANX-4 Sepharose™ Fast Flow, DEAE-Sepharose ™ , and Q-Sepharose-XL™ DEAE Sepharose Fast Flow (GE Healthcare).

[0051] In a further embodiment, the anion exchange medium is selected from sepharose-based quaternary ammonium anion exchange medium such as Q-filters or Q-resin.

[0052] In a further embodiment of the invention, the anion-exchange medium is immobilized on an individualized carrier wherein such a carrier is a column, cartridge or portable filtering system which can be used for transport or storage of the medium/nucleoprotein bound complex.

[0053] In another embodiment of the present invention, periodic analysis of miRNA sequences present, for example, in the urine samples of the same person can give early information about a pathological process in a particular organ or tissue. For example, miRNA122 is synthesized in liver only and increases in its amount may be a marker of hepatitis or another liver pathology. Alzheimer's syndrome can be accompanied by increases in the concentration of miRNA specifically expressed in neurons.

[0054] In another embodiment, more detailed monitoring of tissue-specific miRNA in the bodily fluid sample of the patient will be useful for estimation of a severity of the disease and for evaluation of effectiveness of therapeutic efforts.

[0055] In yet another embodiment, in combination with analysis of tumor-specific mutations the data on the tissue-specific miRNA can help in determination of tumor localization.

[0056] Other aspects of the instant invention relate to diseases caused by or accompanied by changes in a specific miRNA(s) expression. The described technology will help in diagnosis of such type pathologies.

[0057] In yet another embodiment, the application of the instant method may be extended to monitoring pharmacokinetics of synthetic siRNA in the patient's urine to enhance optimization of the siRNA drug design.

[0058] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, suitable methods and materials are described below. In

cases of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples described herein are illustrative only and are not intended to be limiting.

EXAMPLES

[0059] The examples are presented in order to more fully illustrate the various embodiments of the invention. These examples should in no way be construed as limiting the scope of the invention recited in the appended claims.

Example 1

Extraction of miRNA from urine:

[0060] *Urine collection:* For these experiments, urine specimens from patients or volunteers were collected in a sterile 110 ml urine collection cup and were immediately supplemented with EDTA up to final concentration between 10 and 150 mM, preferably 50 mM. Specimens were stored in 10 - 50 ml aliquots at-80°C. Optional filtration of urine was carried out on Stericup™ (Millipore, Vacuum Driven Filtration System, 0.45 µ Durapore™ filter) immediately after specimen collection before the EDTA was added.

[0061] Q *Binding:* In a 50 ml tube 20 mL of urine was diluted with equal volume of 50 mM EDTA (pH 8.0) and 50 mM Tris-HCl (pH 8.0) which was then supplemented with 1-2 ml of Q-Sepharose™ (GE Healthcare; 17-0510-10) slurry and rigorously mixed 10 - 30 min at room temperature. The resin, with bound nucleic acids, was collected by centrifugation at 2000g for 5 minutes at room temperature in a table top clinical centrifuge using a swing bucket rotor. All bull 500 µl of supernatant was removed by aspiration. The resin pellet was resuspended in the remaining supernatant and transferred to a Micro Bio-Spin Chromatography Column (Bio-Rad) or equivalent, which was operated either by centrifugation of vacuum. The resin in the column was washed three times with 500 µl 2xSSC (300 mM NaCl/30 mM sodium citrate (pH 7.0)) or with buffer with comparable ionic strength (*e.g.* 300 mM NaCl or LiCl). Nucleic acids can be eluted from Q-Sepharose with high ionic strength (*e.g.* 1 M NaCl or LiCl) but the methods described below preserves RNA better.

[0062] *Elution from Q-Sepharose™ and TRIzol™ Phase Separation:* Bound nucleic acids were further eluted with 500 µl of TRIzol™ reagent (Invitrogen). The extraction of nucleic acids from TRIzol was carried out according manufacturer's recommendations. Briefly, for phase separation TRIzol eluate was supplemented with 100 µl chloroform, mixed vigorously, incubated at room temperature for 3 - 5 minutes and centrifuged at 12,000xg for 15 min at 4°C. While avoiding touching the interphase, 300 µl of the upper phase was transferred into a fresh centrifuge tube. Then the nucleic acids were precipitated or additionally cleaned and desalted on a silica column.

[0063] *Nucleic acid precipitation:* For nucleic acid precipitation, the above described preparation was supplemented with 1 µl of 20 mg/mL glycogen (Roche) and 300 µl of 100% isopropyl alcohol. Nucleic acids were collected by centrifugation, the pellet was washed twice with 200 µl of 70% ethanol, allowed to air dry for 5 min at room temperature, and then the nucleic acids were dissolved in 30 µl of 0.1 mM EDTA/1x RNA Secure (Ambion). The samples were incubated at 60 °C for 10 min to inactivate any residual RNase activity.

[0064] *Silica Column cleaning of nucleic acids:* For binding to a silica column (Qiagen PCR clean columns or equivalent) 3 volumes of 96% ethanol were added to nucleic acid preparation from the TRIzol upper phase, and, after 3 minutes incubation at room temperature, the mixture was loaded onto the column. The column was washed twice with 500 µl 2 M LiCl/80% ethanol and twice with 500 µl 80% ethanol. Nucleic acids were eluted with 50 µl of 0.1 mM EDTA/1x RNA Secure (Ambion). The samples were incubated at 60 °C for 10 min to inactivate any residual RNase.

[0065] *DNase I and RNase A Digestion:* To verify the nucleic acid identity of the material extracted from urine with the above described protocol, the instant prep was digested with DNase I and/or RNase A. DNase I digestion was carried out in the DNase I Reaction Buffer (NEB) containing 2 units of RNase free DNase I (NEB). RNase A digestion was performed in TE buffer supplemented with 50 ng/mL boiled RNase A. Samples were incubated at 37 °C for 60 min and after addition of loading dye samples were subjected to electrophoresis on 5% polyacrylamide 1x TBE gels and stained with 1/10000 diluted SYBR® Gold (Invitrogen). As shown in Figure 1, the isolated material represents low molecular weight nucleic acids, mainly RNA and their fragments. In addition, (see Figure 1), for comparison nucleic acids from Q-resin were eluted by 3 M NaCl, lanes 2 and 3, and Trizol™, lanes 4 and 5.

[0066] In the Figure 1, lanes I and 5, represent nucleicacids isolated with high salt and TriZol elution from Q-Sepharose, respectively; lanes 2 and 6; 3 and 7; 4 and 8, represent nucleic acids after treatment with DNAse, RNAse, or DNAse plus RNAse, respectively.

[0067] Also, to demonstrate existence and molecular size of RNA, RNA aliquots of purified nucleic acids were digested with DNasel, lanes 3 and 5.

Extraction of RNA from serum

[0068] For these experiments, 1.2 ml of TRIzol LS were added to 0.4 ml of serum, and the mixture was centrifuged 10 at 14,000 rpm. The supernatant was transferred into a 2 ml Eppendorf tube, 0.3 ml of chloroform was added, and the mixture was shaken for 15 seconds. After centrifugation at 14,000 rpm for 15 min, the supernatant was transferred into a 5 ml tube and ethanol was added up to final concentration of 70%. The mixture was loaded on a Quiagen Quick column on a vacuum manifold, and the column was washed twice with 0.5 ml of 2M LiCl-80% EtOH, once with 0.5 ml of 80% ethanol-80 mM sodium acetate (pH 5.0), and finally with 0.5 ml of 95% ethanol. The column was centrifuged in 1.5 ml Eppendorf tube 3 min at 14,000 rpm, and RNA was eluted with 40 µl H_2O.

Example 2

[0069] This Example demonstrates that miRNA, from dying cells, cross the kidney barrier and may be detected in the urine of a patient.

Detection of human miRNA molecules in urine

[0070] Micro RNA species that were analyzed in this example can be grouped in three distinct types, namely ubiquitous miRNAs, which are expressed in all or multiple tissues, tissue-specific miRNAs, and miRNAs in which expression is significantly altered in a particular tissue or cell type. As shown by Table 1, 20 different miRNAs were obtained from urine of 16 healthy volunteers and enrolled donors and later detected by real time RT-PCR using commercially available miRNA expression analysis kit (ABI). Corresponding synthetic miRNA oligonucleotides were used as standards. Reactions were carried out strictly as recommended by the supplier.

Table 1. Detected miRNA

SEQ ID NO:	ID	Sequence	Expression
1	hsa-miR-127	UCGGAUCCGUCUGAGCUUGGCU	Brain overexpressed
2	hsa-miR-153	UUGCAUAGUCACAAAAGUGA	Brain overexpressed
3	hsa-miR-129	CUUUUUGCGGUCUGGGCUUGC	Brain-specific
4	hsa-miR-137	UAUUGCUUAAGAAUACGCGUAG	Brain overexpressed
5	hsa-miR-218	UUGUGCUUGAUCUAACCAUGU	Ubiquitous, Brain overexpressed
6	hsa-miR-219	UGAUUGUCCAAACGCAAUUCU	Brain-specific
7	hsa-miR-128a	UCACAGUGAACCGGUCUCUUUU	Brain-specific
8	hsa-miR-9	UCUUUGGUUAUCUAGCUGUAUGA	Brain overexpressed
9	hsa-miR-138	AGCUGGUGUUGUGAAUC	Brain, Thyroid
10	hsa-miR-134	UGUGACUGGUUGACCAGAGGG	Brain and several other tissues
11	hsa-miR-124a	UUAAGGCACGCGGUGAAUGCCA	Brain-specific
12	hsa-miR-122a	UGGAGUGUGACAAUGGUGUUUGU	Liver-specific
13	hsa-miR-133a	UUGGUCCCCUUCAACCAGCUGU	Heart and Muscle overexpressed
14	hsa-miR-1	UGGAAUGUAAAGAAGUAUGUA	Heart and Muscle overexpressed
15	hsa-miR-335	UCAAGAGCAAUAACGAAAAAUGU	Ubiquitous

SEQ ID NO:	ID	Sequence	Expression
16	hsa-miR-16	UAGCAGCACGUAAAUAUUGGCG	Ubiquitous
17	hsa-miR-215	AUGACCUAUGAAUUGACAGAC	Small intestine and colon overexpressed
18	hsa-miR-135b	UAUGGCUUUUCAUUCCUAUGUG	Placenta-overexpressed
19	hsa-miR-517c	AUCGUGCAUCCUUUUAGAGUGU	Placenta-overexpressed
20	hsa-miR-518e	AAAGCGCUUCCCUUCAGAGUGU	Placenta-overexpressed

[0071] All three types of miRNA were detected in most preps of urinary RNA. The highest copy numbers were characteristic of ubiquitous miRNA. However, tissue-specific miRNA or miRNA over-expressed in a particular tissue or cell type were also detectable. It has been unequivocally demonstrated that a portion of miRNA from dying cells is not degraded but appears in the bloodstream and is finally excreted into urine.

Example 3

[0072] The Example demonstrates that miRNA from human nasopharyngeal carcinoma (NPC) cells can cross the patient's kidney barrier and can be detected in patient's urine by real time RT-PCR.

Virus-derived miRNA in urine

[0073] It is known that some viruses also encode and produce miRNAs. Since Epstein-Barr virus (EBV) is involved in development of nasopharyngeal carcinoma (NPC), the instant system was used to find out if viral miRNA from NPC cells can reach a patient's urine and be detected there. Urine samples from NPC patients were collected and stored according to the procedures described in the Example 1 of this application. EBV infection was confirmed by the detection of virus-specific DNA sequences in urine. Urine collected from healthy donor was negative for EBV specific DNA sequences. Two EBV-specific miRNAs BART3-3p and BART1-3p were analyzed in this study:

BART3-3P CGC ACC ACU AGU CAC CAG GUG U SEQ ID NO:21

BART1-3P UAG CAC CGC UAU CCA CUA UGU CU SEQ ID NO:22

[0074] Reverse transcription was performed in 15 µl, one tenth of the RT reaction was subjected to PCR amplification using JumpStart DNA polymerase from Sigma. The following primers were used at 500 nM concentration:

ID	Sequence	SEQ ID NO:
BART3-3PRT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CAC CT	23
BART1-3PRT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GAC AT	24
BART3-3PF	CGC CGC ACC ACT AGT CAC	25
BART1-3PF	CGC TAG CAC CGC TAT CCA	26
miRNACR	GTG CAG GGT CCG AGG T	27

[0075] Products were analyzed in 15% polyacrylamide gel (PAAG).

[0076] As demonstrated by Figure 2, both BART3 and BART 1 miRNA species were detected in urine from NPC patients but not in the urine sample from a healthy donor. These data again indicate that miRNA from dying cells located outside of urinary system can be detected in the urine. In the Figure 2, lane 1, represents markers; lanes 2 and 3, represent patients with nasopharyngeal carcinoma, lanes 4 and 5, represent control patients, and lane 6, represents positive control, which represents respective synthetic miRNAs.

Example 4

[0077] This example demonstrates that the neuronal death caused by stroke can be registered in *vivo* by measurements of the concentrations of neuron-specific miRNA in the patient's urine.

Brain stroke diagnosis

[0078] For these experiments, patients with brain stroke were investigated for analysis of changes in concentrations of brain-specific miRNAor miRNA which are over-expressed in brain, after stroke. Since currently it is not known in what brain cell types and in what brain areas these miRNA are expressed, 9 different brain specific miRNA were studied.

[0079] ***Patients:*** Urine samples were collected from patients accepted at a hospital through the emergency room. Diagnosis of brain stroke was based on clinical symptoms. Urine samples were collected at 12 and 24 hours after the stroke. Control urine samples were donated by age matched volunteers but without stroke symptoms. Samples were collected and stored according to the procedures described in the Example 1 of this application.

[0080] *miRNA species:* miRNA from urine was extracted according to the procedure described in the Example 1. An amount of RNA equivalent to that isolated from 675 µl of urine underwent reverse transcription PCR and 1/10 of the RT-PCR mixture underwent final real time PCR, which was carried out using the protocol provided by the manufacturer. Data obtained were normalized for individual kidney filtration rates by re-calculation per creatinine concentration in urine. For these experiments, urine samples collected from healthy donors from same age group were used as baseline. Different miRNA species are presented as follows:

1. A. hsa-mir-128a
2. B. hsa-mir-9
3. C. hsa-mir-127
4. D. hsa-mir-137
5. E. hsa-mir-129
6. F. hsa-mir-219
7. G. hsa-mir-218

[0081] Results summarized in Figures 3A to 3G clearly demonstrate that after brain stroke, there is a significant increase in the levels of several brain specific miRNA (128a, 129, 218, 219)reflecting kinetics of the brain cell death.

Example 5

[0082] This example demonstrates that kinetics of the miRNA concentrations in patient's urine after stroke provides information about disease outcome.

Brain stroke monitoring

[0083] For the experiments, patients with brain stroke were investigated for analysis of correlation between changes in concentrations of brain-specific miRNA and disease development.

[0084] ***Patient:*** Urine samples were collected from patients accepted at a hospital through the emergency room. Diagnosis of brain stroke was based on clinical symptoms and MRI analysis. Urine samples were collected at 12, 24, 48 hours and a week after the stroke. Patient clinical status was evaluated 30 days after stroke. Control urine samples were donated by age matched volunteers but without stroke symptoms. Samples were collected and stored according to the procedures described in the Example 1 of this application.

[0085] *miRNA species:* miRNA from urine was extracted according to the procedure described in Example 1 and analyzed with

TaqMan miRNA assays (Applied Biosystems). An amount of RNA equivalent to that isolated from 400 µl of urine underwent reverse transcription PCR and 1/10 of the RT-PCR mixture underwent final real time PCR, which was carried out using the protocol provided by the manufacturer. Data obtained were normalized for individual kidney filtration rates by re-calculation per creatinine concentration in urine. For these experiments, urine samples collected from healthy donors from same age group were used as baseline.

[0086] Results summarized in Figures 4A and B clearly demonstrate that the dynamics of changes in Tr-miRNA 129 and Tr-miRNA 219 after brain stroke are different in different patients and correlates with the disease development. The increase in neuronal death a week after stroke in patient #3 corresponds to worsening in the patient clinical status. At the same time two patients, whose transrenal neuron-specific miRNA had tendency to normalization, demonstrated significant improvement.

Example 6

Alzheimer's disease diagnosis

[0087] Alzheimer's disease is a progressive neurological disease that is caused by the death of neurons, particularly in the cortex and hippocampus. The diagnosis is based on neurological examination and the exclusion of other causes of dementia whereas the definitive diagnosis can be made only at autopsy. The instant invention demonstrates that excessive neuronal death characterizing Alzheimer's disease may be monitored by measuring levels of specific brain miRNAs isolated from the patient's urine.

[0088] For these experiments, patients diagnosed with Alzheimer's disease were investigated for analysis of changes in concentrations of brain-specific or over-expressed miRNA as a result of neuronal death.

[0089] *Patients:* Urine and serum samples were collected from patients diagnosed with various stages of the Alzheimer's disease. Control urine and serum samples were donated by age matched volunteers but without symptoms of Alzheimer's disease. Samples were collected and stored according to the procedures described in the Example 1 of this application. Some urine samples were filtered after collection as described in Example 1 to delete cells and cell debris.

[0090] *miRNA species:* RNAfrom urine and serum was extracted according to the procedures described in the Example 1.

[0091] In one set of experiments an amount of RNA equivalent to that isolated from 750 µl of urine underwent reverse transcription PCR and 1/10 of the RT-PCR mixture underwent final real time PCR, which was carried out using the protocol provided by the manufacturer (Applied Biosystems). Data obtained were normalized for individual kidney filtration rates by re-calculation per creatinine concentration in urine. Figure 5 clearly demonstrates that concentrations of several brain specific miRNAs is increased in the urine of Alzheimer's patients.

[0092] In another set of experiments, RNA isolated from filtered urine or serum was analyzed. An amount of RNA equivalent to that isolated from 0.6 ml of urine or 50 111 of serum underwent reverse transcription PCR and 1/10 of the RT-PCR mixture underwent final real time PCR, which was carried out using the protocol provided by the manufacturer (Applied Biosystems). Data obtained for urinary miRNA were normalized for individual kidney filtration rates by re-calculation per creatinine concentration in urine. Data obtained for plasma miRNA were normalized per ubiquitous miRNA-16. Figures 6 and 7 show that the levels of some neuron-specific miRNAs are higher in both filtered urine and serum of the Alzheimer's patients compared to age-matched controls.

Example 7

Parkinson's disease

[0093] Parkinson's disease is a degenerative disorder of the central nervous system that often impairs the sufferer's motor skills and speech. The instant invention demonstrates that excessive cellular death of dopaminergic neurons, characterizing Parkinson's disease may be monitored by measuring levels of specific brain miRNAs isolated from the patient's urine.

[0094] For these experiments, patients diagnosed with Parkinson's were investigated for analysis of changes in concentrations of brain-specific miRNA or over-expressed miRNA as a result of neuronal death.

[0095] *Patients:* Urine samples were collected from patients diagnosed with various stages of the Parkinson's disease. Control urine samples were donated by age matched volunteers without symptoms of Parkinson's disease. Samples were collected and stored according to the procedures described in the Example 1 of this application.

[0096] *miRNA* species: For these experiments, RNA from urine was extracted according to the procedure described in the Example 1. Amount of RNA equivalent to that isolated from 750 f-ll of urine underwent reverse transcription PCR and 1/10 of the RT-PCR mixture underwent final real time PCR, which was carried out using the protocol provided by the manufacturer (Applied Biosystems). Data obtained were normalized for individual kidney filtration rates by re-calculation per creatinine concentration in urine. Figure 8 demonstrates that concentrations of several brain specific miRNAs is increased in the urine of the patients with Parkinson disease.

Example 8

Prenatal Testing for Pregnancy-Related or Fetal Diseases

[0097] The principal finding of permeability of the kidney barrier for miRNA molecules opens the way for the use of maternal urine to perform completely noninvasive prenatal diagnosis of congenital diseases. One can perform such a noninvasive screen as follows.

[0098] First, a sample of urine is gathered from a pregnant patient. Where desired, miRNA in the urine sample is then be isolated, purified and/or treated to prevent degradation using methods described above. MiRNA profiling is then performed using quantitative PCR or miRNA array and the data obtained are used to determine different fetal pathologies, as described for other pathologies above.

Comparative Example 9

Down syndrome

[0099] For the experiments, differences in concentrations of brain-specific miRNA in maternal urine between women pregnant with normal and Down syndrome fetuses were investigated.

[0100] *Patients:* Urine samples were collected from pregnant women diagnosed with Down syndrome by amniocentesis. Control urine samples were donated by age matched women with normal pregnancies. Samples were collected and stored according to the procedures described in the Example I of this application.

[0101] *miRNA species:* miRNA from urine was extracted according to the procedure described in the Example 1. An amount of RNA equivalent to that isolated from 750 µl of urine underwent reverse transcription PCR and 1/10 of the RT-PCR mixture underwent final real time PCR, which was carried out using the protocol provided by the manufacturer. Data obtained were normalized per placenta-specific miRNA 518. Figure 9 demonstrates lower concentration the brain-specific miRNA 9 in urine of women pregnant with Down syndrome fetuses compared to urine of women with normal pregnancies, which indicates insufficient cell death compared to respective controls.

REFERENCES CITED IN THE DESCRIPTION

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P a t e n t k r a v :

1. Fremgangsmåde til detektion og kvantificering af celle-, vævs- og/eller organ-specifikke cellefrie miRNAs i kropsvæske til evaluering af *in vivo* celledød i forskellige væv og organer, hvorved in vivo celledød er associeret med en forstyrrelse i et bestemt væv og/eller organ, omfattende:

analysering af en kropsvæskeprøve valgt fra blod, serum og urin, der er opnået fra et individ, for en eller flere specifikke sekvenser af miRNA, hvor analyseringen omfatter trinnet detektering af miRNA med en primer og/eller probe, der i hovedsagen er komplementær med en del af de specifikke miRNA-sekvenser,
hvorved forstyrrelsen er

(a) en patogeninfektion, hvor patogenet fortrinsvis er et virus og, mere fortrinsvis, hvor virusset er et Epstein-Barr-virus,
(b) hjerneslag,
(c) Alzheimers sygdom eller
(d) Parkinsons sygdom.

2. Fremgangsmåde ifølge krav 1, hvorved eksessiv eller utilstrækkelig *in vivo* celledød er associeret med en forstyrrelse i et bestemt væv.

3. Fremgangsmåde ifølge krav 1, hvorved trinnet analysering omfatter en teknik, der er valgt fra gruppen bestående af hybridisering, cycliseringssondereaktion, polymerasekædereaktion, nested polymerase-kædereaktion, PCR til analyse af enkeltstrengskonformationspolymorfismer og ligasekædereaktion.

4. Fremgangsmåde ifølge krav 1, hvorved nukleinsyrenedbrydning i en urinprøve reduceres.

5. Fremgangsmåde ifølge krav 4, hvorved reducering af nukleinsyre-nedbrydning omfatter inhibering af nukleaseaktivitet ved tilsætning af RNAse-inhibitor(er), varmeinaktivering eller ved behandling af urinprøven med en forbindelse valgt fra gruppen bestående af:

guanidin-HCl, guanidinisothiocyanat, N-lauroylsarcosin og natriumdodecyl-sulphat.

6. Fremgangsmåde ifølge krav 1, hvorved en opnået urinprøve har været holdt i blæren i mindre end 12 timer.

7. Fremgangsmåde til detektering i urin af cellefrie miRNAs, der stammer fra andre områder end urinsystemet, hos et individ som følge af en forstyrrelse, der er associeret med eksessiv celledød i et væv eller organ, hvor fremgangsmåden er til evaluering af *in vivo* celledød i forskellige væv og organer, omfattende:

analysering af en urinprøve, der er opnået fra et individ, for en eller flere specifikke sekvenser af miRNA, hvorved analyseringen omfatter detektering af miRNA med en primer og/eller sonde, der i hovedsagen er komplementær med en del af de specifikke miRNA-sekvenser,
hvorved forstyrrelsen er:

(a) en patogeninfektion, hvor patogenet fortrinsvis er et virus og, mere fortrinsvis, hvor virusset er et Epstein-Barr-virus,
(b) hjerneslag,
(c) Alzheimers sygdom eller
(d) Parkinsons sygdom.

DRAWINGS

Figure 1

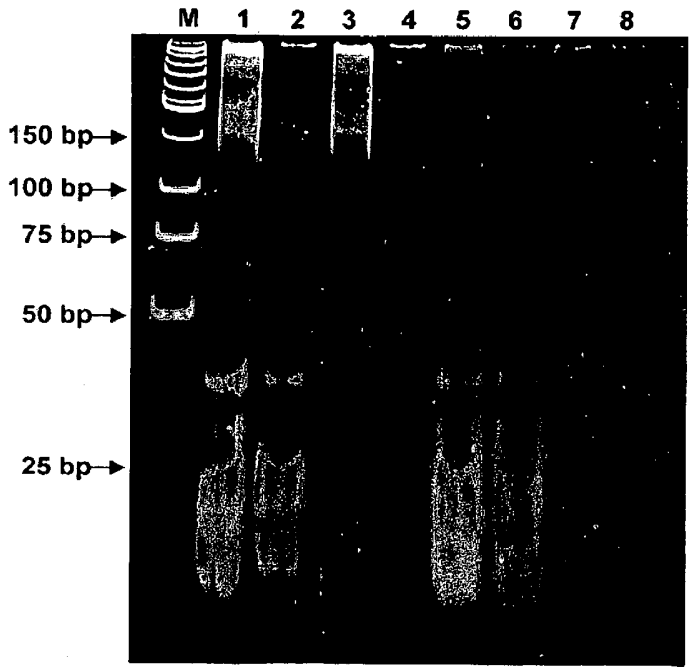


Figure 2

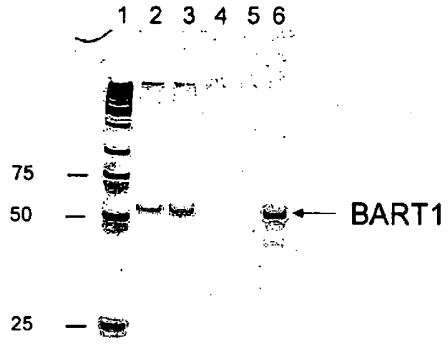
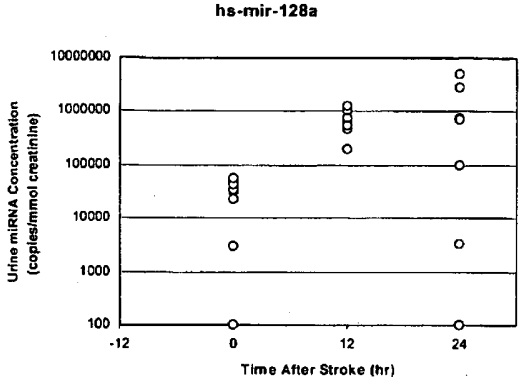
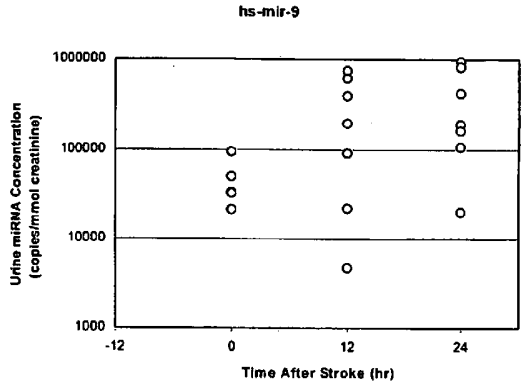


Figure 3

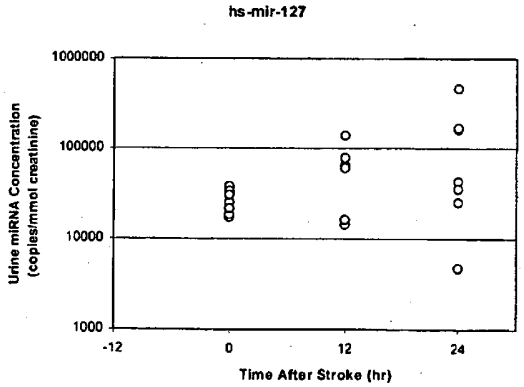
A


hs-mir-128a
Urine miRNA Concentration (copies/mmol creatinine)
10000000
1000000
100000
10000
1000
100
-12
0
12
24
Time After Stroke (hr)

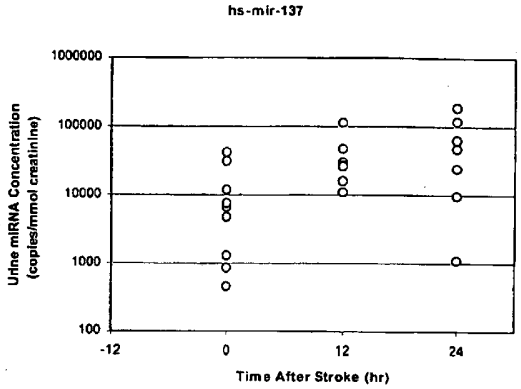
B


hs-mir-9
1000000
100000
10000
1000
Urine miRNA Concentration (copies/mmol creatinine)
-12
0
12
24
Time After Stroke (hr)

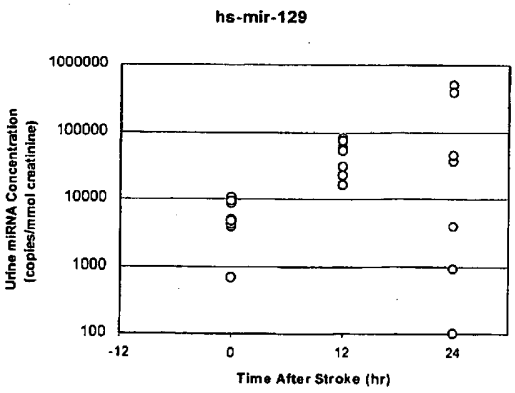
C


hs-mir-127
1000000
100000
10000
1000
Urine miRNA Concentration (copies/mmol creatinine)
-12
0
12
24
Time After Stroke (hr)

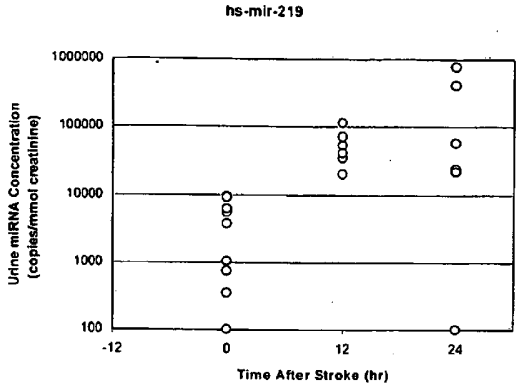
D


hs-mir-137
Urine miRNA Concentration (copies/mmol creatinine)
1000000
100000
10000
1000
100
-12
0
12
24
Time After Stroke (hr)

E


hs-mir-129
Urine miRNA Concentration (copies/mmol creatinine)
1000000
100000
10000
1000
100
-12
0
12
24
Time After Stroke (hr)

F


hs-mir-219
Urine miRNA Concentration (copies/mmol creatinine)
1000000
100000
10000
1000
100
-12
0
12
24
Time After Stroke (hr)

G

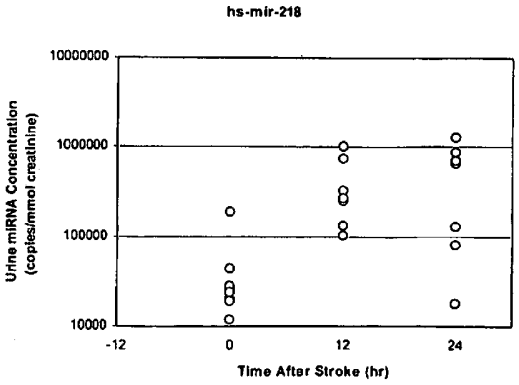

hs-mir-218
Urine miRNA Concentration (copies/mmol creatinine)
10000000
1000000
100000
10000
-12
0
12
24
Time After Stroke (hr)

Figure 4

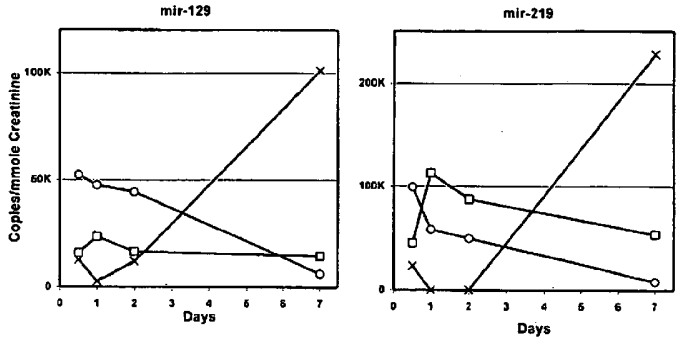
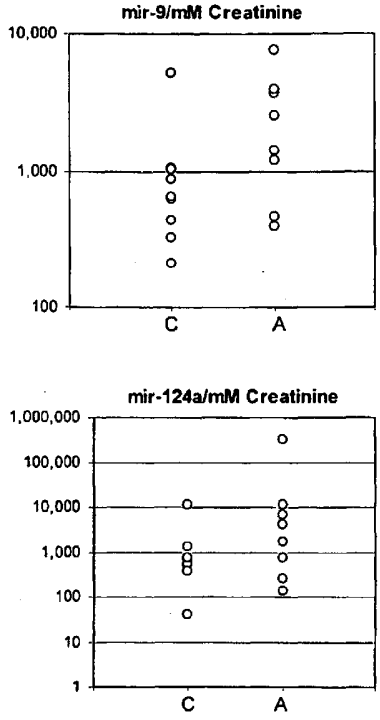
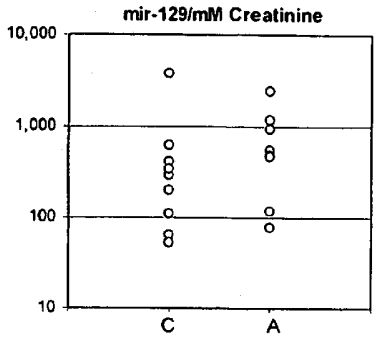
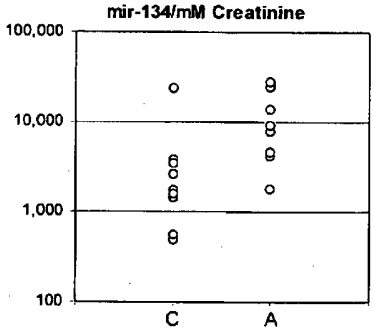

mir-129
mir-219
Copies/mmole Creatinine
100K
50K
0
200K
150K
0
1
2
3
4
5
6
7
Days
Days

Figure 5


mir-9/mM Creatinine
10,000
1,000
100
C
A
mir-124a/mM Creatinine
1,000,000
100,000
10,000
1,000
100
10
1
C
A


mir-129/mM Creatinine
10,000
1,000
100
10
C
A


mir-134/mM Creatinine
100,000
10,000
1,000
100
C
A

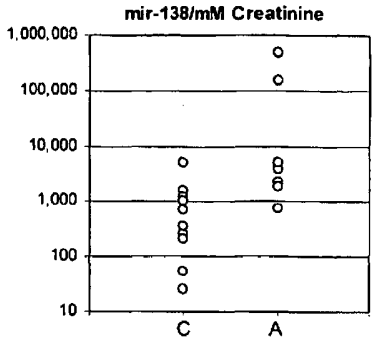
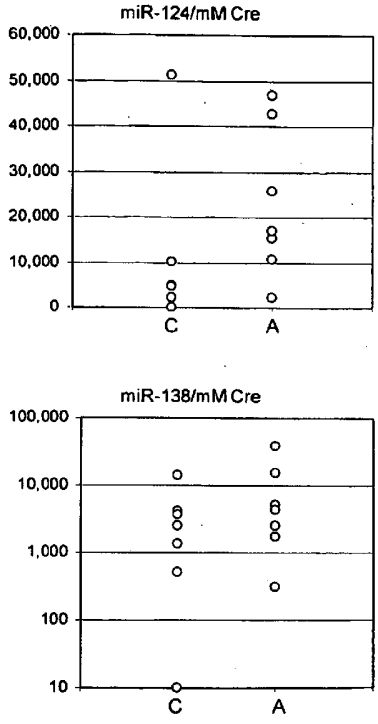

mir-138/mM Creatinine
1,000,000
100,000
10,000
1,000
100
10
C
A

Figure 6


miR-124/mM Cre
60,000
50,000
40,000
30,000
20,000
10,000
0
C
A
miR-138/mM Cre
100,000
10,000
1,000
100
10
C
A

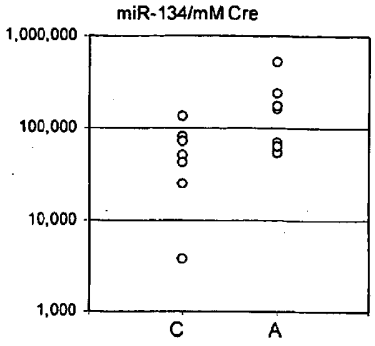
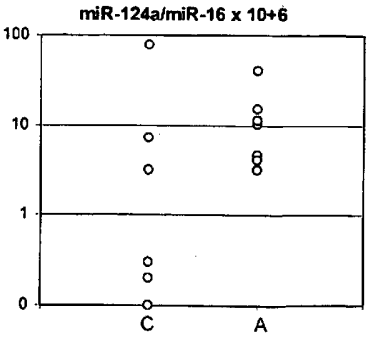
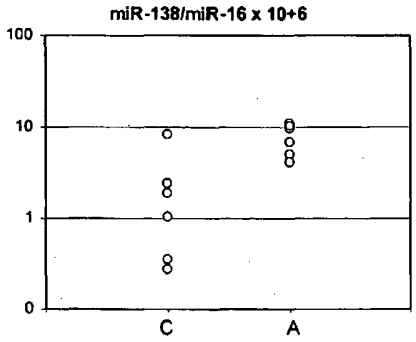

miR-134/mM Cre
1,000,000
100,000
10,000
1,000
C
A

Figure 7


miR-124a/miR-16 x 10+6
100
10
1
0
C
A


miR-138/miR-16 x 10+6
100
10
1
0
C
A

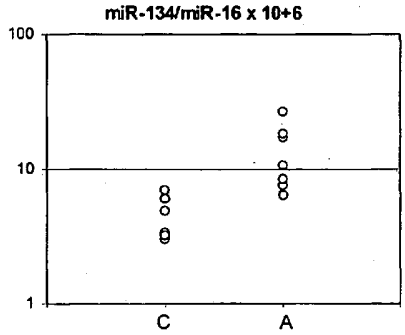
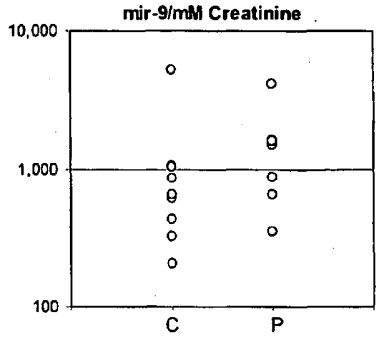
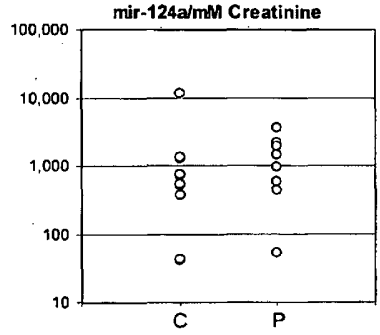
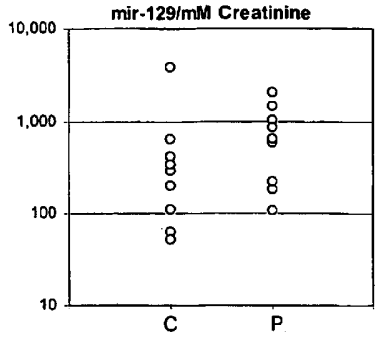

miR-134/miR-16 x 10+6
100
10
1
C
A

Figure 8


mir-9/mM Creatinine
10,000
1,000
100
C
P


mir-124a/mM Creatinine
100,000
10,000
1,000
100
10
C
P


mir-129/mM Creatinine
10,000
1,000
100
10
C
P

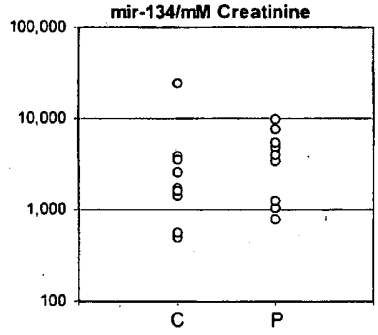
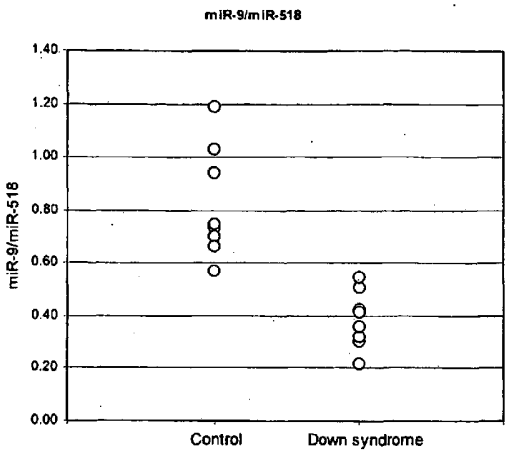

mir-134/mM Creatinine
100,000
10,000
1,000
100
C
P

Figure 9


miR-9/miR-518
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
miR-9/miR-518
Control
Down syndrome