

METHOD FOR ISOLATING SEPARATE TYPES OF MICRO-RNA FROM BIOLOGICAL FLUIDS CONTAINING EXOSOMES

Invention description.

The separation method of specific types of miRNAs from exosome-containing biological fluids.

The invention belongs to molecular biology, in particular, to the separation method of specific types of miRNAs from exosome-containing biological fluids and is intended to separate various types of miRNAs from different exosome-containing biological samples for the assessment of individual exosomal fractions.

Exosomes are small, 20-100 nm, spherical bubbles used for cell interaction. The exosomes contain the complexes of proteins and miRNAs valued as diagnostics markers and can be used for antitumor immune response forming in oncology. However, to use exosomes effectively, it is necessary to get net fraction.

Exosomes are extremely stable in biological fluids, blood, urine, saliva, ascites or cell culture supernatant in the case of culturing. This makes make available non-invasive diagnosis of tumor origin exosomes in plasma and urine.

Exosomes are actively separated by the cells of the immune system – dendritic cells and B cells, by neurons, secretory tissues and tumor cells in particular [K. Denzer et al. 2000, Huber et al. 2005, P. Wewers 2009]. In case of dendritic cells, exosomes are separated. They contain small (8-12 aminoacid) presented polypeptides in complex with the antigen of MHC I class molecules, connecting to the relevant receptors on cytotoxic CD8+ T-cells, NK-cells, as well in complex with MHC II class molecules, containing large (12-16 aminoacid) presented polypeptides, to the relevant receptors cytotoxic CD8+ T-cells. Exosomes separated by tumor cells can interfere with cytotoxic T-cells antitumor immune response forming due to the competition with DC or macrophages exosomes, considering that the concentration of exosomes increases in blood plasma/serum approximately 50 times in case of developed cancer [Taylor et al 2008, Iero 2008].

Exosomes contain MHC I and MHC II class molecules of histocompatibility complex, Rab-proteins of integrins, as well as molecules and patterns of miRNAs defined by tissue specificity and individual characteristics of type of cells. So it has been observed that human urine exosomes contain 295 proteins [Pisitkun et al. 2004]. About 50 proteins are regularly observed in all exosomes [Gonzales et al. 2009]. In case of tumors of tissue-specific pattern of proteins and tumor-specific pattern of miRNAs can be used for early diagnosis or justification of treatment regimens [Baj-Krzyworzeka et al. 2007].

To study tumor-specific exosomes, it is necessary to conduct their custom separation and differentiation. The concentration of specific miRNA is a criterion for exosome selection from specific cells.

There is a method used for the separation of exosomes from biological fluids (see http://tsitologiya.cytspb.rssi.ru/54_5/shtam_ms.pdf), where culture fluid (KC) is gathered and successive centrifugation at 2000 and 10000 g conducted. Then the obtained KC of 500 ml is concentrated with the help of ultra filtration (Centrikon plus-70, 100 kDa, Millipore) up to final volume of 10 ml. In what follows, ultra-centrifugation using the Avanti

30I (JA-30.50 rotor) centrifuge at 100000 g during 2 hours is used in order to separate exosomes from the obtained concentrated KC agents. After centrifugation the supernatant liquid is put into a separate test tube and studied using laser correlation spectroscopy (LCS) to check the absence of exosomal-size particles. The deposit is dissolved in maximal volume of phosphate-saline buffer (PBS) and centrifuged under the same conditions again. The obtained deposit is dissolved in 100 microliter of water (MiliQ) or PBS, separated into aliquots that are frozen at -80°C for further proteomic analysis.

The well-known method does not give an opportunity to separate exosomes into various factions belong to different tissues, and, therefore, to assess the level of miRNA in the selected samples.

The technical effect of the declared method is the possibility to separate individual types of miRNA from exosome-containing biological fluids, in particular, the possibility of separation of specific types, such as tissue-specific and tumor-specific miRNA in the future will give an opportunity to prepare vaccines based on exosomes from tumor cells.

The above stated technical effect is achieved due to the use of the separation method of specific types of miRNAs from exosome-containing biological fluids, including successive centrifugation at 2000 and 10000 g, ultra filtration of the obtained supernatant fluid using rotary concentrator Centrikon plus-70, 100 kDa, Millipore up to the final volume of 10 ml and ultra-centrifugation using the Avanti 30I (JA-30.50 rotor) centrifuge at 100000 g during 2 hours, the dissolution of the obtained deposit in PBS, re-centrifugation under the same conditions and the dissolution of the obtained deposit in 100 microliter of water. According to the present invention, then the obtained solution goes through free flow electrophoresis in bunkers for anode and cathode stabilization using FFE System with the further selection of separate fractions under the following conditions: the total time of free flow electrophoresis is 5 minutes, the impressed voltage – 900 V, current – 34 mA, the sample and marker are injected at a flow rate of 2.5 ml/h and 280 ml/h respectively, the range is conducted in FF-ZE cyclical interval at 85 ml/h and the fractioned sample is eluted at 320 mp/h, the following buffer is used for anode stabilization: 1,5 M of thiourea + 6 M of urea + 100 mM of H_2SO_4 + 25 mM of 2-pyridine-propanol + 300 mM of 2-pyridine-ethanol.

The medium for the division has the following composition: 1,8 M of thiourea + 6 M urea + 55 mM of MOPS + 55 mM of 2-pyridine-propanol; the following buffer is used for cathode stabilization: 1,8 M of thiourea + 76 M of urea + 180 mM of NaOH + 25 mM of Tris + 35 mM of MOPS + 280 mM of EPPS, afterwards, separate fractions are ultra-centrifuged again using the Avanti 30I (JA-30.50 rotor) centrifuge at 100000 g during 2 hours. Then the exudation of miRNAs from every separate fraction is conducted, i.e. 5 mM of K proteinase is added to every separate fraction that is incubated at 56 C within one hour, then it is applied to the Exiqon column with the further centrifugation at 2000 g. Then the column is flushed three times using the flushing buffer. Each flushing is followed by the further centrifugation at 2000 g using the eppendorf 5104 centrifuge. Then 20 microliter of nuclease free water is applied to the column which is left for 10 minutes at room temperature and then centrifuged at 3000 g. It is followed by the reverse transcription reaction using reverse transcriptase (Exiqon), namely 8 microliter of the reagent is added to 8 microliter of the sample and incubated during one hour at 60 C. Afterwards, the reaction is stopped by heating up to 95 C during 5 minutes.

Then the polymerase chain reaction with a real-time detection using 7500 Applied Biosystems with primers on specific types, such as tissue-specific and tumor-specific miRNA, are used to define specific types of miRNA, namely: miR-141, miR-200a, miR-200c, miR-203, miR-205, miR-214, miR-17-3p, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-301a, miR-326, miR-331-3p, miR-432, miR-438, miR-574-3p, miR-625*, miR-92a, miR-584, miR-517c, miR-378, miR-520f, miR-142-5p, miR-451, miR-518d, miR-29a, miR-650, miR-151, miR-19b, miR-29c.

Afterwards, the use of GenEx qPCR software allows calculating the number of each of the studied miRNAs separated from each faction. Then the data is represented in the form of the chart to compare profiles of the expression of miRNA in samples from different tissues.

Exemplary research:

600 ml of urine taken from a healthy donor comes through successive centrifugation at 2000 and 10000 g, then the obtained supernatant liquid of 500 ml is concentrated using ultra filtration (Centrikon plus-70, 100 kDa, Millipore) up to the final volume of 10 ml. Afterwards, to separate exosomes, it is necessary to use ultra-centrifugation with the help of the Avanti 30I (JA-30.50 rotor) centrifuge at 100000 g during 2 hours.

After centrifugation the supernatant liquid is put into a separate test tube and studied using laser correlation spectroscopy (LCS) to check the absence of exosomal-size particles. The deposit is dissolved in maximal volume of phosphate-saline buffer (PBS) and centrifuged under the same conditions again. The obtained deposit is dissolved in 100 microliter of water (MiliQ) or PBS. Then the analyzed sample goes through free flow electrophoresis in free stream in bunkers using FFE System (FFE Service GmbH) under the following conditions: the total time of free flow electrophoresis is 5 minutes, the impressed voltage – 900 V, current – 34 mA.

The sample and the marker are injected at a flow rate of 2,5 ml/h and 280 ml/h respectively. The range is conducted in FF-ZE cyclical interval at 85 ml/h and the fractioned sample is eluted at 320 mp/h. The following buffer is used for anode stabilization: 1,5 M of thiourea + 6 M of urea + 100 mM of H₂SO₄ + 25 mM of 2-pyridine-propanol + 300 mM of 2-pyridine-ethanol.

The medium for the division has the following composition: 1,8 M of thiourea + 6 M urea + 55 mM of MOPS + 55 mM of 2-pyridine-propanol; the following buffer is used for cathode stabilization: 1,8 M of thiourea + 76 M of urea + 180 mM of NaOH + 25 mM of Tris + 35 mM of MOPS + 280 mM of EPPS, with the following collection of separate fractions, ultra-centrifugation of samples from separate pools using ultra-centrifugation with the help of the Avanti 30I (JA-30.50 rotor) centrifuge at 100000 g during 2 hours.

Then the separation of miRNAs from every separate fraction is conducted using the following method: 5 mM of K proteinase is added to every separate fraction that is incubated at 56 C within one hour, then it is applied to the Exiqon column (in order to the purification of miRNAs, the sample is applied to the column) with the further centrifugation at 2000 g using the eppendorf 5104 centrifuge. Then the column is flushed three times using the flushing buffer. Each flushing is followed by the further centrifugation at 2000 g using the eppendorf 5104 centrifuge. Then 20 microliter of nuclease free water is applied to the column

which is left for 10 minutes at room temperature and then centrifuged at 3000 g using the eppendorf 5104 centrifuge. It is followed by the reverse transcription reaction using reverse transcriptase (Exiqon), namely 8 microliter of the reagent is added to 8 microliter of the sample and incubated during one hour at 60 C. Afterwards, the reaction is stopped by heating up to 95 C during 5 minutes.

Then the polymerase chain reaction with a real-time detection using 7500 Applied Biosystems with primers on the following miRNAs is used: miR-141, miR-200a, miR-200c, miR-203, miR-205, miR-214, miR-17-3p, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-301a, miR-326, miR-331-3p, miR-432, miR-438, miR-574-3p, miR-625*, miR-92a, miR-584, miR-517c, miR-378, miR-520f, miR-142-5p, miR-451, miR-518d, miR-29a, miR-650, miR-151, miR-19b, miR-29c. The results are analyzed with the help of GenEx qPCR software and represented in the form of a table (see fig.1-Separation of miRNAs separated from 500 ml of urine of a healthy donor per pool. The concentration is represented as log with the formation of two copies of miRNAs in microliter).

Then 600 ml of urine taken from a patient with breast cancer comes through successive centrifugation at 2000 and 10000 g, then the obtained supernatant liquid of 500 ml is concentrated using ultra filtration (Centrikon plus-70, 100 kDa, Millipore) up to the final volume of 10 ml. Afterwards, to separate exosomes, it is necessary to use ultra-centrifugation with the help of the Avanti 30I (JA-30.50 rotor) centrifuge at 100000 g during 2 hours.

After centrifugation the supernatant liquid is put into a separate test tube and studied using laser correlation spectroscopy (LCS) to check the absence of exosomal-size particles. The deposit is dissolved in maximal volume of phosphate-saline buffer (PBS) and centrifuged under the same conditions again. The obtained deposit is dissolved in 100 microliter of water (MiliQ) or PBS. Then the analyzed sample goes through free flow electrophoresis in free stream in bunkers using FFE System (FFE Service GmbH) under the following conditions: the total time of free flow electrophoresis is 5 minutes, the impressed voltage – 900 V, current – 34 mA.

The sample and the marker are injected at a flow rate of 2,5 ml/h and 280 ml/h respectively. The range is conducted in FF-ZE cyclical interval at 85 ml/h and the fractioned sample is eluted at 320 mp/h. The following buffer is used for anode stabilization: 1,5 M of thiourea + 6 M of urea + 100 mM of H₂SO₄ + 25 mM of 2-pyridine-propanol + 300 mM of 2-pyridine-ethanol.

The medium for the division has the following composition: 1,8 M of thiourea + 6 M urea + 55 mM of MOPS + 55 mM of 2-pyridine-propanol; the following buffer is used for cathode stabilization: 1,8 M of thiourea + 76 M of urea + 180 mM of NaOH + 25 mM of Tris + 35 mM of MOPS + 280 mM of EPPS, with the following collection of separate fractions, ultra-centrifugation of samples from separate pools using ultra-centrifugation with the help of the Avanti 30I (JA-30.50 rotor) centrifuge at 100000 g during 2 hours.

Then the separation of miRNAs from every separate fraction is conducted using the following method: 5 mM of K proteinase is added to every separate fraction that is incubated at 56 C within one hour, then it is applied to the Exiqon column (in order to the purification of miRNAs, the sample is applied to the column) with the further centrifugation at 2000 g us-

ing the eppendorf 5104 centrifuge. Then the column is flushed three times using the flushing buffer. Each flushing is followed by the further centrifugation at 2000 g using the eppendorf 5104 centrifuge. Then 20 microliter of nuclease free water is applied to the column which is left for 10 minutes at room temperature and then centrifuged at 3000 g using the eppendorf 5104 centrifuge. It is followed by the reverse transcription reaction using reverse transcriptase (Exiqon), namely 8 microliter of the reagent is added to 8 microliter of the sample and incubated during one hour at 60 C. Afterwards, the reaction is stopped by heating up to 95 C during 5 minutes.

Then the polymerase chain reaction with a real-time detection using 7500 Applied Biosystems with primers on the following miRNAs is used: miR-141, miR-200a, miR-200c, miR-203, miR-205, miR-214, miR-17-3p, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-301a, miR-326, miR-331-3p, miR-432, miR-438, miR-574-3p, miR-625*, miR-92a, miR-584, miR-517c, miR-378, miR-520f, miR-142-5p, miR-451, miR-518d, miR-29a, miR-650, miR-151, miR-19b, miR-29c. The results are analyzed with the help of GenEx qPCR software and represented in the form of a table (see fig.1-Separation of miRNAs separated from 500 ml of urine of a healthy donor per pool. The concentration is represented as log with the formation of two copies of miRNAs in microliter).

Tables 1 and 2 show that the pools of 61-66 miRNAs (miR-17-3p, miR-106a, miR-146, miR-155, miR-191, miR-192) are observed in the patient's urine with pulmonary adenocarcinoma and are not observed in the healthy person's urine. It indicates that only exosomes from tumor cells of pulmonary adenocarcinoma penetrate into these pools. Consequently, the claimed method allows separating net fractions of specific types of miRNAs, including tissue-specific and tumor-specific miRNAs from exosome-containing biological fluids.

Claim

- 1) A method for separation of particular types of micro-RNA from biological fluids containing exosomes, comprising sequential centrifugation in 2,000 and 10,000 g mode, ultrafiltration of the resulting supernatant fluid on Centricon plus-70 centrifugal concentrator, 100 kilodaltons, Millipore to a final volume of 10 ml, and ultracentrifugation in Avanti 30I centrifuge (JA-30.50 rotor) in 100,000 g mode for 2 hours. Then the resulting precipitate was dissolved in phosphate-saline buffer and re-centrifuged under the same conditions. Then the resulting precipitate was dissolved in 100 µl of water, characterized in that further the resulting solution was subjected to free flow electrophoresis in bunkers for anodic and cathodic stabilization at FFE System device followed by collection of the individual fractions under the following conditions: the total time of electrophoresis was 5 minutes, the applied voltage of 900 V and the current of 34 mA. The sample and the marker were introduced at a flow rate of 2.5 ml/h and 280 ml/h, respectively. Running was conducted at a cyclic ZE-FF interval at 85 ml/h and the fractionated sample was eluted at 320 ml/h. The following buffer was used for anodic stabilization: 1,5M of thiourea + 6M of urea + 100 mM of H₂SO₄ + 25 mM of 2-pyridin-propanol + 300 mM of 2-pyridine-ethanol. The medium for separation has the following composition: 1,8 M of thiourea + 6 M of urea + 55 mM of MOPS + 55 mM of 2- pyridine-propanol. The following buffer was used for cathodic stabilization: 1.8 M of thiourea + 76 M of urea + 180 mM of NaOH + 25 mM of Tris + 35 mM of MOPS + 280 mM of EPPS. Then, the separate fractions were re-ultracentrifuged at Avanti 30I centrifuge (JA-30.50 rotor) in 100,000 g mode for 2 h, then micro-RNA is separated from each separate fraction. Namely, 5 µM of proteinase K was added in each separate fraction, incubated at 56 °C for 1 hour, then applied to Exiqon column, followed by centrifugation at 2,000 g, then the column was washed 3 times with wash buffer. After each washing, centrifugation was conducted at 2,000 g at Eppendorf 5104 centrifuge. Then 20 µl of no-nuclease water is applied to the column, then the column is left for 10 minutes at room temperature and then centrifuged at 3,000 g, and then reverse transcription reaction is conducted using reverse transcriptase (Exiqon) namely, 8 µl of the reagent was added to 8 µl of the sample and incubated for 1 hour at 60 °C, after which the reaction was stopped by heating to 95 degrees for 5 minutes.