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(54) Title: METHOD FOR EARLY DETECTION AND MONITORING OF DISEASES BY ANALYSIS OF CELL-SURFACE-BOUND NUCLEIC ACIDS

(57) Abstract: The invention belongs to the field of diagnostic medicine, to be more exact to the field of development of noninvasive methods of early detection of different sickness, like precancerous state, early stages of cancer development, pathologies of pregnancy, monitoring of efficacy of anticancer therapy, etc. The method based on investigation of cell-surface-bound extracellular nucleic acids from human blood, namely the blood is divided into plasma and cellular fractions, cellular fraction is further divided into leucocytes and erythrocytes, cell-surface-bound extra-cellular nucleic acids are eluted form cell surface with PBS-EDTA treatment or treatment of cells with trypsin solution, eluted nucleic acids are isolated with convenient method and analyzed for presence of at least two specific sequences associated with illness of interest with use of corresponding method of investigation of nucleic acids such as PCR analysis, multiplex PCR, hybridization assay or other methods of investigation of specific sequences of nucleic acids. The method enables to increase the reliability of early detection of the diseases concerned with abnormal functioning of genetic apparatus of cells, due to increase of sensitivity of detection of specific DNA and RNA sequences in the fraction of nucleic acids associated with cell surface of blood cells in comparison with nucleic acids isolated from plasma fraction. This is especially important for early detection of early stages of pathologies when the most part of nucleic acids circulating in the blood are associated with cell surface of blood cells.

WO 2005/017197 A2

Method for early detection and monitoring of diseases by analysis of cell-surface-bound nucleic acids

5 The invention belongs to the field of diagnostic medicine and therapy monitoring. It is based on the development of non-invasive methods for early detection of human diseases including pre-cancerous states, early stages of cancer development, pathologies of pregnancy, monitoring of efficacy of anticancer therapy, etc.

10 It is an object of the invention to provide a method of early detection and monitoring of diseases. It is also an object of the invention to provide a method for the purpose of early detection and monitoring of diseases that is non-invasive. It is another object of the invention to provide a method that allows for the early detection of cancer of different genesis. It is furthermore an object of the invention to provide a method for the early detection in the 15 pathology of pregnancy and it is an object of the invention to provide a method for the monitoring of the efficacy of anticancer therapy.

The method according to the invention is based on the investigation of cell-surface-bound extra-cellular nucleic acids from human blood. Blood samples are divided into plasma and 20 cellular fractions. The cellular fraction is further subdivided into leucocytes and erythrocytes. Cell-surface-bound extra-cellular nucleic acids are eluted from cell surface with PBS-EDTA or by treatment of cells with trypsin solution. Eluted nucleic acids are isolated with and analysed for the presence of at least two specific markers (nucleotide sequences) associated with the disease or parameter of interest by using analytical methods such as PCR, multiplex 25 PCR, hybridisation assay or other methods of investigation of specific sequences of nucleic acids.

The method enables one to increase the reliability and sensitivity of early detection of diseases or therapeutic schemes. This strategy shows improved sensitivity of the detection of 30 specific DNA and RNA sequences in the fraction of nucleic acids associated with cell surface of blood cells when compared with nucleic acids isolated from the plasma fraction. This is especially important for the reliable detection of early stages of pathological processes at which the most part of nucleic acids circulating in the blood are associated with cell surface of

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blood cells. It is important to note that this methodology is non-invasive and, thus, the potential risk by the diagnosis itself is substantially minimized when compared to invasive methods.

5 The method according to the invention allows the isolation of nucleic acids, obtained from the cell-surface of blood-circulating cells, as diagnostic markers:

1. A patient's blood sample is being separated into plasma and cellular fraction.

10 2. The cellular fraction is further divided into erythrocytes and leukocytes.

3. Nucleic acids associated with the cell surface of leukocytes are eluted by treatment with PBS-EDTA.

15 4. The eluted nucleic acids are isolated by methods known in the state of the art (e.g. with a kit from Qiagen or any other suitable laboratory protocol).

5. The composition of these nucleic acid preparations and the absolute and relative amounts can be analysed with any suitable method known in the state of the art, e.g. PCR, 20 Multiplex-PCR, hybridisation and sequencing methods.

In more detail the method of early diagnosis of diseases induced by abnormal functioning of cellular genomes comprises sampling of blood, dividing the blood into plasma and cellular fractions, isolating extracellular nucleic acids (exNA), revealing specific sequences of nucleic 25 acids by means of polymerase chain reaction with subsequent analysis of the presence or absence of specific sequences in total PCR products, which differ from existing methods by the fact that cell-surface-bound extracellular nucleic acids are used as a source of extracellular nucleic acids instead of exNA isolated from plasma fraction, whereby the cellular fraction is divided into leucocytes and erythrocytes, cell-surface-bound extracellular nucleic acids are 30 subsequently eluted from cell surface, exNA are isolated from eluates and these exNA are used for analysis of at least two specific sequences of exNA distinctive for the disease.

In a preferred embodiment of the invention the cell-surface-bound nucleic acids are eluted by treating the cells with 10 volumes of PBS with 5 mM EDTA at 4 °C with subsequent pelleting of the cells by centrifugation and collection of the supernatant, followed by the elution with 0,25 % trypsin solution, subsequent inactivation of the enzyme with trypsin inhibitor, centrifugation and collection of the supernatant.

Description of the tables

The tables enclosed in the description summarize the results of an analysis using the method according to the invention and underline the impact of such method on the clinical diagnosis of diseases.

Table 1 shows the correlation between the symptom lung cancer and increased amounts of extra-cellular and cell-surface-bound nucleic acids (from leukocytes and from erythrocytes). Both groups were sampled from a lung cancer risk group and healthy donors. The number in percentages show to which extent the samples were marked positive for the gene-markers “APC” and “RASSF1A”. The cell-surface-bound nucleic acids are divided into subcellular fractions for erythrocytes and leukocytes and further distinguished by their method of elution (PBS-EDTA or trypsin treatment).

Table 2 shows that the nucleotide sequences „c-myc“ and „c-erbB2“ are detectable in preparations of cell-surface-bound nucleic acids of leukocytes and erythrocytes in 9 % of patients with breast cancer.

Table 3 shows that the nucleotide sequences CK19“ and „CEA are detectable in preparations of cell-surface-bound nucleic acids of leukocytes and erythrocytes as well as in cell-free plasma-DNA in a colon cancer risk group.

Table 4 shows an increase of DNA-concentration in plasma and of cell-surface-bound DNA of leukocytes and erythrocytes in samples of pregnant women with differing degree of pre-eclampsia.

Table 1. Frequency of APC and RASSF1A genes promoter methylation in patients of lung cancer risky group and in healthy don

Analysed groups of patients	Gene	Year	exDNA in blood plasma	Cell-surface-bound exDNA			
				Eluted from erythrocytes		Eluted from leucocytes	
				PBS/EDTA	Trypsin	PBS/EDTA	Trypsin
Lung cancer risk group, N=24	APC	1999	12,51%	41,7%	37,53%	20,85%	16,68%
		2000	16,68%	37,53%	21,19%	12,51%	0
	RASSF1A	1999	16,68%	50,04%	41,7%	25,02%	16,68%
		2000	20,85%	41,7%	21,19%	20,85%	8,34%
	B 1999-2003 – lung sarcoma was found in 62,5% patients from risk group, furthermore all patients with high level of promoter methylation were in that group						
Healthy donors, N=21	APC	1999	0	4,17%	0	4,17%	0
		2000	4,17%	0	0	0	0
	RASSF1A	1999	0	0	0	0	0
		2000	0	0	0	0	0

le 2. Frequency of c-myc and c-erbB2 expression in patients with breast cancer (N= 32).

Gene	Plasma DNA	Cell surface bound DNA	
		Erythrocytes	Leucocytes
c-myc	0	9%	9%
c-erbB2	0	9%	9%

Table 3. Frequency of CK19 and CEA genes expression in patients of colon cancer risky group and in healthy donors.

Analysed patients	Gene	Plasma DNA	Cell-surface-bound DNA	
			Erythrocyte	Leucocytes
Risky group, N=38	CK19	26,31%	60,53%	52,63%
	CEA	23,68%	57,89%	47,37%
Control group, N=20	CK19	15%	20%	15%
	CEA	20%	15%	15%

Table 4. Quantitative analysis of fetal and total extra-cellular DNA levels in normal pregnancies and pregnancies with pre-eclampsia.

Checkup groups	Pregnancies with male fetus	Source of DNA	Levels of plasma DNA (genome equivalent per ml of maternal blood)	Levels of cell surface bounded DNA (genome equivalent per ml maternal blood)	
				Erythrocyte	Leucocytes
Control (N=48)	45	fetal	308,5* (0-1537)	462 (0-2396)	349 (0-1876)
		total**	22342,8 (400-226453)	30352,5 (572-34269,7)	24676 (409-27887,9)
Pregnancies with pre-eclampsia. (N=24)	20	fetal	805,3 (0-3864,8)	1046,5 (0-5468,8)	887,76 (0-4567,9)
		total	63113,3 (1224-455545,8)	97771,5 (1843,4-694989)	80976,78 (1456-60435)
Pregnancies with severe pre-eclampsia. (N=8)	8	fetal	3028,65 (347-6995,4)	4481,44 (453-9648,96)	3564 (407-7869,7)
		total	217786 (1324-744545)	328534,5 (1934,3-1100592)	287654,8 (1675-978657,7)

* average value
** both maternal and fetal extracellular DNA

Claims

1. Method of early diagnosis of diseases induced by abnormal functioning of cellular genomes, comprising sampling of blood, dividing the blood into plasma and cellular fractions, isolating extracellular nucleic acids (exNA), revealing specific sequences of nucleic acids by means of polymerase chain reaction with subsequent analysis of the presence or absence of specific sequences in total PCR products, which differ from existing methods by the fact that cell-surface-bound extracellular nucleic acids are used as a source of extracellular nucleic acids instead of exNA isolated from plasma fraction, whereby the cellular fraction is divided into leucocytes and erythrocytes, cell-surface-bound extracellular nucleic acids are subsequently eluted from cell surface, exNA are isolated from eluates and these exNA are used for analysis of at least two specific sequences of exNA distinctive for the disease.

2. Method according to claim 1, characterized by a two-stage elution of exNA from the surface of leucocytes comprising the steps of eluting exNA by treatment of cells with 10 volumes of PBS supplied with 5mM EDTA at 4 °C, with subsequent pelleting of cells by centrifugation and collection of supernatant followed by elution of exNA with 0,25 % trypsin solution, with subsequent inactivation of the enzyme with trypsin inhibitor, centrifugation and collection of the supernatant.

3. Method according to claim 1, characterized in isolating the exNA by increased glass-milk protocol.

4. The use of the method according to claim 1 for early detection of cancer and / or pathologies of pregnancy.