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(54) Title: METHOD AND KIT FOR DETECTING, DIAGNOSTING OR CONTROLLING THE TREATMENT OF OVARIAN CANCER

(57) Abstract: The present invention pertains to a procedure and a kit for the detection, diagnosis or monitoring of ovarian cancer in humans. This procedure is based on the feature that one recognizes the presence or absence in a human blood sample of at least two different mRNAs that code for various members of the group comprising tumor marker proteins EGFR, CEA, CK20, GA733-2, MUC1, HER2, MUC4, and CA12-5 and a conclusion is drawn from this in regard to the presence of ovarian carcinoma cells in the blood sample, and thus in regard to possible metastasis.



METHOD AND KIT FOR DETECTING, DIAGNOSING OR
CONTROLLING THE TREATMENT OF OVARIAN CANCER

The present invention pertains to a procedure and a kit for the detection, diagnosis or monitoring of ovarian cancer in humans.

The ability to detect a relapsing malignant tumor in a timely manner via the occurrence of metastasizing tumor cells in the blood is relevant in cancer after-care. So-called "tumor markers" are determined quantitatively at the protein level (immunologically or enzymatically) in the blood, or in other bodily fluids, in cancer patients when using current test methods.

These detection procedures are suitable only to a limited extent for tumor diagnosis, or monitoring/after-care, since increased tumor marker values can also be produced by non-tumor diseases (e.g. in-

flammation of the gastrointestinal tract, cirrhosis of the liver, viral infections), heavy smoking, or as a result of pregnancy.

5 The currently used diagnostic methods are inexact when one is dealing with the evaluation of the malignant potency of residual tumors after chemotherapy has been carried out in the metastasizing stages. Some clinical studies indicate the prognostic importance of disseminated tumor cells. However, numerous methodological aspects are critical and have not been adequately standardized so far. Thus, detection methods for occult-or residual-metastasis have to be found that permit timely classification into the various primarily curative therapeutic options.

10 The effort to improve the chances of healing is currently accompanied, on the one hand, by the search for and the use of new tumor markers and, on the other hand, by increasing sensitivity of the methods that are used.

20 The problem of the present invention is to make available a procedure and a kit with which the detection of circulating tumor cells, the diagnosis or monitoring of ovarian cancer is possible in a simple, safe and repeatable manner.

25 This problem is solved by the procedure in accordance with Claim 1 and with the kit in accordance with Claim 30 and the microarray in accordance with Claim 48. Advantageous further developments of the procedure, of the kit, and of the microarray are given in the pertinent dependent claims.

30 In accordance with the invention, the presence

or absence of at least two different mRNAs from the group comprising tumor marker proteins EGFR, CEA, GA733-2, MUC1, HER2, CA12-5 and MUC4 are tested in a human blood sample by means of the kit in accordance with the invention or by means of the procedure in accordance with the invention. This means selection of any particular combination of two tumor markers named above and recognition of their mRNA in a blood sample. Although at least two of the markers mentioned above are detected according to the present invention, any further marker, which is not mentioned here, may or may not be detected additionally.

Preferably a sample is considered positive if at least one of the at least two tumor markers mentioned above is detected to be present in the sample. Thus, even if only one of these markers is detected, the sample is considered as containing ovarian circulating tumor cells.

Since the mRNAs of the markers that have been described are not normally present in over-expressed form in the blood of healthy persons, a direct correlation is found between a positive RT-PCR result detection of a sample for these tumor markers and circulating tumor cells in the blood that can lead to metastasis. Thus, the present invention provides a highly sensitive and highly specific method for diagnosis of circulating tumor cells, i.e. ovarian circulating tumor cells, in a sample, in particular in ovarian cancer patients, thereby diagnosing or monitoring ovarian cancer in humans.

Since individual markers can be expressed differently in a therapy-dependent manner, and ovarian cancer exhibits pronounced heterogeneity in the ex-

pression pattern of the ovarian cancer cells, it is expedient to examine a combination of tumor markers in order to recognize all the tumor cells that are circulating in the blood. As a result of this, tumor cells can also be recognized when the expression of a particular marker is relatively slight in a patient or in a stage of the disease, which could otherwise lead to a supposedly negative result. However, the use of additional markers usually encounters limits if mononuclear blood cells exhibit background expression ("illegitimate transcription") that impedes exact analysis.

Thus the following advantageous combination of markers is proposed in accordance with the invention for the recognition of ovarian cancer cells.

At least the two markers in at least one of the following groups:

GA733-2 and MUC1;
GA733-2 and HER2;
GA733-2 and CEA;
GA733-2 and CA12-5;
GA733-2 and EGFR;
GA733-2 and MUC4;

or

at least the three markers in at least one of the following groups:

GA733-2, MUC1 and HER2;
GA733-2, MUC1 and CA12-5;
GA733-2, MUC1 and EGFR;
GA733-2, MUC1 and CEA;
GA733-2, MUC1 and MUC4;
GA733-2, CA12-5 and EGFR;

GA733-2, CA12-5 and CEA;
GA733-2, CA12-5 and HER2;
GA733-2, CA12-5 and MUC4;
GA733-2, HER2 and EGFR;
5 GA733-2, HER2 and CEA;
GA733-2, HER2 and MUC4;
GA733-2, EGFR and CEA;
GA733-2, EGFR and MUC4;
GA733-2, CEA and MUC4;

10 or

at least the markers in at least one of the following
groups of marker combinations

GA733-2, HER2, CEA and EGFR;
15 GA733-2, HER2, MUC1 and CA12-5;
GA733-2, HER2, MUC1, CA12-5, EGFR and CEA;
GA733-2, MUC1, CA12-5 and EGFR;
GA733-2, HER2, MUC4 and EGFR;
GA733-2, HER2, MUC4 and CEA;

20

or further combinations of at least markers compris-
ing at least marker GA733-2 as first marker, at least
one marker of the group of markers EGRF, CEA, MUC1,
HER2, CA12-5 and MUC4 as second marker, at least one
25 marker of the group of markers EGRF, CEA, MUC1, HER2,
CA12-5 and MUC4 as third marker different from the
second marker and at least one marker of the group of
markers EGRF, CEA, MUC1, HER2, CA12-5 and MUC4 as
forth marker different from the second marker and
30 different from the third marker.

Advantageously, to each of the combinations of two,
three or more markers cited in the preceeding text
the further marker CA12-5 may be added.

35

Surprisingly, the combination of the three markers

GA733-2, HER2 and MUC1, which was already successfully used in the *AdnaTest BreastCancer* kits for detection of circulating breast cancer cells, turned out to also be an advantageous combination of markers for the detection of circulating tumor cells in ovarian cancer. In contrast, the combination used in the *AdnaTest Colon Cancer* kits was much less effective. The combination of these three markers can be preferably supplemented by additional markers from the list of markers provided in claim 1, especially CA12-5.

Further, the combination of markers GA733-2, MUC1 and EGFR turned out to be a further most advantageous marker combination for detection of circulating tumor cells associated with ovarian cancer. This combination of markers may be supplemented by further markers as mentioned above.

Use can be made of the primers indicated in the following table for the amplification of segments of the markers.

Tumor marker - primer	Sequence 5' → 3'	PCR product
GA733-2 sense	AATCGTCAATGCCAGTGACTTCA	395 bp
GA733-2 antisense	TAACGCGTTGTGATCTCCTTCTGA	
EGFR sense	AGTCGGGCTCTGGAGGAAAAGAAA	163 bp
EGFR antisense	GATCATAATTCCTCTGCACATAGG	
CEA sense	AGAAATGACGCAAGAGCCTATGTA	231 bp
CEA antisense	AACTTGTGTGTGTTGCTGCGGTAT	
MUC1 sense	TCAGCTTCTACTCTGGTGACACAAC	299 bp
MUC1 antisense	TGGTAGTAGTCGGTGCTGGGATCT	
HER2 sense	CCCAGTGTGTCAACTGCAGCCAGT	265 bp
HER2 antisense	CAGATGGGCATGTAGGAGAGGTCA	
MUC4 sense	TCAACGCCTCGGTGGCATAAC	296 bp
MUC4 antisense	CTGTCACATCGCGCACGTCT	
CA12-5 sense	ACACATTCCGCTTCTGCCTGGTCA	432 bp
CA12-5 antisense	CCCCGGTGTGGTGCCTGTT	

Internal control		
Aktin sense	CTGGAGAAGAGCTACGAGCTGCCT	111 bp
Aktin antisense	ACAGGACTCCATGCCCAGGAAGGA	

The designations of the markers are explained in the following table.

Gene or gene product	Gene
Human carcinoma-associated antigen GA733-2 gene	GA733-2
Human epidermal growth factor receptor (EGFR) gene	EGFR
Human carcinoembryonic antigen (CEA) gene	CEA
Homo sapiens mucin 1 (MUC1)	MUC1
Homo sapiens C-erb B2/neu protein (ERBB2) gene	HER-2
Homo sapiens mucin 16 (MUC16)	CA12-5
Homo sapiens mucin 4 (MUC4)	MUC4

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In the case of using RT-PCR systems for the detection of tumor cells, specificity is a critical point because of the very high amplification rate. The least contamination, e.g. via extraneous RNA or illegitimate transcription, can in this way falsify the result.

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An increase in specificity as a result of concentrating the tumor cells relative to the blood cells and, at the same time, an increase in sensitivity in the detection of tumor cells can be achieved (detection rate of 1 tumor cell per 10^7 mononuclear blood cells) by using immunocytochemistry with monoclonal antibodies that react against tumor cell antigens. In this way the tumor cells are separated by means of specific antibodies or an antibody mixture of mononuclear blood cells. Separation can take place by means of magnetic particles (Dynabeads, Dynal) to which the antibodies are bound. This is described in greater detail in the following section.

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Eukaryotic cells carry a plurality of different molecules on their cell surface. The combination of expressed surface molecules differs depending on the origin and the function of the individual cell, so that patterns are produced that are cell-type specific. Antibodies are utilized in order to recognize these cell-type specific patterns. Antibodies bind with high specificity to their antigen, namely to selected surface molecules in this case. This property is utilized in order to recognize cells and to differentiate them from one another by means of specific antibody binding on the basis of their cell-type specific patterns.

The expression of special surface proteins differentiates tumor cells from non-transformed cells of this cell type. Since, in the case of tumor cells, this special pattern of surface antigens also differs from the patterns that are typical of blood cells, tumor cells can be differentiated in the blood. In order to identify tumor cells, antibodies that specifically recognize these special surface proteins are utilized as tools. This specific antibody binding becomes usable for various analyses and separation methods.

In addition to recognizing cells via their surface epitopes, it is also possible to separate recognized cells from non-recognized ones because of the intensive binding of immunoglobulins that are specially selected for this purpose.

1. Separation principle based on the liquid phase;
e.g. continuous flow cytometry or fluorescence activated cell sorting

Antibodies are coupled to fluorescent dyes for the purpose of continuous flow cytometry analysis. Isolated cells are individually led past a light source (laser) in a constant stream of liquid. The fluorescent dyes bound to the antibodies are excited during illumination of the cells, and they irradiate light of a particular wavelength. The irradiated light is detected, and the measured signal is stored in digital form. The light signal can be assigned to individual cells. The antibody-labeled cell is recognized in this way, and can now be separated from other cells. The cells are isolated in extremely small drops for separation purposes. After recognizing the antibody-labeled cell, the drops in question are deflected into a collection container.

2. Separation principle based on the solid phase;
e.g. magnetic separation:

Antibodies are coupled to magnetic or pseudo-magnetic particles for the purpose of magnetic separation. After introducing the magnetic or pseudomagnetic particles to a magnetic field, the particles migrate in this magnetic field. During movement in this magnetic field, the cells to which the coupled antibodies are bound are carried along, and separated from other cells.

Thus, in order to recognize tumor cells by means of magnetic separation, antibodies are covalently coupled to magnetic or pseudomagnetic particles that possess a defined number of chemically activated sites on their surface. The separation specificity is determined by the specificity of the antibodies. A blood sample that contains tumor cells is

mixed with antibody-coupled magnetic or pseudomag-
netic particles; the particles and blood then move
relative to one another. Those (tumor) cells which
are recognized by the antibodies (that are bound to
the solid phase) and which are firmly bound to them
follow the movement of the particles. As a result, it
is possible to withdraw, from the blood, the parti-
cles with the cells that are bound to them (e.g. to-
ward the wall of the separation vessel) upon applying
a magnetic or pseudomagnetic field. The blood that
has been tumor-cell depleted in this way can be ex-
changed for other solutions, whereby the cells that
have been separated via magnetic or pseudomagnetic
particles remain behind, and are available for addi-
tional applications until the point in time of
switching off/removing the magnetic field.

Use can advantageously be made of specific an-
tibody mixtures in order to recognize the tumor
cells, whereby these mixtures have either been opti-
mized with respect to tumor cells in a general man-
ner, or they have been specifically optimized with
respect to breast cancer cells as well. For example,
a combination of antibody against the epithelial cell
marker Ber-EP4 with different other antibodies di-
rected against another epitope of the same antigen
(MOC31) or against different tumor cell associated
antigens (anti-MUC1 antibodies, anti-EGFR antibodies,
anti-CA12-5 antibodies) is used in order to increase
the efficacy of the recognition and selection step
and not to lose any but to recover most or all tumor
cells present in the sample.

If a mixture of antibodies is used to recognize
and select tumor cells, any cell marked by at least
one of the antibodies can be selected in order to in-

crease sensitivity of the recognition and selection step and select all cells which might be tumor cells.

Recognition that is specially directed toward ovarian cancer cells can be achieved via an additionally optimized antibody mixture in accordance with the table below. This is based on the selective expression of certain surface proteins, whereby ovarian cancer cells are differentiated from other cancer cells. Surprisingly, it was found, that the antibody combination used in the *AdnaTest BreastCancerSelect* kit turned out to be the most efficient one for selection of circulating ovarian cancer cells, whereas the antibody mix used in the *AdnaTest ColonCancerSelect* kit was inefficient, although both mixtures contained the same anti-EPCAM antibody.

Antigen	Clone (Provider)	Antibody Concentration in 5 ml blood
EpCAM	2D3 (AdnaGen AG)	0.375 µg/100 µl beads
EpCAM	Moc31 (Acris)	1.3125 µg/100 µl beads
HER2	10E9 (AdnaGen AG)	0.625 µg/100 µl beads
MUC1	HMPV (BD)	2.0 µg/100 µl beads

In comparison to the antibodies when used separately in each case, such antibody mixtures show increased sensitivity in terms of cell recognition and cell separation, independent of the method used.

The diagnosis kit in accordance with the invention and the procedure in accordance with the invention also make it possible to subsequently use the sorted and separated cells further as desired. For example, these can be inserted into a suitable cell culture medium where they can be cultivated in situ.

Since the cells are intact following separation, the properties of the cell membrane and of the cell nucleus are also conserved. This opens up the possibility of microscopically investigating the expression of additional surface markers, and of carrying out chromosome analyses as well. The sorted cells are applied to microscope slides for this purpose. The detection of additional surface markers can take place cytochemically or via fluorescence microscopy. Likewise, genetic analyses can be carried out such as, for example, chromosome analyses by means of FISH (fluorescence in situ hybridization), or via karyogram compilation or by gene expression analysis.

In the following, examples for detection and characterization of circulating tumor cells in patients with primary ovarian cancer are shown by the inventive method of molecular profiling.

Patients and Methods

2 x 5 ml blood were obtained from ovarian cancer patients before (n=30) and after (n=23) completion of chemotherapy, CTC were immunomagnetically enriched from blood by targeting different epitopes of EpCAM and MUC1, which was followed by isolation of their mRNA with oligo-dT magnetic beads.

Enrichment was done using two different sets of antibodies, i.e.

set (*AdnaTest BreastCancerSelect*, Adnagen AG, Langenhagen, Germany) designated "BR" containing one antibody directed against GA733-2 (EpCAM) and two antibodies directed against MUC1 (HMPV and GP1.4).

set (*AdnaTest ColonCancerSelect*, Adnagen AG, Langenhagen, Germany) designated "CO" containing 2

antibodies directed against GA733-2 (EpCAM and MOC31).

5 In each assay, cells labeled by at least one of the antibodies were selected for enrichment.

10 The mRNA fraction was analyzed for EpCAM, MUC1, MUC4, CA12-5, HER-2, EGFR and CEA transcripts by multiplex RT-PCR with the *AdnaTests ColonCancerDetect* and *BreastCancerDetect* (AdnaGen AG), containing primers for amplification of transcripts of GA733-2, MUC1 and HER2 (*AdnaTest BreastCancerDetect* designated "BR") or GA733-2, CEA, EGFR (*AdnaTest ColonCancerDetect* designated "CO").

15 CA12-5 and MUC4 transcripts are both assayed in a separate RT-PCR. The PCR products were analyzed by capillary electrophoresis on the Agilent Bioanalyzer 2100. Blood of healthy donors as controls was used to confirm the specificity of the tests.

25 In total, 52 patients with ovarian primary tumor were analyzed for circulating tumor cells (CTC) in samples of peripheral blood. In these patients, a total of 14 patients (27 %) were found positive for CTC.

30 The following table shows the detection results using specific combinations of the *AdnaTest CancerSelect* kits and the *AdnaTest CancerDetect* kits. In the table, the first designation (BR, CO) determines the immunomagnetic selection step and the second designation (BR, CO) determines the expression profiling step, e.g. BR-CO designation as a selection step using *AdnaTest BreastCancerSelect* and a profiling step using *AdnaTest ColonCancerDetect*.

Patient#	Max BR_BR	Max BR_CO	Max CO_CO	Max CO_BR	Max ALL
1	4.91	0.13	0	3.03	4.91
2	1.67	0	0	0.13	1.67
3	0	0	1.51	0	1.51
4	0.42	0	0	0.76	0.76
5	0.54	-	-	-	0.54
6	0.45	0.53	0.39	0	0.53
7	0.5	-	-	-	0.5
8	0.43	0	0	0.14	0.43
9	0.09	-	-	0.39	0.39
10	0.29	-	-	-	0.29
11	0	0.21	0.27	0	0.27
12	0	0	0.26	0	0.26
13	0.17	-	-	-	0.17
14	0.16	0	0	0.16	0.16
Detected positive	10	2	4	4	14
Total positive	14	9	9	10	14
Percentage	71 %	22 %	44 %	40 %	100%

100% recovery is considered when all transcripts were tested for (Max ALL).

5 Surprisingly, already the use of *AdnaTest BreastCancerDetect* provides for a detection rate of at least 40 % and in combination with *AdnaTest BreastCancerSelect* a detection rate of 71 %.

10 Using *AdnaTest ColonCancerDetect*, no such detection rates could be obtained.

15 Obviously, it is the marker combination GA733-2, MUC1 and HER2 of *AdnaTest BreastCancerDetect* kit, which provides improved performance to detect CTC's

in patients with primary ovarian cancer. This could further be improved by combination with an immunoseparation using antibodies for EpCAM, HMPV and GP1.4.

5

Sensitivity could be further improved by detection of additional patients if additionally to analysis with the *AdnaTest BreastCancerDetect* kit (containing markers GA733-2, MUC1 and HER2) the samples were analyzed for CA12-5 and/or EGFR transcripts as shown in the following table.

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	number of positive patient samples out of 50 samples	percentage of positive patient samples
<i>AdnaTest BreastCancerDetect</i> kit	10	20 %
CA12-5	5	10 %
EGFR	3	6 %
<i>AdnaTest BreastCancerDetect</i> kit + CA12-5	13	26 %
<i>AdnaTest BreastCancerDetect</i> kit + EGFR	13	26 %
<i>AdnaTest BreastCancerDetect</i> kit + CA12-5 + EGFR	16	32 %

Therein samples from 50 ovarian cancer patients were tested for CTC using the *AdnaTest BreastCancerDetect* kit as described in the manufacturers instructions. Additionally, the cDNA obtained from these samples was tested for expression of marker CA12-5 and/or expression of marker EGFR. Using *AdnaTest BreastCancerDetect* kit alone, 10 out of 50 patient

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samples (20%) were positive tested for CTC. Analysing expression of markers CA 12-5 or EGFR alone, 5 out of 50 patient samples (10%, CA12-5) and 3 out of 50 patient samples (6%, EGFR) were tested positive for CTC. However, evaluating the combination of *AdnaTest BreastCancerDetect* kit and CA12-5, i.e. considering the sample to be positive if at least one, or respectively only one, of the markers contained in this marker combination turned out to be positive, 13 out of 50 patient samples (26 %) were tested positive for CTC. Evaluating the combination of *AdnaTest BreastCancerDetect* kit and EGFR, also 13 out of 50 patient samples (26 %) were tested positive for CTC. Evaluating the combination of *AdnaTest BreastCancerDetect* kit, EGFR and CA12-5, 16 out of 50 patient samples (32 %) were tested positive for CTC. This is a further major improvement in detection rate of CTC in peripheral blood of patients with ovarian cancer.

Further, superior sensitivity was found by detecting a combination of markers GA733-2, MUC1, EGFR and CA12-5. This is demonstrated in fig. 1. Fig. 1 shows an electrophoresis gel with a ladder and an analyzed sample of peripheral blood. Therein 5 SKOV cells were spiked into a sample volume of 5 ml. Fig. 1 further shows bands for markers CA12-5 at 432 base pairs, GA733-2 at 395 base pairs, MUC1 at 299 base pairs, EGFR at 163 base pairs and actin at 114 base pairs. Any of these markers tested positive in this specific sample, allowing for even higher sensitivity than 5 cells per 5 ml peripheral blood.

Claims

- 5 1. Procedure for the detection, diagnosis or monitoring of ovarian cancer in humans, characterized in that the presence or absence of at least two different mRNAs is recognized in a human blood sample, whereby these mRNAs code for at least two members of the group comprising tumor marker proteins EGFR, CEA, GA733-2, MUC1, HER2, CA12-5 and MUC4 and conclusions are drawn from this in regard to the presence of ovarian carcinoma cells in the sample of blood, and hence in regard to possible metastasis.
- 10
- 15
- 20 2. Procedure in accordance with Claim 1, characterized in that the presence or absence is recognized of members of at least one group of the following groups of marker:
- GA733-2 and MUC1;
GA733-2 and HER2;
GA733-2 and CEA;
25 GA733-2 and CA12-5;
GA733-2 and EGFR;
GA733-2 and MUC4;
- or
- 30 GA733-2, MUC1 and HER2;
GA733-2, MUC1 and CA12-5;
GA733-2, MUC1 and EGFR;
GA733-2, MUC1 and CEA;
35 GA733-2, MUC1 and MUC4;

GA733-2, CA12-5 and EGFR;
GA733-2, CA12-5 and CEA;
GA733-2, CA12-5 and HER2;
GA733-2, CA12-5 and MUC4;
5 GA733-2, HER2 and EGFR;
GA733-2, HER2 and CEA;
GA733-2, HER2 and MUC4;
GA733-2, EGFR and CEA;
GA733-2, EGFR and MUC4;
10 GA733-2, CEA and MUC4;

or

GA733-2, HER2, CEA and EGFR;
15 GA733-2, HER2, MUC1 and CA12-5;
GA733-2, HER2, MUC1, CA12-5, EGFR and CEA;
GA733-2, MUC1, CA12-5 and EGFR;
GA733-2, HER2, MUC4 and EGFR;
GA733-2, HER2, MUC4 and CEA;
20

or further combinations of at least markers comprising at least marker GA733-2 as first marker, at least one marker of the group of markers EGRF, CEA, MUC1, HER2, CA12-5 and MUC4 as second
25 marker, at least one marker of the group of markers EGRF, CEA, MUC1, HER2, CA12-5 and MUC4 as third marker different from the second marker and at least one marker of the group of markers EGRF, CEA, MUC1, HER2, CA12-5 and MUC4 as forth
30 marker different from the second marker and different from the third marker.

3. Procedure in accordance with one of the preceding claims, characterized in that the presence
35 or absence of at least three or at least four

different mRNAs is recognized.

4. Procedure in accordance with one of the preceding claims, characterized in that the mRNA associated with the gene of CA12-5 is recognized.

5. Procedure in accordance with one of the preceding claims, characterized in that tumor cells from the blood sample are separated or concentrated, and the detection procedure is done using these tumor cells.

6. Procedure in accordance with the preceding claim, characterized in that the ovarian carcinoma cells are separated or concentrated by means of antibodies or antibody derivatives which are generally specific for tumor cells, and/or by means of antibodies or antibody derivatives which are specific for ovarian carcinoma cells, or by means of mixtures of such antibodies and/or antibody derivatives.

7. Procedure in accordance with the preceding claim, characterized in that the antibodies or antibody derivatives used for separating ovarian tumor cells have binding sites that bind to the epitopes of an epithelial antigen, of an epithelial membrane antigen and/or of the antigen MUC1.

8. Procedure in accordance with the preceding claim, characterized in that use is made of at least one antibody of the group comprising antibodies 2D3, MOC-31, 10E9 and HMPV, or of a mix-

ture of two, three or four of these antibodies,
as the antibody.

- 5 9. Procedure in accordance with one of claims 6 to
8, characterized in that the ovarian carcinoma
cells are separated or concentrated by means of
antibodies bound to magnetic particles.
- 10 10. Procedure in accordance with one of claims 6 to
9, characterized in that the ovarian carcinoma
cells are separated or concentrated by means of
fluorescence-activated continuous flow cytome-
try, density gradient centrifugation, and/or
centrifugation following erythrocyte lysis.
- 15 11. Procedure in accordance with Claim 10, charac-
terized in that leukocytes in the blood sample
are pelletized by centrifugation.
- 20 12. Procedure in accordance with Claim 10, charac-
terized in that the RNA-containing components of
the sample are concentrated via lysis of the
erythrocytes that are contained in it, together
with subsequent pelletization of the nonlysed
- 25 leukocytes.
- 30 13. Procedure in accordance with Claim 10, charac-
terized in that the RNA-containing components
are concentrated by at least one density gradi-
ent centrifugation of the blood sample in order
to separate and harvest the mononuclear blood
cells that are contained in it.

14. Procedure in accordance with one of Claims 10 through 13, characterized in that the harvested mononuclear blood cells are labeled with fluorescence-labeled antibodies or antibody derivatives, and are separated and harvested by means of fluorescence-activated cell sorting (FACS) of the sample.
15. Procedure in accordance with one of Claims 10 through 14, characterized in that the mononuclear cells from the harvested fraction are lysed, and the mRNA is separated.
16. Procedure in accordance with one of claims 1 to 4, characterized in that the RNA (total RNA or mRNA) is isolated directly and in a conventional manner from the whole blood sample.
17. Procedure in accordance with the preceding claim, characterized in that DNA digestion is carried out subsequently to isolation of the RNA.
18. Procedure in accordance with one of the preceding claims, characterized in that the harvested mRNA is reverse transcribed into cDNA, and the presence or absence of the cDNA that is assigned to the tumor marker protein is recognized.
19. Procedure in accordance with the preceding claim, characterized in that at least one predetermined segment of the cDNA is replicated by means of polymerase chain reaction ("PCR").

20. Procedure in accordance with the preceding claim, characterized in that one or more oligonucleotide pairs from the group of pairs which comprise or consist of the sequences of the following group of sequences are used for replicating the cDNA, the group comprising the following pairs of sequences:

AATCGTCAATGCCAGTGTACTTCA and

TAACGCGTTGTGATCTCCTTCTGA,

AGTCGGGCTCTGGAGGAAAAGAAA and

GATCATAATTCCTCTGCACATAGG,

AGAAATGACGCAAGAGCCTATGTA and

AACTTGTGTGTGTTGCTGCGGTAT,

TCAGCTTCTACTCTGGTGCACAAC and

TGGTAGTAGTCGGTGCTGGGATCT,

CCCAGTGTGTCAACTGCAGCCAGT and

CAGATGGGCATGTAGGAGAGGTCA,

TCAACGCCTCGGTGGCATAC and

CTGTCACATCGCGCACGTCT,

ACACATTCCGCTTCTGCCTGGTCA and

CCCCGGTGTGGTGCCTGTT.

21. Procedure in accordance with one of the preceding claims, characterized in that the mRNA of the protein β -actin is determined for internal control purposes.

22. Procedure in accordance with the preceding claim, characterized in that the mRNA for β -actin is reverse transcribed into cDNA, and a segment of the cDNA is replicated by means of a

polymerase chain reaction.

23. Procedure in accordance with the preceding claim, characterized in that an oligonucleotide pair is used for replicating the cDNA of the β -actin, whereby the oligonucleotides of the pair comprise or consist of the following sequences:

CTG GAG AAG AGC TAC GAG CTG CCT and
ACA GGA CTC CAT GCC CAG GAA GGA.

24. Procedure in accordance with one of Claims 19 through 23, characterized in that the replicated cDNA segment is digested via suitable restriction enzymes, and the presence or absence of the mRNA coding for a tumor marker protein is determined by means of the cDNA fragments that are produced.

25. Procedure in accordance with one of Claims 19 through 24, characterized in that gel electrophoresis of the PCR products is carried out in order to detect the amplified cDNA segments.

26. Procedure in accordance with one of Claims 19 through 24, characterized in that fragment analysis is carried out in order to detect the amplified cDNA segments.

27. Procedure in accordance with one of Claims 19 through 24, characterized in that, during the course of the polymerase chain reaction, the fluorescence produced by the products is recognized, and the formation of products is recog-

nized (fluorescence-based real time PCR).

28. Procedure in accordance with one of Claims 19
through 24, characterized in that use is made of
5 a nucleotide microarray in accordance with one
of Claims 48 to 50 in order to detect the mRNA
or cDNA.

29. Procedure in accordance with the preceding
10 claim, characterized in that the PCR product is
applied to a nucleotide microarray in accordance
with one of Claims 48 to 50 in order to detect
the amplified cDNA.

30. Diagnosis kit for the detection, diagnosis or
15 monitoring of ovarian cancer via at least two
different pairs of oligonucleotides (reverse
primers, forward primers), whereby the two oli-
gonucleotides of each pair are suitable as prim-
20 ers for amplification by means of a polymerase
chain reaction of, in each case, one of the two
complementary strands of for the two pairs dif-
ferent DNA segments that are being sought, and
whereby the DNA segments that are being sought
25 are selected from the group comprising as mem-
bers cDNA associated with the genes EGFR, CEA,
GA733-2, MUC1, HER2, CA12-5 and MUC4.

31. Diagnosis kit in accordance with Claim 30, char-
30 acterized in that it contains at least three
different pairs of oligonucleotides (reverse
primers, forward primers), whereby the two oli-
gonucleotides of each pair are suitable as prim-
ers for amplification by means of a polymerase
35 chain reaction of, in each case, one of the two

complementary strands of for the three pairs
each different DNA segments that are being
sought, and whereby the DNA segments that are
being sought are selected from the group com-
prising as members cDNA associated with various
tumor marker proteins EGFR, CEA, GA733-2, MUC1,
HER2, CA12-5 and MUC4.

32. Diagnosis kit in accordance with Claim 30 or
Claim 31, characterized in that the DNA segments
that are being sought comprise members of at
least one group of the following groups of
marker combinations:

GA733-2 and MUC1;
GA733-2 and HER2;
GA733-2 and CEA;
GA733-2 and CA12-5;
GA733-2 and EGFR;
GA733-2 and MUC4;

or

GA733-2, MUC1 and HER2;
GA733-2, MUC1 and CA12-5;
GA733-2, MUC1 and EGFR;
GA733-2, MUC1 and CEA;
GA733-2, MUC1 and MUC4;
GA733-2, CA12-5 and EGFR;
GA733-2, CA12-5 and CEA;
GA733-2, CA12-5 and HER2;
GA733-2, CA12-5 and MUC4;
GA733-2, HER2 and EGFR;
GA733-2, HER2 and CEA;
GA733-2, HER2 and MUC4;
GA733-2, EGFR and CEA;

GA733-2, EGFR and MUC4;

GA733-2, CEA and MUC4;

or

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GA733-2, HER2, CEA and EGFR;

GA733-2, HER2, MUC1 and CA12-5;

GA733-2, HER2, MUC1, CA12-5, EGFR and CEA;

GA733-2, MUC1, CA12-5 and EGFR;

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GA733-2, HER2, MUC4 and EGFR;

GA733-2, HER2, MUC4 and CEA;

or further combinations of markers comprising at least marker GA733-2 as first marker, at least one marker of the group of markers EGRF, CEA, MUC1, HER2, CA12-5 and MUC4 as second marker, at least one marker of the group of markers EGRF, CEA, MUC1, HER2, CA12-5 and MUC4 as third marker different from the second marker and at least one marker of the group of markers EGRF, CEA, MUC1, HER2, CA12-5 and MUC4 as forth marker different from the second marker and different from the third marker.

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33. Diagnosis kit in accordance with one of claims 30 to 32, characterized in that it contains at least four different pairs of oligonucleotides (reverse primers, forward primers), whereby the two oligonucleotides of each pair are suitable as primers for amplification by means of a polymerase chain reaction of, in each case, one of the two complementary strands of for the four pairs each different DNA segments that are being sought, and whereby the DNA segments that are being sought comprise the cDNA associated with tumor marker proteins selected from the group

30

35

comprising tumor marker proteins EGFR, CEA, GA733-2, MUC1, HER2, CA12-5 and MUC4.

34. Diagnosis kit in accordance with one of Claims 30 to 33, characterized in that the two oligonucleotides of a pair from the group of pairs comprise or consist of one of the sequences of the following group of sequences in a pair-wise manner, the group of sequences comprising the following pairs of sequences:

AATCGTCAATGCCAGTGTACTTCA and
TAACGCGTTGTGATCTCCTTCTGA,

AGTCGGGCTCTGGAGGAAAAGAAA and
GATCATAATTCCTCTGCACATAGG,

AGAAATGACGCAAGAGCCTATGTA and
AACTTGTGTGTGTTGCTGCGGTAT,

TCAGCTTCTACTCTGGTGCAACAAC and
TGGTAGTAGTCGGTGCTGGGATCT,

CCCAGTGTGTCAACTGCAGCCAGT and
CAGATGGGCATGTAGGAGAGGTCA,

TCAACGCCTCGGTGGCATAC and
CTGTCACATCGCGCACGTCT,

ACACATTCCGCTTCTGCCTGGTCA and
CCCCGGTGTGGTGCCTGTT.

35. Diagnosis kit in accordance with one of claims 30 to 34, characterized in that it contains an additional pair of oligonucleotides that are suitable as primers for the amplification of at least one segment of one of the two complementary strands of the cDNA associated with the protein β -actin for internal control purposes.

36. Diagnosis kit in accordance with the preceding claims, characterized in that, in order to amplify the cDNA associated with β -actin, it contains a pair of oligonucleotides comprising or consisting of the following sequences:

CTG GAG AAG AGC TAC GAG CTG CCT and
ACA GGA CTC CAT GCC CAG GAA GGA.

37. Diagnosis kit in accordance with one of claims 30 to 36, characterized in that, in each case, at least one of the two oligonucleotides of a pair of oligonucleotides is labeled with fluorophores.

38. Diagnosis kit in accordance with the preceding claim, characterized in that the oligonucleotides of different pairs are labeled with different fluorophores.

39. Diagnosis kit in accordance with one of Claims 30 to 38, characterized in that it contains the substances required for carrying out a polymerase chain reaction.

40. Diagnosis kit in accordance with the preceding claims, characterized in that it contains the following as the substances required for carrying out a polymerase chain reaction: a buffer solution, magnesium chloride, deoxynucleotide triphosphates as well as a heat-stable polymerase.

- 5 41. Diagnosis kit in accordance with the preceding claim, characterized in that it contains a polymerase from *Thermus aquaticus* (Taq polymerase) as the heat-stable polymerase.
- 10 42. Diagnosis kit in accordance with one of Claims 30 to 41, characterized in that, as a positive control, it contains a DNA sample with the DNA segment that is being sought in each case.
- 15 43. Diagnosis kit in accordance with one of Claims 30 to 42, characterized in that it contains: instructions for carrying out the polymerase chain reaction and/or instructions for carrying out a fragment analysis.
- 20 44. Diagnosis kit in accordance with one of Claims 30 to 43, characterized in that it contains a schematic arrangement for evaluating the measurement results.
- 25 45. Diagnosis kit in accordance with one of Claims 30 to 44, characterized in that it contains a microarray (DNA chip), whereby the array has a number of cells (fields) that are separated from one another, and an oligonucleotide, which hybridizes with the DNA segment that is being
30 sought, is arranged in at least one cell of the microarray.

46. Diagnosis kit in accordance with the preceding claim, characterized in that an additional oligonucleotide arranged in at least one additional cell of the microarray, and the sequence of the nucleotide arranged in said cell differs from the sequence of the additional oligonucleotide.

47. Diagnosis kit in accordance with one of the two preceding claims, characterized in that, in each case, an oligonucleotide is arranged in at least two cells, whereby the oligonucleotides arranged in different cells hybridize in each case with the different DNA segments that are being sought.

48. Microarray for the detection, diagnosis or monitoring of ovarian cancer, e.g. a DNA chip, with an arrangement of several cells that are separated from one another, characterized in that, in each case, different oligonucleotides are arranged in at least two cells, whereby these oligonucleotides hybridize with a DNA segment that is part of the cDNA associated with two different members of the group comprising tumor marker proteins EGFR, CEA, GA733-2, MUC1, HER2, CA12-5 and MUC4.

49. Microarray in accordance with Claim 48, characterized in that different oligonucleotides are arranged in two to four cells in each case, whereby these oligonucleotides hybridize in each case with two to four different DNA segments, which are part of the cDNA of, in each case, various tumor marker proteins that are selected

from the group comprising tumor marker proteins EGFR, CEA, GA733-2, MUC1, HER2, CA12-5 and MUC4.

50. Microarray in accordance with Claim 48 or Claim 49, characterized in that different oligonucleotides are, in each case, arranged in at least two cells, whereby these oligonucleotides hybridize with DNA segments that are part of the cDNA associated with genes from the groups of genes coding for tumor markers comprising the following groups of tumor marker combinations

GA733-2 and MUC1;
GA733-2 and HER2;
GA733-2 and CEA;
GA733-2 and CA12-5;
GA733-2 and EGFR;
GA733-2 and MUC4;

or

GA733-2, MUC1 and HER2;
GA733-2, MUC1 and CA12-5;
GA733-2, MUC1 and EGFR;
GA733-2, MUC1 and CEA;
GA733-2, MUC1 and MUC4;
GA733-2, CA12-5 and EGFR;
GA733-2, CA12-5 and CEA;
GA733-2, CA12-5 and HER2;
GA733-2, CA12-5 and MUC4;
GA733-2, HER2 and EGFR;
GA733-2, HER2 and CEA;
GA733-2, HER2 and MUC4;
GA733-2, EGFR and CEA;
GA733-2, EGFR and MUC4;
GA733-2, CEA and MUC4;

or

GA733-2, HER2, CEA and EGFR;
GA733-2, HER2, MUC1 and CA12-5;
GA733-2, HER2, MUC1, CA12-5, EGFR and CEA;
GA733-2, MUC1, CA12-5 and EGFR;
GA733-2, HER2, MUC4 and EGFR;
GA733-2, HER2, MUC4 and CEA;

or further combinations of markers comprising at least marker GA733-2 as first marker, at least one marker of the group of markers EGRF, CEA, MUC1, HER2, CA12-5 and MUC4 as second marker, at least one marker of the group of markers EGRF, CEA, MUC1, HER2, CA12-5 and MUC4 as third marker different from the second marker and at least one marker of the group of markers EGRF, CEA, MUC1, HER2, CA12-5 and MUC4 as forth marker different from the second marker and different from the third marker.

51. Use of a diagnosis kit, a microarray, and/or a procedure in accordance with one of the preceding claims for the diagnosis of diseases or metastasis, or for monitoring, in cases of ovarian cancer.

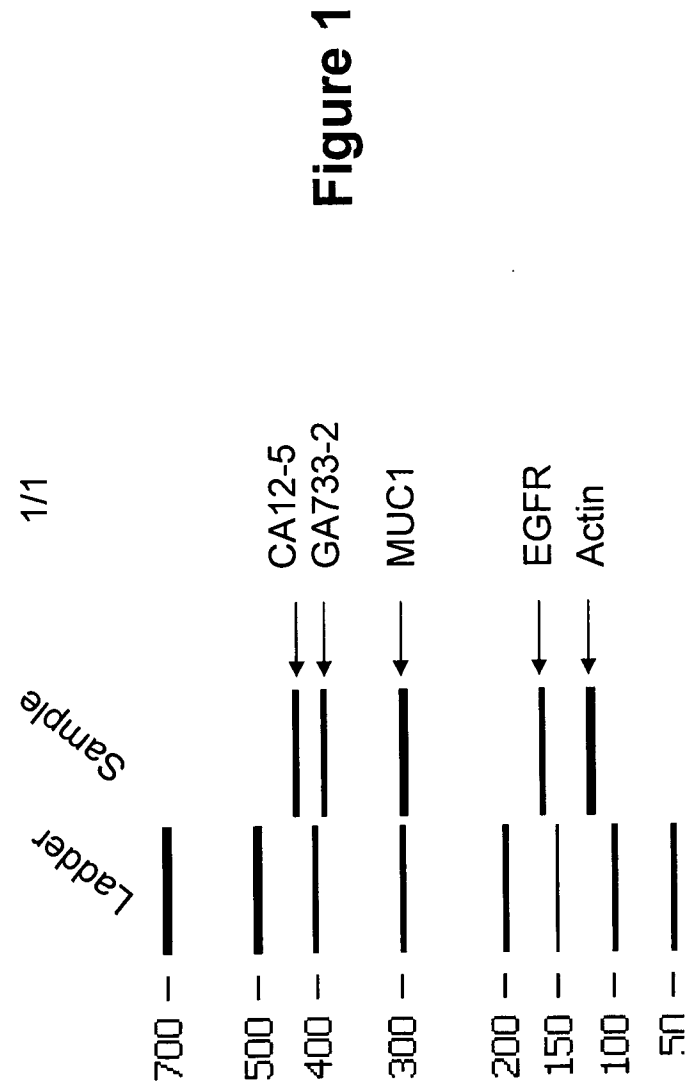


Figure 1 shows a ladder and an analyzed 5 ml blood sample, spiked with five SKOV cells. The figure shows the marker CA12-5 (432 base pairs, GA733-2 395 base pairs, MUC1 (299 base pairs and EGFR (163 base pairs). Also actin (114 base pairs) is shown.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/000755

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	ALLARD W J ET AL: "Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases" CLINICAL CANCER RESEARCH 20041015 US, vol. 10, no. 20, 15 October 2004 (2004-10-15), pages 6897-6904, XP002489234 ISSN: 1078-0432 abstract	1-29,51



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/000755

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/000755

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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P,X	WO 2008/118798 A (UNIV PITTSBURGH [US]; LISOVICH ALEXSANDR [US]; LOKSHIN ANNA [US]) 2 October 2008 (2008-10-02) claims 1,3,4,7-10,13 -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2009/000755

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