(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 June 2010 (24.06.2010)

PCT

(10) International Publication Number
WO 2010/070124 A1

(51) International Patent Classification:
G01N 33/574 (2006.01)

(21) International Application Number:
PCT/EP2009/067582

(22) International Filing Date:
18 December 2009 (18.12.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
08172217.5 18 December 2008 (18.12.2008) EP

(71) Applicant *(for all designated States except US)*: **UNIVERSITE LIBRE DE BRUXELLES** [BE/BE]; Avenue Franklin Roosevelt 50 CP 161, B-1050 Bruxelles (BE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **SOTIRIOU, Christos** [GR/BE]; Avenue Brugmann 358, B-1080 Bruxelles (BE). **IGNATIADIS, Michail** [GR/GR]; Tobazi 17, GR-54644 Thessaloniki (GR).

(74) Agent: **PRONOVEM - OFFICE VAN MALDEREN**; Avenue Josse Goffin 158, B-1082 Brussels (BE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/070124 A1

(54) **Title**: DIAGNOSTIC METHOD AND KIT OF CIRCULATING TUMOR CELLS

(57) **Abstract**: The present invention is related to a method and a diagnostic kit for an improved detection (diagnosis) of early breast cancer by combining screening mammography together with HER2-positive circulating tumor cells (CTCs) possibly present in a mammal subject (including a human patient).

DIAGNOSTIC METHOD AND KIT OF CIRCULATING TUMOR CELLS

Field of the invention

[0001] The present invention is related to a method and a diagnostic kit for an improved detection (diagnosis) of early breast cancer by combining screening mammography together with HER2-positive circulating tumor cells (CTCs) possibly present in a mammal subject (including a human patient).

Background of the invention

[0002] Among epithelial cancers, breast cancer is the most common cancer in women in Western countries.

[0003] The presence of CTCs in the blood of a cancer patient is associated with a worse prognosis.

[0004] Veridex corp developed a test kit for the isolation of CTC in the peripheral blood of a patient to determine the prognosis of patients with metastatic breast, colorectal or prostate cancer at any time. The test offers an objective, quantitative, real-time reading of tumor information so that oncologists can provide optimum care for their patients. This test is based on an isolation of cells via their epithelial proteins, such as Epithelial cell adhesion molecule (EPCAM) and/or cytokeratins 8, 18 or 19. In brief, CTC are bound and enriched on beads derivatized with anti EPCAM antibodies, followed by a labelling using anti CD45 (negative control; leukocyte marker), a labelling for the nuclei (positive control: all

cells) and cytokeratins (CTC). However, this test is not yet accepted for diagnostic use: its sensitivity remains to be demonstrated in clinical diagnostics.

[0005] Another test sold by Veridex allows HER2 tumor-phenotyping. This test also is for research-use only. More particularly, this test may lack of sensitivity and of quantitative measurements.

[0006] These tests are sold to monitor a woman diagnosed with breast cancer in order to follow if said woman has circulating tumor cells and to follow any change in the number of CTC, however there is no indication on the use of these tests for women not previously diagnosed as having a breast cancer, or who are suspected of having a breast cancer and/or who are at higher risk for developing breast cancer like a women with a pre-invasive cancer lesion or an abnormal mammogram.

[0007] Rack et al. launched a trial evidencing prognostic significance of CTCs in early breast cancer (SUCCESS trial), >1 CTC/23 ml of blood was detected by the CellSearch® System in 9.5% and 8.7% of 1500 node-positive and high risk node-negative early breast cancer patients before and after adjuvant chemotherapy, respectively. After a 12-month median follow up, detection of >1 CTC/23 ml after but not before adjuvant chemotherapy was associated with shorter disease-free and overall survival (Rack B. et al, Proc. Am.Soc.Clin.Oncol. vol 26: abstr 503 2008). However, the CTC positivity rate was low (<10%).

Aims of the invention

[0008] The present invention aims to provide new detection method and tools that do not present the drawbacks of the state of the art.

[0009] The present invention aims to provide an improved diagnostic of the HER2 state of (putative)

circulating tumor cells in a (woman) patient, especially in an early breast cancer woman patient.

Summary of the invention

[0010] The present invention discloses method and tools presenting (compared to the methods and tools of the state of the art) an improved sensitivity or specificity of an hyperproliferative disorder (cancer) detection, in (woman) patients considered as free from (not affected by) epithelial (breast cancer) or suffering from early epithelial cancer such as breast cancer, ovarian cancer or bladder cancer, possibly combined with standard imaging methods and devices, such as mammography or magnetic resonance imaging (MRI).

[0011] The present diagnostic method may advantageously be applied to biological samples obtained from women presenting pre-invasive lesions in breast cancer like hyperplasias or ductal or lobular carcinoma in situ or for women at higher risk of developing breast cancer, possibly based on the gail model (Gail MH et al J Natl Cancer Inst 81(24):1879-86, 1989).

[0012] The present diagnostic method may also be applied to a patient (woman) that is not presently diagnosed as having a cancer (an epithelial cancer such as breast cancer).

[0013] Advantageously, the present diagnostic method may be applied to a patient (woman) at higher risk for developing epithelial cancer (breast, bladder, ovarian cancer, preferably a breast cancer), such as a woman with an abnormal mammogram.

[0014] More precisely, the present diagnostic method may be applied to a woman patient with a pre-invasive (cancer) lesion, preferably selected from the group

consisting of atypical hyperplasia, Ductal Carcinoma in Situ (DCIS) and Lobular Carcinoma in Situ (LCIS).

[0015] Alternatively, the present diagnostic method may be applied to a patient (woman) at higher risk for developing metastatic (breast) cancer, such as a human (woman) suffering from an early (invasive) epithelial cancer (breast, bladder or ovarian cancer, preferably a breast cancer).

[0016] More precisely, the method and tools of the present invention are related to a diagnostic method and kit of the HER2 state of (putative) circulating tumor cells in breast cancer (woman) patients.

[0017] The method and kit of the present invention are applied to biological samples obtained from patients not previously diagnosed as having a (breast) cancer or a predisposition to develop a (breast) cancer.

[0018] The method and kit of the present invention are preferably applied to biological samples obtained from patients who are suspected of having a (breast) cancer.

[0019] The method and kit of the present invention are preferably applied to biological samples obtained from patients who are at higher risk for developing (breast) cancer, like patients with a pre-invasive (cancer) lesion or an abnormal mammogram.

[0020] Alternatively, the diagnostic of the HER2 state of putative circulating tumor cells in a (woman) patient is done upon biological samples obtained from patients suffering from early (breast) cancer.

[0021] Less preferably, the diagnostic of the HER2 state of putative circulating tumor cells in a (woman) patient may also be done upon biological samples obtained from patients suffering from metastatic (breast) cancer.

[0022] The present invention relates to a cancer diagnostic method, which comprises the step of:

- a) submitting a patient blood sample to an enrichment of putative circulating tumor cells;
- b) labelling the retained cells of step a)

for one, two, three or four epithelial marker, preferably cytokeratin 8 and/or cytokeratin 18 and/or cytokeratin 19 and/or mammaglobin;

and for HER2 state;

- c) deducing and possibly quantifying a presence of circulating tumor cells in the sample, via a positive signal for epithelial marker detection and
- d) establishing an HER2 state of circulating tumor cells in the said sample.

[0023] The method of the invention advantageously comprises a last step (such as step e in the above-mentioned method) of deducing whether a patient is at risk of (present a risk of) developing a cancer (or of having a worsening risk of developing a cancer) by quantifying the number of CTCs measured and/or of HER2-positive CTC possibly present in the blood sample (obtained from this patient).

[0024] Advantageously, this risk is increasing in the case of more than 1, 2, 3, 4, 5, 6, 7, 8, 9 or even more than 10 CTC are measured in a blood sample (preferably a 25 ml blood sample).

[0025] Advantageously, this risk is increasing in the case of at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or even more than 10 HER-2 positive CTC are measured in this blood sample (preferably of 25 ml blood sample).

[0026] Preferably, this risk is increasing in the case of more than 2, 3, 4, 5, 6, 7, 8, 9 or even more than 10 CTC or at least 1, HER2 positive CTC are measured in this blood sample (preferably a 25 ml blood sample).

[0027] The method of the invention advantageously comprises one or more enrichment step(s) of epithelial cells that may be present in a blood sample of a patient.

[0028] Preferably, recognition of this membrane marker is done by the use of an antibody or its specific hypervariable fragment both being directed against this marker of a membrane-marker of epithelial cells, this marker being preferably EpCAM.

[0029] Alternatively, the enrichment step of epithelial cells (in a blood sample) is based on centrifugation, preferably using Ficoll gradient.

[0030] Another preferred enrichment step consist on the use of cell filters (in a blood sample) able to retain epithelial cells, but not blood cells.

[0031] Advantageously, two enrichment methods of CTC are performed (sequentially) in the method of the invention.

[0032] The method of the invention advantageously further comprises the step of sorting non-epithelial cells based on a recognition of the membrane-marker not expressed by epithelial cells, preferably the extracellular part (portion) of a protein, being preferably antigen CD45.

[0033] The method of the invention optionally comprises the step of labelling epithelial proteins and/or mRNA expressed in breast tissue, being preferably mammaglobins.

[0034] The method of the invention advantageously comprises the step of labelling epithelial proteins and/or mRNA expressed in epithelial tissue, these proteins and/or mRNA being preferably cytokeratin 8, cytokeratin 18 and/or cytokeratin 19.

[0035] Advantageously, these cytokeratin 8, cytokeratin 18 and/or cytokeratin 19 are labelled by an (one) antibody (or a specific hypervariable fragment

thereof or a mixture of antibodies) that specifically recognizes (that is (are) directed against) these three cytokeratin 8, cytokeratin 18 and cytokeratin 19 proteins.

[0036] Alternatively, an (one) antibody specific for a (one) protein selected from the group consisting of cytokeratin 8, cytokeratin 18 and cytokeratin 19 is used in the method and kit of the invention.

[0037] Alternatively, a specific detection of both cytokeratin 8 and cytokeratin 18 is performed.

[0038] Alternatively, a specific detection of both cytokeratin 8 and cytokeratin 19 is performed.

[0039] Alternatively, a specific detection of both cytokeratin 18 and cytokeratin 19 is performed.

[0040] Alternatively, a specific detection of both cytokeratin 8, cytokeratin 18 and cytokeratin 19 is performed.

[0041] Alternatively cytokeratin 8 and/or cytokeratin 18 and/or cytokeratin 19 mRNA and/or mammaglobin is identified and/or quantified using PCR.

[0042] The present invention concerns a cancer diagnostic and possibly monitoring method (possibly combined with standard imaging methods such as mammography or magnetic resonance imaging (MRI)) and comprising the step of:

- a) submitting a biological sample such as a fluid (preferably a blood sample) from a human (woman) patient, to an enrichment of putative circulating tumor cells, preferably upon anti-EPCAM antibodies (or specific hypervariable portion thereof) retention,
- b) labelling the obtained (retained) cells from step a) with non-epithelial markers, being preferably anti-CD45 antibodies (or specific hyper variable fragments thereof),
- c) labelling the obtained (retained) cells with epithelial markers, being preferably anti-cytokeratin (8,

18 or 19) antibodies (or a specific hyper variable fragment thereof),

- d) quantifying Her2 content, preferably via the labelling with of HER2 protein anti-HER2 antibodies (or specific hyper variable fragments thereof)
and/or with Her2 mRNA content
and/or with Her2 DNA content and/or with Her2 mRNA-related sequence (micro-RNA).

- e) deducing and possibly quantifying the presence of circulating tumor cells, via a positive signal for cytokeratin detection and a negative signal for CD45 detection,

- f) establishing the HER2 state (profile or level) of circulating tumor cells present in the sample.

[0043] The present invention concerns a cancer diagnostic and possibly monitoring method (possibly combined with standard imaging methods such as mammography or magnetic resonance imaging (MRI)) and comprising the step of:

- a) submitting a biological sample such as a fluid (preferably a blood sample) from a human (woman) patient, to an enrichment of putative circulating tumor cells, preferably upon centrifugation in Ficoll or through filtering,
- b) optionally labelling the obtained (retained) cells from step a) with non-epithelial markers, being preferably anti-CD45 antibodies (or specific hyper variable fragments thereof),
- c) measuring the obtained (retained) cells with epithelial markers, being preferably cytokeratin (8, 18 or 19) mRNA content and/or mammaglobin mRNA content,
- d) quantifying Her2 content, preferably via the labelling with of HER2 protein anti-HER2 antibodies (or specific hyper variable fragments thereof)

and/or with Her2 mRNA content
and/or with Her2 DNA content and/or with Her2 mRNA-related sequence (micro-RNA),

- e) deducing and possibly quantifying the presence of circulating tumor cells, via a positive signal for cytokeratin detection and a negative signal for CD45 detection,

- f) establishing the HER2 state (profile or level) of circulating tumor cells present in the sample.

[0044] The present invention concerns a cancer diagnostic and possibly monitoring method (possibly combined with standard imaging methods such as mammography or Magnetic Resonance Imaging (MRI)) and comprising the step of:

- a) submitting a biological sample, such as a fluid (preferably a blood sample) obtained from a human (woman) patient, to an enrichment of putative circulating tumor cells;

- b) labelling (preferably a first part of) the obtained (retained) cells with epithelial markers, being preferably anti-cytokeratin (8, 18 or 19) antibodies (or a specific hyper variable fragment thereof),

- c) deducing and possibly quantifying the presence of circulating tumor cells (preferably in the first part of this biological sample), via a positive signal for cytokeratin detection and a negative signal for CD45 detection,

- d) quantifying Her2 content of the cells of step c), preferably via the labelling with of HER2 protein with anti-HER2 antibodies (or specific hyper variable fragments thereof)

- e) submitting a biological sample, such as a fluid (preferably a blood sample) obtained from a human (woman)

patient to an enrichment of putative circulating tumor cells,

- f) measuring the obtained (retained) cells (in steps a and/or e) with epithelial markers, being preferably cytokeratin (8, 18 or 19) mRNA content and/or mammaglobin mRNA content,

- g) deducing and possibly quantifying the presence of circulating tumor cells, via a positive signal for cytokeratin (8, 18, 19) or mammaglobin detection,

- h) quantifying Her2 content, preferably via the measurement of Her2 mRNA content;

- i) establishing the HER2 state (profile or level) for every circulating tumor cell identified in steps b-c or in steps f-g and possibly deducing the presence of Her2 positive CTC.

[0045] Advantageously, the said method(s) further comprises a step of labelling the obtained (retained) cells from step a) with non-epithelial markers, being preferably anti-CD45 antibodies (or specific hyper variable fragments thereof),

[0046] Alternatively, the diagnostic method of the invention comprises the steps of:

- a) submitting a biological sample preferably a fluid (blood sample) to an enrichment of putative circulating tumor cells, preferably on anti-EPCAM antibodies (or specific hyper variable fragments thereof) retention, and preferably upon absence of binding to anti CD45 antibodies (or specific hyper variable fragments thereof);

- b) quantifying HER2 protein expression via the labelling with anti-HER2 antibodies (or specific hyper variable fragments thereof) for every circulating tumor cell enriched and

- c) deducing whether these circulating tumor cells present HER2+ characteristics (HER2+ state).

[0047] Alternatively, the diagnostic method of the invention comprises the steps of:

-submitting a biological fluid (blood sample) to an enrichment of putative circulating tumor cells, preferably on anti-EPCAM antibodies (or specific hyper variable fragments thereof) retention,

-preferably label and/or sort the said enriched cells with anti CD45 antibodies (or specific hyper variable fragments thereof);

- quantifying mRNA and/or gene amplification of HER2 for every enriched putative circulating tumor cell and

- deducing whether these putative circulating tumor cells present HER2-positive (i.e. HER2+ characteristics; HER2+ state).

[0048] The present invention is also related to the tools for performing the essential steps of the method of the invention, especially related to a detection and possibly monitoring kit that comprises anti-EPCAM and anti-HER2 antibodies (or specific hyper variable portions thereof), and possibly anti-CD45 and/or anti-cytokeratin (8, 18 or 19) and/or mammaglobin antibodies (or specific hyper variable fragments thereof).

[0049] The present invention is further related to a cancer diagnostic kit, which comprises an insoluble solid support, such as beads coupled with (grafted with or fixed to) anti-EpCAM antibodies or specific hyper variable fragments thereof and anti-HER2 antibodies or specific hyper variable fragments thereof and/or means (primers and possibly detection probe) for Her2 mRNA quantification.

[0050] The cancer diagnostic kit of the invention may further comprise anti-CD45 antibodies and antibodies recognising at least one cytokeratin selected from the group consisting of cytokeratin 8, cytokeratin 18 and cytokeratin 19.

Detailed description of the invention

[0051] Her2 (i.e. neu; also known as ErbB-2; Seq. ID. NOs. 11 and 12) stands for Human Epidermal growth factor 2 and is a protein giving higher aggressiveness in breast cancers. ErbB 2 is a receptor tyrosine kinase of the ErbB family. It is closely related in structure to the epidermal growth factor receptor. ErbB 2 oncoprotein is detectable in a proportion of breast and other adenocarconomas, as well as transitional cell carcinomas. In the case of breast cancer, expression determined by immunohistochemistry has been shown to be associated with poor prognosis. The sequence of human Her2 protein, mRNA, gene and RNA-related sequences may be alternatively found using available databases.

[0052] This protein has no ligand binding domain of its own and therefore cannot bind growth factors. However, it does bind tightly to other ligand-bound EGF receptor family members to form a heterodimer, stabilizing ligand binding and enhancing kinase-mediated activation of downstream signalling pathways.

[0053] Allelic variations at amino acid positions 654 and 655 of isoform a (positions 624 and 625 of isoform b) have been reported.

[0054] Amplification and/or overexpression of this gene has been reported in numerous cancers, including breast and ovarian tumors.

[0055] Alternative splicing results in several additional transcript variants, some encoding different isoforms. All variants of Her2 are encompassed in the present invention.

[0056] CD45 (Seq. ID. NOs. 7 and 8) was originally called leukocyte common antigen. CD45 is a family of single chain transmembraneous glycoproteins consisting of at least

four isoforms (220, 205, 190, 180 kDa) which share a common large intracellular domain. Their extracellular domains are heavily glycosylated. The different isoforms are produced by alternative messenger RNA splicing of three exons of a single gene on chromosome 1. CD45 is expressed on cells of the human hematopoietic lineage (including hematopoietic stem cells) with the exception of mature red cells. CD45 is not detected on differentiated cells of other tissues. Antibodies recognising a common epitope on all of the isoforms are termed CD45 antibodies. However, every variants are encompassed in the present invention.

[0057] Cytokeratins are intermediate filament keratins found in the intracytoplasmic cytoskeleton of epithelial tissue.

The present patent application refers to the use of the mRNA and/or protein sequence of human cytokeratin 8 (SEQ. ID. Nos. 1 and 2) that may alternatively be found using databases and/or to the use of mRNA and/or protein sequence of human cytokeratin 18 (Seq. ID. Nos. 3 and 4) that may alternatively be found using databases and/or to the use of mRNA and/or protein sequence of human cytokeratin 19 (Seq. ID. Nos 5 and 6) that may alternatively be found using databases.

[0058] Epithelial Cell Adhesion Molecule (EpCAM; SEQ. ID. NOs. 9 and 10) is a 40kD cell surface antigen. This antigen has been identified independently by a number of groups, and has been known by a variety of names. Several monoclonal antibodies have been raised against EpCAM. EpCAM is a Type 1 transmembrane glycoprotein. It is expressed on the basolateral membrane of cells by the majority of epithelial tissues, with the exception of adult squamous epithelium and some specific epithelial cell types including hepatocytes and gastric epithelial cells.

[0059] EpCAM expression has been reported to be a possible marker of early malignancy, with expression being increased in tumour cells, and *de novo* expression being seen in dysplastic squamous epithelium. EPCAM is a cell surface molecule.

[0060] EPCAM accession number is NM_002354. Its Entrez gene id is 4072.

[0061] The present invention will be explained in more details in the following non-limiting example.

[0062] The skilled person may develop alternative without being outside the scope of the present invention. More particularly, the skilled person may find and/or use and/or develop other means to enrich circulating tumor cells, to label them and to assess HER2 state in these cells.

[0063] This can be done using antibodies, fragments of antibodies, other protein or peptides having an affinity for circulating tumor cells, or any ligand able to enrich and/or bind and/or label circulating tumor cells.

[0064] Her2 expression state can be determined by labelling and/or quantifying the translated protein (e.g. using specific antibodies or hyper variable fragments of antibodies), by measuring its mRNA content, the content of RNA-related sequences (e.g. micro-RNA), or quantification of gene copies number.

[0065] Her2 expression state is concluded as positive if at least one, preferably at least two, more preferably at least three, or the four of the test selected from the list consisting of quantification of the HER2 translated protein, of the Her2 mRNA content, of Her2 RNA-related sequences (e.g. micro-RNA) or of Her2 gene duplication is/are positive.

[0066] Her2 expression state is advantageously determined by the combined quantification of the translated HER2 protein and of the Her2 mRNA content.

[0067] Alternatively, Her2 expression state is determined by the combined quantification of the translated HER2 protein and of Her2 gene duplication.

[0068] Preferably, Her2 state of CTC is determined by the combined quantification of the HER2 protein and of the Her2 DNA by FISH (Fluorescence after in situ hybridization) in the same CTC.

[0069] Her2 state of CTC may alternatively be obtained by quantification of mRNA sequences (mRNA transcripts) and of the microRNA level of CTC.

[0070] Her2 state of CTC may be obtained by quantification of the HER2 protein and of the microRNA level of CTC.

[0071] By a patient, it is meant every a person susceptible to have and/or to develop a (breast) cancer.

[0072] By an insoluble solid support, it is meant every surface that may be grafted with (fixed by) antibodies.

[0073] Beads are an example of insoluble solid support.

[0074] The preferred beads present in the kit according to the invention are sedimentable.

Example 1:

[0075] CTCs have been detected using the CellSearch® System in early and metastatic breast cancer (BC). In this study, the inventors first aimed to analyze CTCs for HER2 expression in different stages of BC progression from ductal/lobular carcinoma in situ (DCIS/LCIS) to early and

metastatic BC. This test was also applied to healthy woman as negative control and possible detection of non-diagnosed breast cancer.

[0076] The skilled person may use and/or develop other tests to isolate CTC, and to evaluate their Her2 state.

Patients and Methods:

[0077] 20 ml of peripheral blood per woman was analyzed from healthy women, DCIS/LCIS or early BC patients, whereas 7.5 ml of blood was analyzed from metastatic BC patients. The presence of CTCs as well as HER2 expression was assessed with the CellSearch® System (Veridex, USA). After immunomagnetic enrichment with an anti-Epcam-antibody, cells were labeled with anti-cytokeratin (8, 18, 19), anti-HER2 and anti-CD45 antibodies.

[0078] CTCs were defined as Cytokeratin-positive/CD45-negative cells, whereas HER2+ CTCs were defined as Cytokeratin-positive/CD45-negative/HER2-positive cells.

Results:

[0079] The presence of CTCs and HER2+ CTCs in healthy women, DCIS/LCIS, early and metastatic BC is depicted in table 1.

	Women studied	Women with CTCs			Women with HER2+ CTCs
		1 CTC	2CTCs	>2CTCs	≥1 CTC
Healthy women	47	7	4	0	0
DCIS/LCIS	40	8	2	1	8
Early	37	5	2	5	11
Metastatic	21	3	1	8	7

Table 1: identification and Her2 characterization of circulating tumor cells in healthy woman, precancerous and cancerous patients.

[0080] Her2 state can alternatively be measured by PCR, for instance using nested PCR with
Forward primer1 (SEQ.ID.NO.13): TCC TCC TCG CCC TCT TGC ;
Forward primer2 (SEQ.ID.NO.14): AGC CGC GAG CAC CCA AGT;
Reverse primer1: (SEQ.ID.NO.15): GCG GGT CTC CAT TGT CTA and
Reverse primer2: (SEQ.ID.NO.16): TTG GTG GGC AGG TAG GTG AGT.

[0081] The inventors also combined both methods.

[0082] Since 1 and 2 CTCs were detected in 7 (14.8%) and 4 (8.5%) of 47 healthy women, respectively, >1CTC or >2CTCs were selected as cutoffs for further analysis. Using the above cutoffs, only 7 (18.9%) and 5 (13.5%) of 37 patients with early BC were considered CTC-positive, respectively.

[0083] Since no HER2+ CTCs were detected in any of the 47 healthy women, a cutoff of 1 HER2+ CTCs was advantageously chosen.

[0084] With this refined cutoff, more women with early BC [11 (29.7%) of 37 women] were considered CTC-positive.

[0085] HER2+ CTCs is thus a more sensitive and specific marker for CTCs positivity than total CTCs counts in early breast cancer. Clinical relevance of the above cutoff for CTCs positivity using HER2+ CTCs detection will be performed in ongoing clinical trials of HER2 positive early breast cancer.

Example 2

Comparison of alternative methods of enrichment and detection of CTC

[0086] The inventors measured CTC of 99 patients having early breast cancer or in DCIS state, using the Cellsearch platform. They observed more than 2 CTC in 8 patients. Then they evaluated the Her2 state of the CTC. They observed at least one Her2 positive CTC in 14 patients. However they did not observe any Her 2 positive CTC in the 74 healthy patients tested.

[0087] The inventors then developed a method to enrich CTC based on Ficoll gradient, using the same blood samples as for the test with the Cellsearch system.

[0088] After the enrichment of CTC in Ficoll, the inventors measured mRNA content of cytokeratin 19 (for instance by PCR using Forward primer: SEQ.ID.NO.17: GCACTACAGCCACTACTACACGA and Reverse primer SEQ.ID.NO.18: CTCATGCGCAGAGCCTGTT) and mammaglobin (for instance in nested PCR using Forward primer1 SEQ.ID.NO.19: CAATCAATCCACAAGTGTCTAA ; Forward primer2 SEQ.ID.NO.20: ACGGATGAAACTCTGAGCAATG; Reverse primer1 SEQ.ID.NO.21: AACATGTATAGCAGGTTTCAAC and Reverse primer2 SEQ.ID.NO.22: CAGTTCTGTGAGCCAAAGGT).

[0089] The inventors deduced the presence of CTCs in 12 patients.

[0090] Surprisingly, the inventors found only partial correlation between the two methods (some blood samples taken from one patient are positive in one method and negative in the other), evidencing complementarities between the two methods.

[0091] The inventors took the advantage to combine the two methods to detect CTC with the measure of Her2 state.

[0092] The inventors then analyzed other epithelial cancers (bladder and ovarian) at early (non metastatic) stages and found HER2 positive CTCs using the diagnostic method of the present invention.

CLAIMS

1. A cancer diagnostic method, which comprises the step of:

- a) submitting a patient blood sample to an enrichment of putative circulating tumor cells;
- b) labelling the retained cells of step a) for at least one epithelial marker, preferably cytokeratin 8 and/or cytokeratin 18 and/or cytokeratin 19 and/or mammaglobin; and for HER2 state;
- c) deducing and possibly quantifying a presence of circulating tumor cells in the sample, via a positive signal for epithelial marker detection and
- d) establishing an HER2 state of circulating tumor cells in the said sample.

2. The method of Claim 1, wherein the said cancer diagnostic is an epithelial (breast) cancer diagnostic.

3. The method of Claims 1 or 2, wherein the patient is not metastatic.

4. The method according to any of the preceding Claims, wherein the patient is free from cancer.

5. The method according to any of the preceding Claims, wherein the patient is a woman at higher risk of breast cancer.

6. The method of Claim 5, wherein the woman at higher risk of breast cancer is a woman with a pre-invasive (cancer) lesion.

7. The method of Claims 5 or 6, wherein the woman at higher risk of breast cancer is a woman with an abnormal mammogram.

8. The method according to any of the preceding Claims 5 to 7, wherein the patients are selected

from the group consisting of patients having atypical hyperplasia, Ductal Carcinoma in Situ (DCIS) and Lobular Carcinoma in situ (LCIS).

9. The method according to any one of the preceding claims 1 to 8, wherein the sample is obtained from human (woman) at higher risk for developing metastatic epithelial (breast) cancer.

10. The method according to any of the preceding claims 1 to 3 and 9, wherein the sample is obtained from human (woman) suffering from an early (invasive) epithelial (breast) cancer.

11. The method according to any of the preceding Claims, wherein the enrichment of putative circulating tumor cells is obtained upon anti-EPCAM antibodies or specific hyper variable fragments thereof.

12. The method according to any of the preceding Claims, wherein the enrichment of putative circulating tumor cells is obtained using centrifugation in Ficoll gradients.

13. The method according to any of the preceding Claims, wherein the enrichment of putative circulating tumor cells is obtained using filtration.

14. The method according to any of the preceding claims, further comprising a step of labelling the enriched cells of step a with anti-CD45 antibodies or specific hyper variable fragments thereof and possibly discard the cells labelled with said anti-CD45 antibodies or specific hyper variable fragments thereof.

15. The method according to any of the preceding claims, wherein the HER2 state is obtained by detection with anti-HER2 antibodies.

16. The method according to any of the preceding claims, wherein the HER2 state is obtained by quantification of Her2 mRNA content.

17. The method according to any of the preceding claims, wherein the HER2 state is obtained by quantification of gene copies number.

18. The method according to any of the preceding Claims, which further comprises a step e) of deducing if the patient is at risk of developing a cancer by quantifying the number of CTCs measured and/or of HER2-positive CTC in the blood sample.

19. The method of Claim 18, wherein the risk is present in the case of more than 1 CTC are measured in the blood sample.

20. The method of Claim 18 or 19, wherein the risk is present in the case of at least 1 HER-2 positive CTC is measured in the blood sample.

21. A cancer diagnostic kit, which comprises an insoluble support, preferably beads, coupled with anti-EpCAM antibodies or specific hyper variable fragments thereof and anti-HER2 antibodies or specific hyper variable fragments thereof and/or means (primers and possibly detection probe) for Her2 mRNA quantification.

22. The kit of claim 21, which further comprise anti-CD45 antibodies or specific hyper variable fragments thereof and antibodies and/or specific hyper variable fragments recognising at least one cytokeratin selected from the group consisting of cytokeratin 8, cytokeratin 18 and cytokeratin 19 and/or mammaglobin.

23. Use of the kit of claim 21 or 22 for a detection of early epithelial (breast) cancer.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/067582

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574

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)
G01N

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, WPI Data, Sequence Search, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2008/113350 A1 (TERSTAPPEN LEON W M M [US]) 15 May 2008 (2008-05-15)	1-2,5,7, 9,11, 14-15, 18-20
Y	paragraph [0009] claims 1,2 examples 1,2	3-4,6,8, 10, 12-13, 16-17, 21-23
X	WO 99/41613 A (IMMUNIVEST CORP [US]; UNIV TEXAS [US]; TERSTAPPEN LEON W M M [US]; RAO) 19 August 1999 (1999-08-19)	1-7, 9-11, 14-15, 18-23
Y	claims 1-7,62-64	8,12-13, 16-17

-/--

[X] Further documents are listed in the continuation of Box C.

[X] 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	Date of mailing of the international search report
16 February 2010	01/03/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Jones, Laura

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/067582

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SLEIJFER ET AL: "Circulating tumour cell detection on its way to routine diagnostic implementation?" EUROPEAN JOURNAL OF CANCER, PERGAMON PRESS, OXFORD, GB, vol. 43, no. 18, 30 October 2007 (2007-10-30), pages 2645-2650, XP022361345 ISSN: 0959-8049 the whole document	1-23
Y	IGNATIADIS MICHAIL ET AL: "Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2 in early breast cancer." CLINICAL CANCER RESEARCH : AN OFFICIAL JOURNAL OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH 1 MAY 2008, vol. 14, no. 9, 1 May 2008 (2008-05-01), pages 2593-2600, XP002568864 ISSN: 1078-0432 the whole document	1-23
Y	WO 2008/131035 A (CELLPOINT DIAGNOTICS INC [US]; KOPF-SILL ANNE R [US]; WU LENA [US]; ST) 30 October 2008 (2008-10-30) the whole document	1-23
Y	PATERLINI-BRECHOT ET AL: "Circulating tumor cells (CTC) detection: Clinical impact and future directions" CANCER LETTERS, NEW YORK, NY, US, vol. 253, no. 2, 10 July 2007 (2007-07-10) , pages 180-204, XP022144282 ISSN: 0304-3835 the whole document	1-23
Y	MOCELLIN S ET AL: "Circulating tumor cells: the 'leukemic phase' of solid cancers" TRENDS IN MOLECULAR MEDICINE, ELSEVIER CURRENT TRENDS, vol. 12, no. 3, 1 March 2006 (2006-03-01), pages 130-139, XP025229876 ISSN: 1471-4914 [retrieved on 2006-03-01] the whole document	1-23

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/067582

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PACHMANN KATHARINA ET AL: "Standardized quantification of circulating peripheral tumor cells from lung and breast cancer" CLINICAL CHEMISTRY AND LABORATORY MEDICINE, vol. 43, no. 6, 2005, pages 617-627, XP009112550 ISSN: 1434-6621 the whole document	1-23
A	JACOB KARINE ET AL: "Circulating tumor cells: detection, molecular profiling and future prospects." EXPERT REVIEW OF PROTEOMICS DEC 2007, vol. 4, no. 6, December 2007 (2007-12), pages 741-756, XP009112584 ISSN: 1744-8387 the whole document	1-23
A	RIETHDORF SABINE ET AL: "Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system." CLINICAL CANCER RESEARCH : AN OFFICIAL JOURNAL OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH 1 FEB 2007, vol. 13, no. 3, 1 February 2007 (2007-02-01), pages 920-928, XP002516029 ISSN: 1078-0432 paragraph [0927]	1-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2009/067582

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [ ] on paper

   [X] in electronic form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [ ] filed together with the international application in electronic form

   [ ] furnished subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2009/067582

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2008113350 A1	15-05-2008	NONE	
WO 9941613 A	19-08-1999	AU 760560 B2	15-05-2003
		AU 2763699 A	30-08-1999
		BR 9907852 A	24-10-2000
		CA 2320418 A1	19-08-1999
		EP 1062515 A1	27-12-2000
		JP 2002503814 T	05-02-2002
		JP 3834326 B2	18-10-2006
		JP 2005010177 A	13-01-2005
		JP 2006208399 A	10-08-2006
WO 2008131035 A	30-10-2008	WO 2008131048 A2	30-10-2008